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Syk-dependent Actin Dynamics Regulate Endocytic Trafficking and Processing of Antigens Internalized through the B-Cell Receptor[D] [V]

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Antigen binding to the B-cell receptor (BCR) induces multiple signaling cascades that ultimately lead to B lymphocyte activation. In addition, the BCR regulates the key trafficking events that allow the antigen to reach endocytic compartments devoted to antigen processing, i.e., that are enriched for major histocompatibility factor class II (MHC II) and accessory molecules such as H2-DM. Here, we analyze the role in antigen processing and presentation of the tyrosine kinase Syk, which is activated upon BCR engagement. We show that convergence of MHC II- and H2-DM–containing compartments with the vesicles that transport BCR-uptaken antigens is impaired in cells lacking Syk activity. This defect in endocytic trafficking compromises the ability of Syk-deficient cells to form MHC II-peptide complexes from BCR-internalized antigens. Altered endocytic trafficking is associated to a failure of Syk-deficient cells to properly reorganize their actin cytoskeleton in response to BCR engagement. We propose that, by modulating the actin dynamics induced upon BCR stimulation, Syk regulates the positioning and transport of the vesicles that carry the molecules required for antigen processing and presentation.

INTRODUCTION

Mature resting B lymphocytes capture antigens (Ag) via their specific B-cell receptor (BCR), which corresponds to a surface immunoglobulin (Ig) coupled to a signaling module formed by the Igα/Igβ dimer (Cambier *et al.*, 1994; Reth and Wienands, 1997). Productive BCR-Ag interaction initiates a complex signaling cascade (reviewed in Niiro and Clark, 2002) that starts with the activation of tyrosine kinases from the src family, especially Lyn. Src kinases phosphorylate the ITAM motif of Igα and Igβ, leading to the recruitment and subsequent activation of the Syk tyrosine kinase. Syk activates the downstream signaling pathways that ultimately lead to proliferation and activation of B lymphocytes, which can then initiate the development of germinal centers (GCs). To complete GC formation, B lymphocytes must present internalized Ag onto major histocompatibility factor class II (MHC II) molecules to primed CD4 T-cells, a process referred to as T-B cooperation (McHeyzer-Williams *et al.*, 2000; Mitchison, 2004).

MHC II molecules assemble shortly after synthesis in the endoplasmic reticulum (ER) with a type II transmembrane protein, the invariant chain (Ii), which directs their trafficking to endocytic compartments for them to be loaded with antigenic peptides (reviewed in Bryant and Ploegh, 2004). Such peptides are derived from the degradation of internalized Ag by endocytic proteases, which must also cleave Ii to free MHC II molecules for peptide loading, a reaction catalyzed by the chaperone molecule H2-DM (reviewed in Lennon-Dumenil *et al.*, 2002 and Watts, 2001). Successful Ag processing therefore relies on the after directional membrane trafficking events: 1) Ag internalization and targeting into endocytic compartments, 2) MHC II-Ii complexes, proteases and H2-DM convergence toward this incoming pool of Ag, and 3) export of MHC II-peptide complexes to the cell surface.

Ensuring these key events of protein trafficking is an essential function of Ag receptors such as the BCR (reviewed in Vascotto *et al.*, 2007b). BCR engagement is accompanied by a dramatic reorganization of MHC II–containing compartments, which change from discrete peripheral vesicles to a massive central cluster that is essentially composed of multivesicular lysosomal-like compartments wherein the Ag, MHC II and accessory molecules concentrate together for processing (Siemasko *et al.*, 1998; Drake *et al.*, 1999; Siemasko and Clark, 2001; Lankar *et al.*, 2002; Boes *et al.*,

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2004; Vascotto *et al.*, 2007a). Although Igα/β phosphorylation is not necessary for Ag uptake, the cytosolic tails of Igα and Igβ ITAM motifs cooperatively and synergistically interact to optimize the trafficking and maintenance of BCR-Ag complexes into lysosomes devoted to Ag processing (Bonnerot *et al.*, 1995; Cheng *et al.*, 1995; Li *et al.*, 2002). Accordingly, transfection of a dominant negative form of the ITAM-associated kinase Syk was shown to inhibit MHC II processing and presentation (Lankar *et al.*, 1998).

Translocation of BCR-Ag complexes into lipid rafts triggers clathrin phosphorylation by activated Src kinases and is needed for efficient Ag internalization (Stoddart *et al.*, 2002) and targeting to MHC II–containing lysosomes (Cheng *et al.*, 1999, 2001). Analysis of lipid raft dynamics by time-lapse microscopy showed that BCR engagement induces their coalescence into a localized portion of the plasma membrane, an event that relies on actin cytoskeleton remodelling (Hao and August, 2005; Gupta *et al.*, 2006). BCR stimulation has indeed been shown to induce the dynamic reorganization of the actin cytoskeleton, including a fast depolymerization phase followed by polarized repolymerization (Hao and August, 2005). The importance of actin dynamics in Ag trafficking and processing was further demonstrated by showing that actin depolymerizing reagents decrease the efficiency of BCR-Ag internalization and convergence with MHC II-Ii complexes into H2-DM–containing lysosomes (Barois *et al.*, 1998; Brown and Song, 2001). In addition, we have recently identified the actin-based motor protein Myosin II as being necessary for MHC II molecules and BCR-uptaken Ag to concentrate together in lysosomes devoted to Ag processing (Vascotto *et al.*, 2007a).

Syk-deficient mice lack mature B lymphocytes as a result of developmental arrest at the pro-B stage (Cheng *et al.*, 1995; Turner *et al.*, 1997). We therefore took advantage of a mouse B lymphoma cell line deficient for Syk to unravel the role of this kinase in Ag processing and presentation. We found that Syk is required for efficient formation of MHC II-peptide complexes from BCR-uptaken Ag. Indeed, B-cells that lack Syk activity show alterations in endocytic trafficking, which hamper the convergence of MHC II– and H2-DM–containing vesicles with those that transport BCR-uptaken Ag. Altered endocytic trafficking results from the inability of Syk mutants to properly reorganize their actin cytoskeleton upon BCR-engagement. Syk therefore emerges as a key regulator of the interactions between endocytic vesicles and actin filaments, such interactions being essential for the processing and presentation of BCR-internalized Ag.

MATERIALS AND METHODS

Cells

The mouse B lymphoma IIA1.6 cell line is a FcγR-defective variant of A20 cells and has the phenotype of quiescent mature B-cells expressing surface IgG2a (previously described in Lankar *et al.*, 1998). Cells were maintained in RPMI 1640 supplemented with 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2–mercaptoethanol (2-ME), 5 mM sodium pyruvate, and 10% fetal calf serum (FCS) as previously described (Lankar *et al.*, 2002). The Syk-deficient clone (ΔSyk) was identified from a population of IIA1.6 B lymphoma cells (wild-type [WT] cells; Amigorena *et al.*, 1992; Bonnerot *et al.*, 1992; Yokozeki *et al.*, 2003). To obtain Syk-reconstituted clones, Syk-deficient cells were electroporated with the pNTH2 expression vector containing the cDNA encoding wild-type (^{WT}Syk) or a kinase-inactive mutant form of Syk (K395R mutant in the ATP-binding domain, ^{K395R}Syk; Yokozeki *et al.*, 2003). The T-cell LMR75 hybridoma is specific of the LACK peptide 156–173 associated to I-A^d (Malherbe *et al.*, 2000) and the T-cell B9.1 hybridoma recognize I-E^d/HEL$_{108-116}$. Primary B-cells were purified from spleen of I-A$_\beta$-green fluorescent protein (GFP) knockin (referred to as MHC II-GFP) mice (Boes *et al.*, 2002) as previously described (Vascotto *et al.*, 2007a).

Antibodies and Reagents

The following primary antibodies (Ab) were used for immunofluorescence, cytofluorometry, and/or immunoblot experiments: rabbit anti-mouse H2-DM, rabbit anti-MHCII (JV2; Driessen *et al.*, 1999), and rabbit anti-I-A^b I-A$_\beta$ (Lankar *et al.*, 2002), the biotinylated 2C44 mAb restricted to I-A^d/LACK$_{156-173}$ complexes and anti-mouse CD107a (LAMP-1; BD Biosciences, San Jose, CA). We used the following secondary antibodies (all F(ab')$_2$ for immunofluorescence analysis) : Cy3-conjugated donkey anti-goat and Cy5-conjugated donkey anti-rabbit (both from Jackson ImmunoResearch, West Grove, PA), Alexa488-conjugated donkey anti-rat, Alexa488-conjugated anti-goat, and Alexa488-conjugated anti-rabbit (all three from Molecular Probes, Eugene, OR), anti-rabbit conjugated to horseradish peroxidase (HRP; Ozyme, Saint-Quentin en Yvelines, France). To amplify the signal obtained from biotinylated 2C44, the tyramide amplification kit containing streptavidin conjugated to HRP and Alexa546-tyramide was used, following the manufacturer's instructions (TSA Kit, Molecular Probes). Actin was stained using phalloidin conjugated to FluoroProbes 547 (FluoroProbes, Interchim, Lyon, France). Optimal concentrations of the inhibitors piceatannol and cytochalasin D (Sigma, St. Louis, MO) were determined based on the manufacturer's instructions and cell viability as assessed by flow cytometry.

Antigen Presentation Assays

Ag presentation assays were performed by culturing 2×10^4 WT, ΔSyk, ^{K395R}Syk, or ^{WT}Syk cells together with 2×10^4 specific T-cell hybridomas for 18–20 h in the presence of various concentrations of Ag (HEL or LACK) complexed mixed with F(ab')$_2$ goat anti-mouse IgG and with a 3.2× volume of nanoparticles (NP-anti-IgG-Ag). Nanoparticles (NP, 8-nm diameter, Fe_2O_3) were kindly provided by C. Ménager (Laboratoire Liquides Ioniques et Interfaces Chargées, Université Pierre et Marie Curie). The release of IL-2 by T-cell hybridomas was determined by a CTL.L2 proliferation assay, as previously described (Amigorena *et al.*, 1992). Each point represents the average of triplicate samples that varied by <5%.

B-Cell Activation

For BCR activation experiments using nanoparticles, cells were activated with 10 μg/ml F(ab')$_2$ goat anti-mouse IgG mixed to 10 μg/ml the p40 LACK protein and with a 3.2× volume of NP (NP-anti IgG-LA CK) at 37°C. For BCR cross-linking activation, cells were activated with 10 μg/ml F(ab')$_2$ goat anti-mouse IgG premixed to 20 μg/ml donkey anti-goat IgG for 20 min at 37°C.

Antigen Internalization Assays

WT, ΔSyk ^{K395R}Syk, or ^{WT}Syk cells (5×10^5) were washed once with PBS and then resuspended in internalization buffer (RPMI 1640, 5% FCS, 10 mM glutamine, 5 mM sodium pyruvate, 50 mM 2-ME, and 10 mM HEPES, pH 7.4) at a density of 10^6 cells/ml. Cells were incubated with 10 μg/ml F(ab')$_2$ goat anti-mouse IgG premixed to 20 μg/ml donkey anti-goat IgG for 30 min at 4°C. Cells were washed twice with cold internalization buffer to remove the excess ligand and incubated at 37°C for 0–60 min. Internalization was stopped by incubating the cells on ice and adding cold PBS plus 3% bovine serum albumin (BSA). To detect receptors remaining on the cell surface, cells were stained on ice with Cy5-anti-goat IgG, washed twice with PBS plus 3% BSA. Flow cytometry was performed on a FACScan, and the data were analyzed with Flojo software (BD Biosciences). The percent of BCR on the cell surface was calculated as follows: (MFI at 37°C)/(MFI at 4°C) × 100.

Immunofluorescence

WT cells (2×10^5) and ΔSyk, ^{K395R}Syk, and ^{WT}Syk cells were activated or not, washed, resuspended in PBS, and plated on poly-L-lysine–coated glass coverslips (12 mm) for 15 min at room temperature (RT). Cells were fixed in 4% paraformaldehyde for 20 min at RT and incubated in PBS plus 1 mM glycine twice for 10 min. Fixed cells were incubated with antibodies in PBS plus 0.2% BSA plus 0.05% saponin for 60 min (primary Abs) and 45 min (secondary Abs). After washing, coverslips were mounted on glass slides using fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). For experiment using NP, cells were plated on poly-L-lysine–coated glass coverslips (12 mm) for 15 min at RT before activation. Cells were washed with RPMI and incubated with 100 μl of LACK-anti-IgG-NP for different time points at 37°C. Cells were then fixed and stained as indicated above. Immunofluorescence images were acquired on a confocal microscope (LSM Axiovert 720, Carl Zeiss MicroImaging, Thornwood, NY) with a 63× 1.4 NA oil immersion objective. Quantifications were performed on acquired confocal images, by counting 100–300 cells per experiment and making an average of 2–3 experiments (as indicated in figure legends). Quantifications were obtained either manually or when specified, by using the MetaMorph program (Universal Imaging, West Chester, PA).

Time-Lapse Analysis

For videomicroscopy, WT or ΔSyk B-cells were transiently transfected with an actin-RFP construct by electroporation with nucleofactor R T16 (Amaxa,

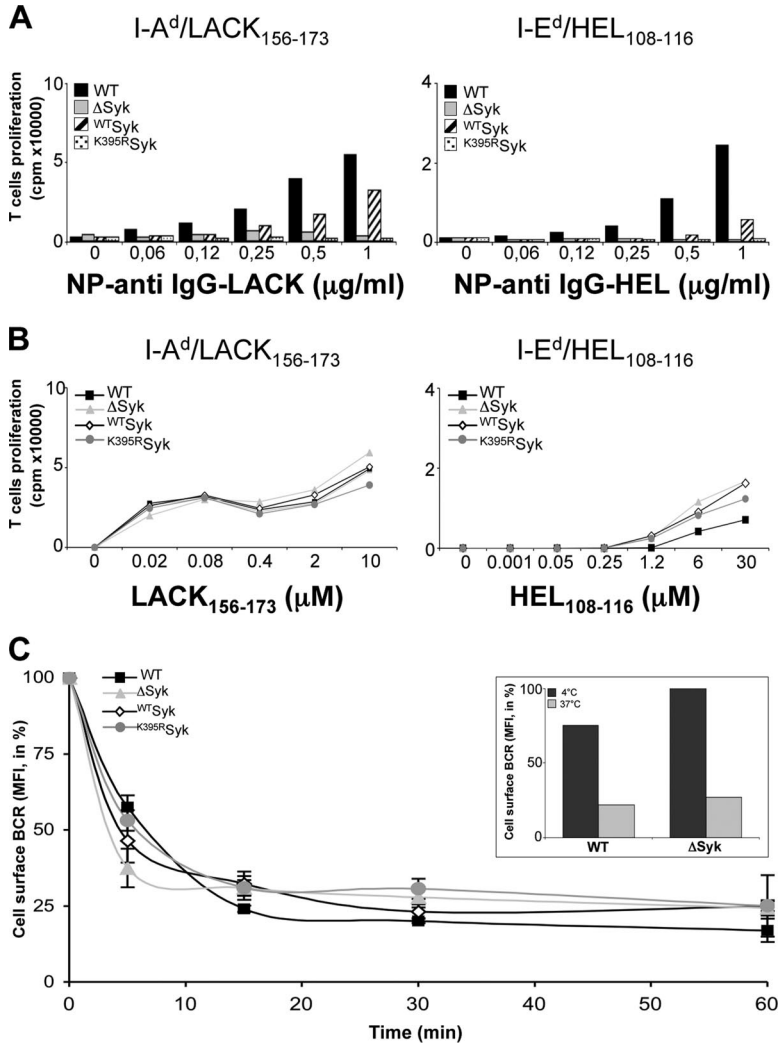

Figure 1. Syk is required for BCR-driven Ag processing and presentation but not for BCR internalization. Ag presentation assays using wild-type (WT), Syk-deficient (ΔSyk), and Syk-deficient cells reconstituted with wild-type Syk (^{WT}Syk) or a kinase-dead Syk mutant (^{K395R}Syk). (A) Syk-sufficient and -deficient cells were incubated with variable amounts of NP-anti-IgG-Ag for 18–20 h, washed, and further cultured with the corresponding T-cell hybridoma during 24 h. T-cell activation was measured as described in *Materials and Methods*. The results show that the activity of Syk is needed for presentation to CD4 T-cells of both LACK and HEL Ag, when internalized through the BCR. (B) Ag presentation assays were performed as in A but using increasing amounts of peptide instead of NP-anti-IgG-Ag. Syk-sufficient and -deficient cells are equally able to activate T-cells under such conditions. (C) BCR internalization kinetics are not altered in Syk-deficient cells. Cells were incubated with polyvalent BCR ligands (see *Material and Methods*) for 30 min at 4°C and chased at 37°C for various time points. To detect the BCR remaining on the cell surface, cells were stained on ice with an anti-goat Cy5-conjugated antibody and analyzed by flow cytometry. BCR-internalized Ag in cells lacking Syk activity is as efficient as in their WT counterparts.

Gaithersburg, MD). Twenty-four hours later cells were attached on poly-L-lysine–coated slides and incubated in a Ludin chamber at 37°C for time-lapse analysis. Images were acquired before or immediately after adding activating ligands, every 40 s during 35 min on a confocal microscope (LSM Axiovert 720; Carl Zeiss MicroImaging) with a 63× 1.4 NA oil immersion objective (Carl Zeiss MicroImaging). Images were deconvolved with MetaMorph (Universal Imaging). Films were reconstructed using MetaMorph 6.2 software.

Immunogold Cryo-Electronmicroscopy

Activated WT and ΔSyk cells, 5×10^6, were fixed in 2% PFA and processed as previously described (Vascotto *et al.*, 2007a).

Immunoprecipitations

WT and ΔSyk cells were stimulated for different time periods (3×10^6 per condition) with NP-anti IgG-LACK as described above, washed, and lysed in NP40 buffer (Tris 20 mM, NaCl 140 mM, NP40 0.5%, EDTA 2 mM, and proteases cocktail inhibitors from Roche, Indianapolis, IN). Lysates were precleared and I-A^d/LACK$_{156-173}$ complexes were immunoprecipitated with protein G-agarose coupled to 5 μg of purified 2C44 mAb. Samples were washed, resuspended in reducing Laemmli sample buffer, boiled, and loaded onto a 12% SDS-PAGE gel (Invitrogen, Carlsbad, CA). Proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA), the membrane incubated with anti-MHCII Abs (JV2 and anti-anti-I-A^b I-A$_\beta$ as described above) and revealed using enhanced chemiluminescence (GE Healthcare Amersham, Piscataway, NJ).

RESULTS

Syk Activity Is Required for BCR-driven Ag Presentation

To evaluate the role of the Syk kinase in BCR-driven Ag presentation, we took advantage of a recently described Syk-deficient (ΔSyk) mouse B lymphoma cell line (Yokozeki *et al.*, 2003). Hen egg lysozyme (HEL) and *Leishmania major* LACK Ag were targeted to BCR uptake by coupling them to nanoparticles (NP) together with an anti-BCR F(ab′)$_2$ (LACK-anti IgG-NP; Vascotto *et al.*, 2007a). LACK-NP were incubated with Syk-sufficient and -deficient cells, and their Ag presentation capacity was assessed using HEL- and LACK-reactive T-cell hybridomas. Under such conditions, no T-cell stimulation was observed with NP coupled to HEL/LACK or to anti-BCR F(ab′)$_2$ alone (Supplementary Figure S1). Syk deficiency severely compromised the presentation of both HEL and LACK Ag (Figure 1A). Importantly, no impact of Syk deficiency on peptide presentation was found (Figure 1B), suggesting that the observed defect in Ag presentation resulted from impaired Ag processing. To verify that defective presentation of BCR uptaken Ag was indeed due to the lack of Syk activity, we assessed the Ag presentation capacity of ΔSyk cells reconstituted with WT (^{WT}Syk) or a kinase-dead mutant form of the enzyme (^{K395R}Syk; Yokozeki *et al.*, 2003). Although expression of WT Syk restored the Ag presentation capacity of Syk-deficient cells, no complementation was observed when expressing the kinase-dead form of the enzyme (Figure 1, A and B). We therefore conclude that the kinase activity of Syk is required for presentation of BCR-capture Ag to CD4 T-cells.

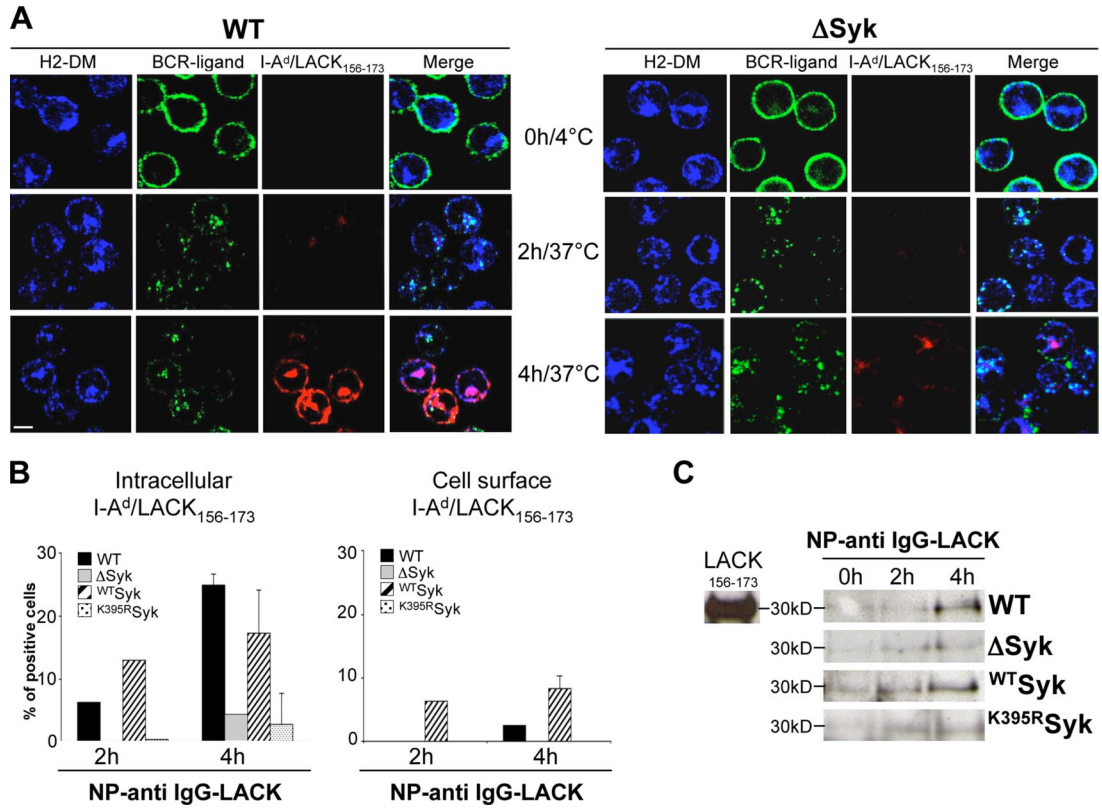

Figure 2. Syk controls the formation of MHC II-peptide complexes from BCR-internalized Ag. (A) Confocal images of WT and ΔSyk cells incubated with NP-anti-IgG-LACK for different time points, fixed ,and stained for H2-DM, BCR-Ligand, and I-A^d/$LACK_{156-173}$ complexes, as described in *Materials and Methods.* Bar, 5 μm. (B) The percentage of cells showing I-A^d/$LACK_{156-173}$complexes in H2-DM compartments or at the cell surface was quantified by counting cells on images obtained from three independent experiments (250–300 cells per condition). Fewer I-A^d/$LACK_{156-173}$ complexes form in the absence of Syk activity. (C) Extracts from cells incubated or not with $LACK_{156-173}$ peptide or NP-anti-IgG-LACK complexes for different time periods were immunoprecipitated with the 2C44 mAb and analyzed by immunoblotting using anti-MHC II β-chain rabbit Abs, as described in Materials and Methods. Syk-deficient cells display reduced 2C44-reactive material confirming the requirement of the kinase for efficient formation of I-A^d/$LACK_{156-173}$ complexes from BCR uptaken LACK Ag.

Syk Is Not Required for BCR-Ag Internalization But Is Essential for Formation of MHC II-Peptide Complexes in Lysosomes

The failure of Syk-deficient cells to efficiently process and present BCR-internalized Ag could result from impaired 1) Ag uptake, 2) formation of MHC II-peptide complexes, 3) association of such complexes to costimulatory molecules, and/or 4) transport to the cell surface. BCR-Ag internalization kinetics measured by cytofluorometry was not reduced in cells lacking Syk activity (Figure 1C), in agreement with a recent publication (Caballero *et al.*, 2006). Hence, defective Ag processing in Syk-deficient cells does not result from impaired BCR-Ag internalization.

To evaluate the capacity of Syk-deficient cells to form and transport MHC II-peptide complexes, we took advantage of the 2C44 "restricted" mAb, which can successfully be used for intracellular staining (Vascotto *et al.*, 2007a). This mAb is specific for complexes composed of I-A^d MHC II molecules loaded with the $LACK_{156-173}$ peptide, but does not recognize any of its free components. As shown above, this epitope of LACK is strictly dependent on Syk for processing and presentation (Figure 1A). I-A^d/$LACK_{156-173}$ complexes became detectable in clusters of H2-DM+ compartments 2 h upon Ag internalization and increased up to 4 h (Figure 2, A and B, for quantifications). The same observation was made when staining for LAMP-1 instead of H2-DM (not shown), indicating that the compartments where I-A^d/$LACK_{156-173}$ complexes formed are of lysosomal origin. Accordingly, H2-DM staining was found to perfectly match LAMP-1 staining in Syk-sufficient and -deficient cells (Supplementary Figure S2). Strikingly, I-A^d/$LACK_{156-173}$ complex formation was dramatically affected in ΔSyk cells, I-A^d/$LACK_{156-173}$ complexes being barely detected 2 h after LACK-NP uptake (Figure 2, A and B). The percentage of lysosomes that stained positive for 2C44 after 4 h was still ~80% reduced in ΔSyk cells (Figure 2, A and B). Intracellular 2C44 labeling intensity was also decreased in ΔSyk cells, suggesting that less I-A^d/$LACK_{156-173}$ complexes per cell were generated in the absence of the kinase. In addition, although some of these complexes were found at the surface of WT cells at 4 h, they remained in lysosomes in the Syk mutant (Figure 2, A and B). Equivalent results were obtained when comparing ΔSyk cells reconstituted with WT or the kinase-dead mutant form of Syk (Figure 2, A and B). These results were further strengthened by using the 2C44 mAb to immunoprecipitate I-A^d/$LACK_{156-173}$ complexes and analyzing the amount of MHC II molecules in the precipitated material: cells lacking Syk activity displayed a sizeable decrease in the total amount of I-A^d/$LACK_{156-173}$ complexes at 4 h upon LACK-NP internalization, compared with Syk-sufficient cells (Figure 2C). We therefore conclude that the kinase activity of Syk is required for efficient formation of MHC II-peptide complexes from BCR uptaken Ag.

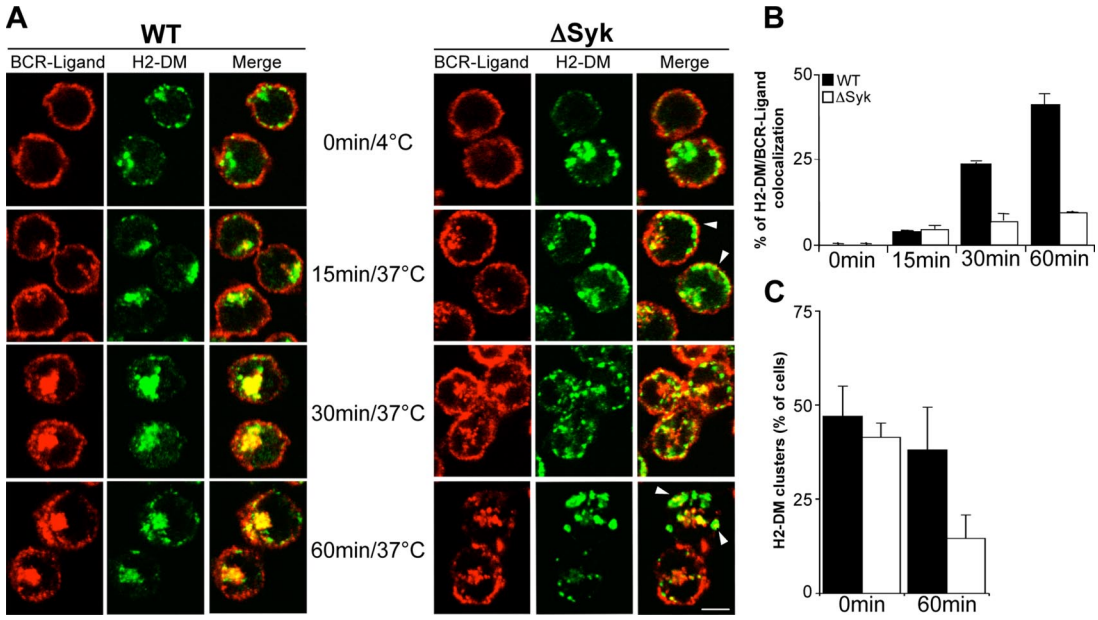

Figure 3. Syk is required for clustering and convergence of H2-DM-conaining lysosomes together with Ag-carrying vesicles. (A) Confocal images of WT and ΔSyk cells activated with BCR polyvalent ligands for different time periods, fixed, and stained for the indicated markers. H2-DM–containing lysosomes do not cluster but rather disperse after BCR stimulation of ΔSyk cells, aberrant H2-DM+ patches accumulating beneath the plasma membrane at 60 min upon BCR engagement (see white arrows; bar, 5 μm). (B) Quantification of colocalization between H2-DM+ or LAMP1+ and BCR-internalized Ag obtained from images of the experiment described in A, using the Metamorph colocalization program (~100 cells per condition, two independent experiments). (C) Quantification of intracellular H2-DM+ or LAMP1+ clusters located at the center of WT and ΔSyk cells. 3-D confocal images from nonactivated or 60 min BCR-stimulated cells were acquired, and the cells harboring H2-DM+ or LAMP1+ central lysosomal clusters were counted. Lysosomal clustering is compromised in Syk-deficient cells (~100 cells per condition, two independent experiments).

Altered Endocytic Trafficking in Syk-deficient Cells

So far, we have shown that Syk regulates both formation and transport to the cell surface of MHC II-peptide complexes from BCR-internalized Ag but has no impact on BCR-Ag internalization. ΔSyk cells may thus be altered in the events of vesicular trafficking that are required for proper processing of such Ag. To address this question, we analyzed the early trafficking events of BCR-Ag complexes in WT and Syk-deficient cells. Confocal images showed that, in WT cells, BCR-Ag complexes started to accumulate in H2-DM+ lysosome clusters located toward the center of the cell, as soon as 15 min after Ag uptake (Figure 3A). Colocalization analysis between the Ag and H2-DM showed a considerable increase up to 60 min after Ag internalization (Figure 3, A and B). A drastically different picture was observed in Syk-deficient cells: H2-DM+ lysosomes did not efficiently cluster toward the cell center upon BCR stimulation but instead dispersed at the cell periphery, where they started to make aberrant patches beneath the plasma membrane (Figure 3, A and C, for quantifications). Importantly, dispersion of H2-DM+ lysosomes in ΔSyk cells resulted in a failure of Ag-carrying vesicles to reach these compartments (Figure 3, A and B, for colocalization quantification). The same observations could be made when using LAMP-1 instead of H2-DM staining, as well as when comparing the ^{WT}Syk and ^{K395R}Syk transfectants (not shown). These results suggest that deficient Ag processing in the absence of Syk activity is likely to result from impaired convergence of H2-DM+/LAMP-1+ lysosomes toward incoming BCR-internalized Ag.

Immunogold labeling on ultrathin cryosections was next performed in order to analyze the convergence between BCR-uptaken Ag and MHC II–containing vesicles. Indeed, the extremely high level of MHC II surface expression in A20-derived B lymphoma cells hampers the study of intracellular MHCII distribution by immunofluorescence. WT BCR-stimulated cells showed the typical intracellular network of tubular and multivesicular compartments wherein BCR-Ag complexes and MHC II molecules accumulate (Figure 4; Lankar *et al.*, 2002; Vascotto *et al.*, 2007a). Relative quantification of gold labeling indicated that 92.0 and 89.5% of labeled Ag and MHC II molecules were found together in these cells, respectively (Table 1). In contrast to WT cells, the amounts of Ag and MHC II molecules that accumulated together in Syk-deficient cells were considerably reduced. Indeed, only 42.3% of Ag and 45.3% of MHC II molecules colocalized in ΔSyk cells (Figure 4 and Table 1). As expected from the immunofluorescence results described in Figure 3, electron microscopy analysis showed that colocalization of Ag and LAMP-1 molecules was also considerably reduced in Syk-deficient cells (see Table 1). We therefore conclude that the Syk tyrosine kinase is required for proper convergence and concentration of BCR-uptaken Ag together with MHC II molecules in H2-DM+/LAMP-1+ lysosomes.

Altered Organization of the Actin Cytoskeleton in BCR-stimulated Cells Lacking Syk Activity

Syk is known to regulate actin dynamics downstream of integrins in various cell types such as platelets, neutrophils, and osteoclasts (Obergfell *et al.*, 2002; Mocsai *et al.*, 2006; Zou *et al.*, 2007). In addition, the actin cytoskeleton was shown to be required for proper convergence of BCR-internalized Ag with MHC II+/H2-DM+-containing vesicles, as well as for clustering of H2-DM+/LAMP-1+ lysosomes toward the center of BCR-activated cells (Barois *et al.*, 1998; Brown and Song, 2001; Vascotto *et al.*, 2007a). We therefore hypothe-

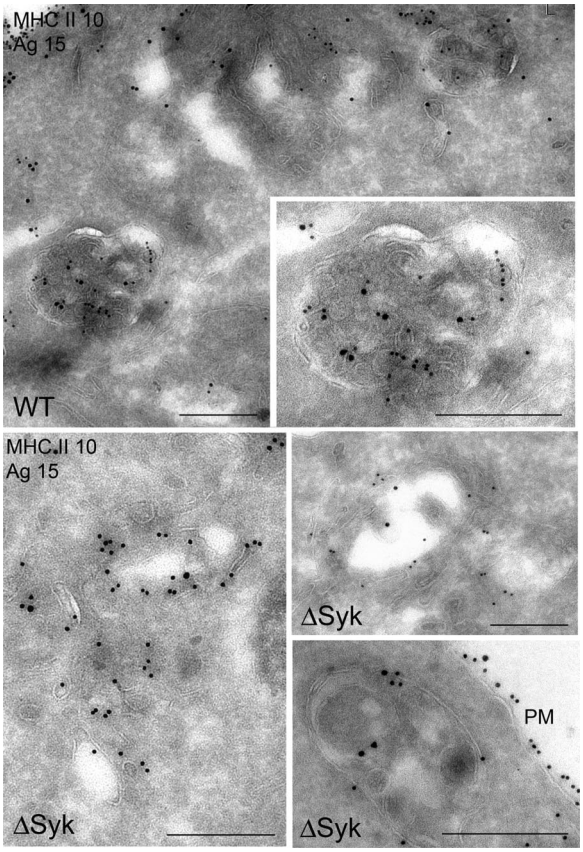

Figure 4. Immunogold labeling of ultrathin cryosections analyzed by electron microscopy. Sixty-minute–activated WT and ΔSyk B-cells were labeled for MHC II (10-nm gold particles) and Ag (15-nm gold particles). Activated WT cells display a network formed by tubular and vesicular lysosomes wherein Ag and MHC II molecules concentrate together. Such compartments are not observed in ΔSyk cells, which preferentially display smaller and sometimes vacuolar lysosomes that fail to efficiently accumulate BCR-uptaken Ag and MHC II molecules together (see Table 1 for quantifications). Bar, 200 nm.

Table 1. Quantification of the immunogold cryo-electronmicroscopy experiment

	WT	ΔSyk
BCR-ligand		
BCR-ligand alone	152	1160
BCR-ligand in MHC II compartments	1754	851
Total BCR-ligand	1906	2011
% of BCR-ligand in MHC II compartments	92	42.3
MHC II		
MHC II alone	523	2730
MHC II in BCR-ligand compartments	4477	2269
Total MHC II	5000	4999
% of MHC II in BCR-ligand compartments	89.5	45.3
BCR-ligand		
BCR-ligand alone	91	194
BCR-ligand in LAMP1 compartments	424	191
Total BCR-ligand	515	385
% of BCR-ligand in LAMP1 compartments	82.3	49,6
LAMP1		
LAMP1 alone	145	600
LAMP1 in BCR-ligand compartments	1105	191
Total LAMP1	1245	791
% of LAMP1 in BCR-ligand compartments	88.7	24.1

Quantification of the immunogold cryo-electronmicroscopy experiment shown in Figure 4. Gold particles corresponding to Ag, MHC II, and LAMP-1 staining observed in single- or double-labeled compartments were counted from ~30 randomly selected cell profiles. The lack of Syk activity reduces the amount of Ag and MHC II as well as Ag and LAMP-1 co-localization.

sized that the defect in endocytic trafficking observed in Syk-deficient cells may result from impaired remodeling of the actin cytoskeleton upon BCR activation. To test this hypothesis, both resting and BCR-stimulated WT and ΔSyk cells were stained with anti-H2-DM Abs together with fluorescent phalloidin, which binds to polymerized actin. In agreement with previous studies showing that activated B lymphocytes display higher levels of polymerized actin (Brown and Song, 2001; Hao and August, 2005), we observed increased phalloidin staining in stimulated WT B-cells (Figure 5A). At the 60-min time point, a patch of actin filaments was found in close association with the central lysosomal cluster in which the Ag and H2-DM colocalize (Figure 5, A and B). In addition, 30-min BCR-stimulated cells often exhibited a polarized actin tail that was more important in size than the actin tail sometimes detected on resting cells (Figure 5A, white arrows). This observation is in agreement with results showing that BCR stimulation triggers a fast actin depolymerization wave that is followed by an event of polarized actin polymerization (Hao and August, 2005).

Cells lacking Syk exhibited a highly disorganized actin network compared with WT cells: 1) the central actin patch was often not detected in 60-min–stimulated ΔSyk cells, and 2) they contained multiple actin protrusions that were distributed in a nonpolarized manner, all around the cell cortex (Figure 5, A and B). Equivalent observations were made in ΔSyk cells expressing the ^{K395R}Syk kinase dead form of the enzyme (not shown). To verify that such conclusion equally applied to primary B lymphocytes, we took advantage of the Syk inhibitor, piceatannol (Geahlen and McLaughlin, 1989). The specificity of the drug was verified by showing that WT B lymphoma cells treated with this drug exhibited a very similar phenotype to the one of Syk-deficient cells: they neither clustered H2-DM+ lysosomes nor polarized their actin cortex upon BCR stimulation (Figure 6A). Piceatannol treatment had a dramatic effect on freshly purified mouse spleen B-cells: although resting cells did not undergo any change after drug treatment, BCR-stimulated cells showed a spectacular disorganization of their actin network (Figure 6B). Indeed, as observed in the Syk mutant, piceatannol-treated cells exhibited actin protrusions that distributed in a nonpolarized manner all around the cell cortex (Figure 6B). In addition, the distribution of Ag-carrying vesicles and lysosomes was strongly altered in piceatannol-treated primary B-cells, which were dispersed at the periphery of the cells rather than clustered together at the cell center (Figure 6B). Hence, as observed in B lymphoma cells, the tyrosine kinase Syk regulates the actin dynamics and positioning of endocytic vesicles in BCR-stimulated primary lymphocytes.

BCR-induced Actin Dynamics Are Impaired in Syk-deficient Cells

These results were further strengthened by performing a comparative time-lapse analysis of actin dynamics in Syk-sufficient and -deficient cells. For this, we transiently expressed an actin-RFP construct in both cell types and imaged the cells on a confocal microscope before and immediately after BCR engagement. No major change in organization of

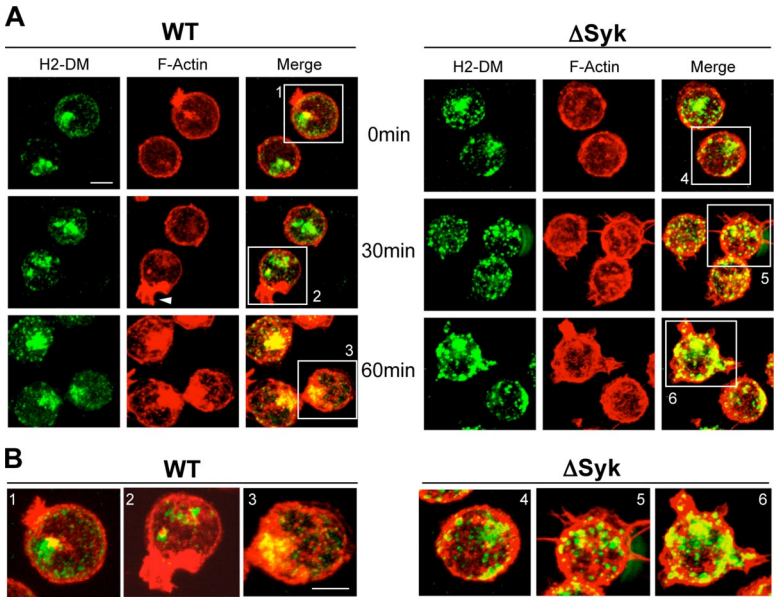

Figure 5. Altered organization of the actin cytoskeleton in BCR-stimulated cells lacking Syk activity. (A and B) 3-D reconstitution of confocal images of WT and ΔSyk cells activated or not by BCR cross-linking and stained for the indicated markers (bar, 5 μm). Syk-deficient cells display a disorganized actin cortex upon BCR stimulation.

the actin cortex was observed in the absence of BCR ligand (not shown). In contrast, BCR-stimulated WT cells showed a rapid polarization of their actin cortex which lasted for ~30 min (Figure 7 and Supplementary Movie S1). In particular, most of actin protrusions were found to concentrate in one pole of the cell, from which they extended toward the extracellular space, resulting in the polarized actin tail observed in immunofluorescence experiments (Figures 5A and 7, Supplementary Movie S1). In contrast, Syk-deficient cells presented minor changes in the actin cytoskeleton rearrangements induced after BCR engagement. Actin-RFP displayed a more homogeneous distribution in Syk-deficient cells compared with WT cells, and this remained during the entire time of image acquisition. Furthermore, the actin cortex of activated Syk-deficient cells did not polarized and actin protrusions extended from all around their cell body (Figure 7, Supplementary Movie S2). Hence, BCR-triggered actin dynamics are altered in the absence of the Syk tyrosine kinase. Together these results suggest that impaired trafficking of BCR-uptaken Ag into MHC II– and H2-DM–containing lysosomes associates to a failure of Syk mutant cells to properly reorganize their actin network upon Ag stimulation.

Inhibition of BCR-induced Actin Polymerization Modifies Endocytic Trafficking

As mentioned above, BCR triggering was found to induce a fast actin depolymerization/repolymerization two-phase response. We therefore analyzed which of these two actin-remodeling events were altered in Syk-deficient cells. The initial phase of actin depolymerization was reported on

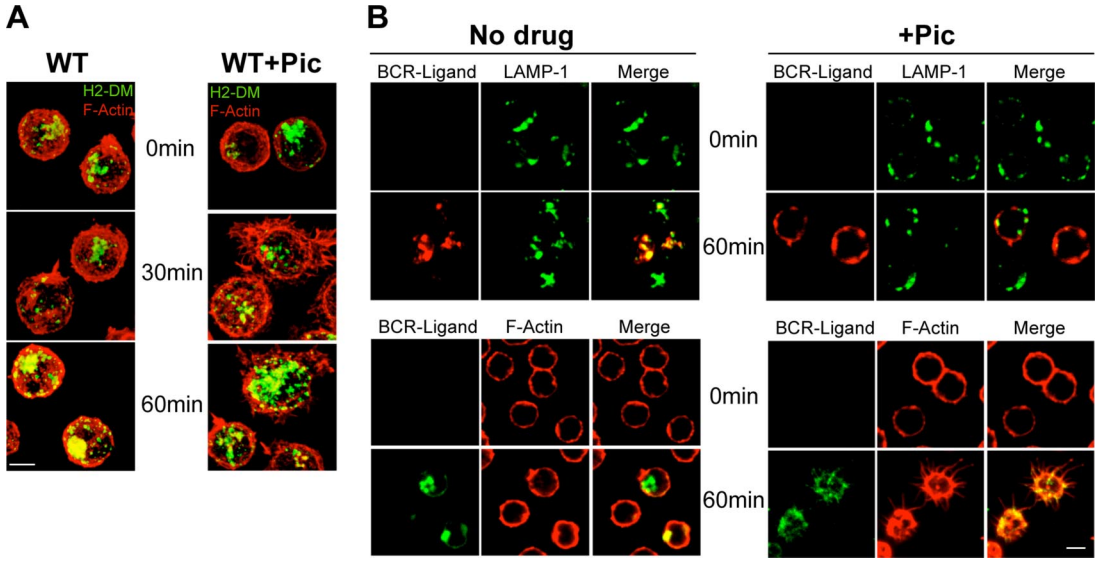

Figure 6. Altered organization of the actin cytoskeleton in BCR-stimulated primary mouse spleen B-cells that lack Syk activity. WT (A) and freshly purified spleen B-cells from I-A_β-GFP knockin mice (B) were treated with the Syk inhibitor piceatannol (10 μM) for 45 min, stimulated with BCR polyvalent ligands for 60 min, fixed, and stained. The actin cortex is highly disorganized in activated Syk-inhibited cells (WT or primary spleen B-cells) and lysosomal vesicles are peripherally distributed rather than clustered at the center of the cells.

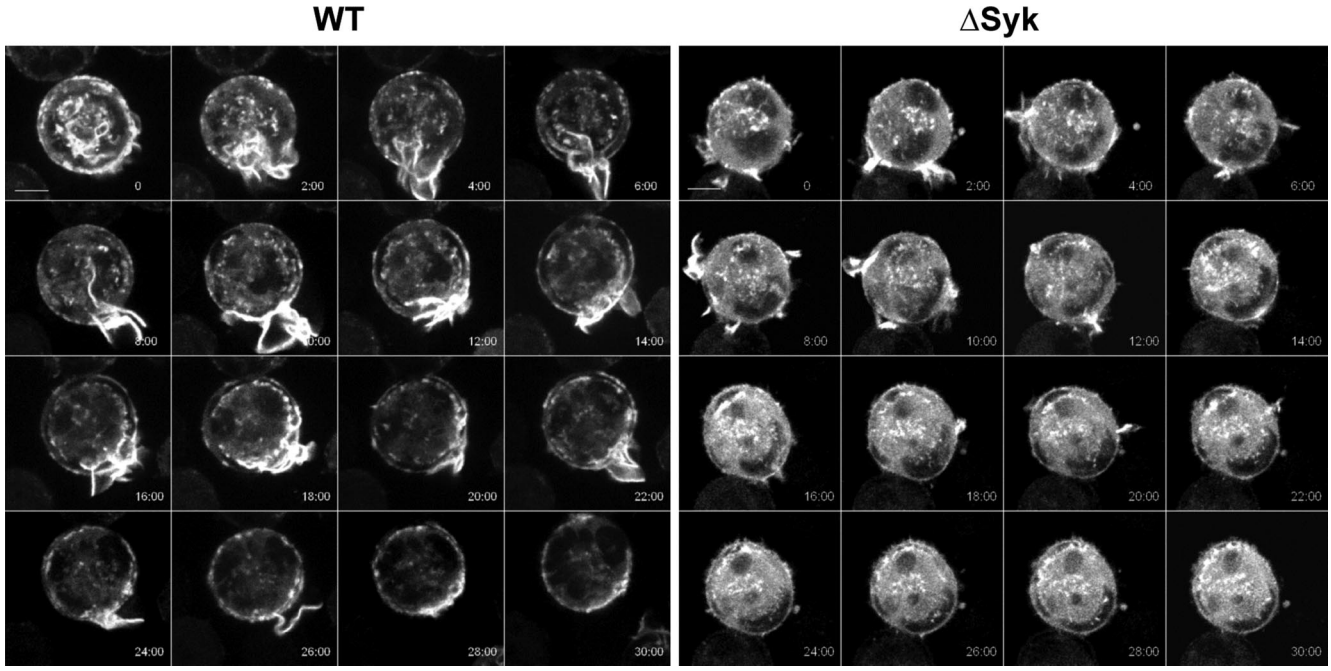

Figure 7. Actin dynamics during BCR-mediated B-cell activation. Confocal images of WT and ΔSyk cells expressing RFP-actin were acquired immediately after BCR cross-linking every 40 s during 35 min, on a confocal microscope (LSM Axiovert 720; Carl Zeiss MicroImaging) with a 63× 1.4 NA oil immersion objective. WT cells show polarization of their actin cortex, actin filaments being concentrated at and extending from one cell pole. In contrast, Syk-deficient cells present a nonpolarized and homogeneously distributed actin network.

individual cells by immunofluorescence (Hao and August, 2005). Indeed, it occurs transiently and as fast as 5 s upon BCR engagement, making it unlikely to take place in a synchronized manner within the cell population. For this reason, although we did observe individual cells showing a depolymerized actin cortex 15 s upon BCR engagement, we could not quantify these observations. However, we made an important set of qualitative observations describing changes in the actin cytoskeleton induced by BCR engagement. In particular, we noticed that a considerable proportion of both WT and Syk-deficient cells were spread out 15 s after BCR engagement (Figure 8A). While most spread WT cells exhibited a lamellipodium surrounding their cell body, the actin network of spread ΔSyk cells rather showed a "spiky" morphology (Figure 8, A and B, for quantifications). This difference became even more apparent at later time points: 1 min upon BCR stimulation WT cells were back to their contracted shape and showed only one or two condensed lamellipodia-like structures emerging from their cell body (Figure 8, A and B, red arrows). Strikingly, Syk-deficient cells failed to contract back after spreading and displayed numerous filopodia-like actin structures all around their cell body (Figure 8, A and B), a picture that was reminiscent of the results obtained in time-lapse experiments (Figure 7 and Supplementary Movie S2). Analyzing the levels of F-actin by cytofluorometry further strengthened

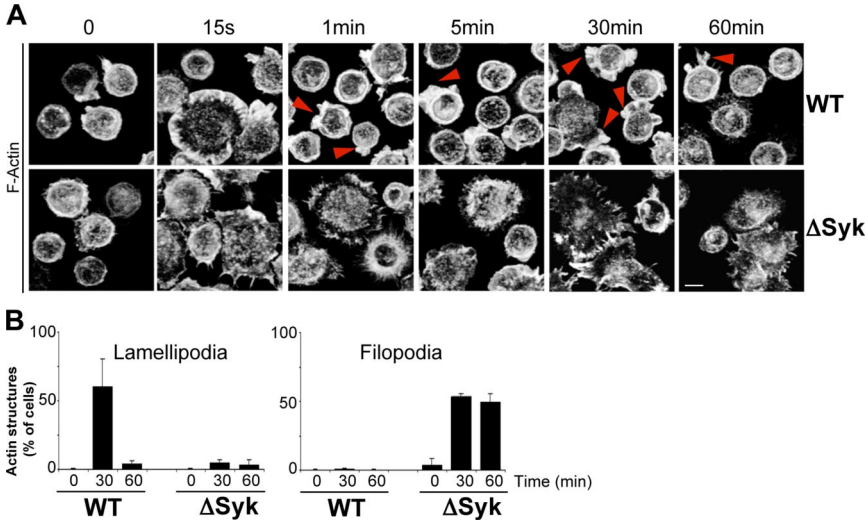

Figure 8. Inhibition of BCR-triggered actin dynamics alters endocytic trafficking. (A) WT and ΔSyk cells were activated by BCR cross-linking for different time periods, immediately fixed, stained with phalloidin-FluoroProbe546 to detect actin filaments, and analyzed by confocal microscopy. 3-D reconstructions are shown. Bar, 5 μm. WT cells undergo spreading a few seconds after BCR engagement and then recover back their contracted shape. The latter event does not occur in Syk-deficient cells, which remain spread, with filopodia surrounding their cell body. (B) Quantification of the percentage of cells showing lamellipodium- and filopodium-like actin structures before and 30 and 60 min after BCR engagement. WT or Syk-deficient cells was counted from confocal 3-D reconstituted images obtained in two independent experiments (150–220 cells per experiment). Although WT cells preferentially exhibit lamellipodia around their cell body, Syk-deficient cells rather show filopodia-like actin extensions.

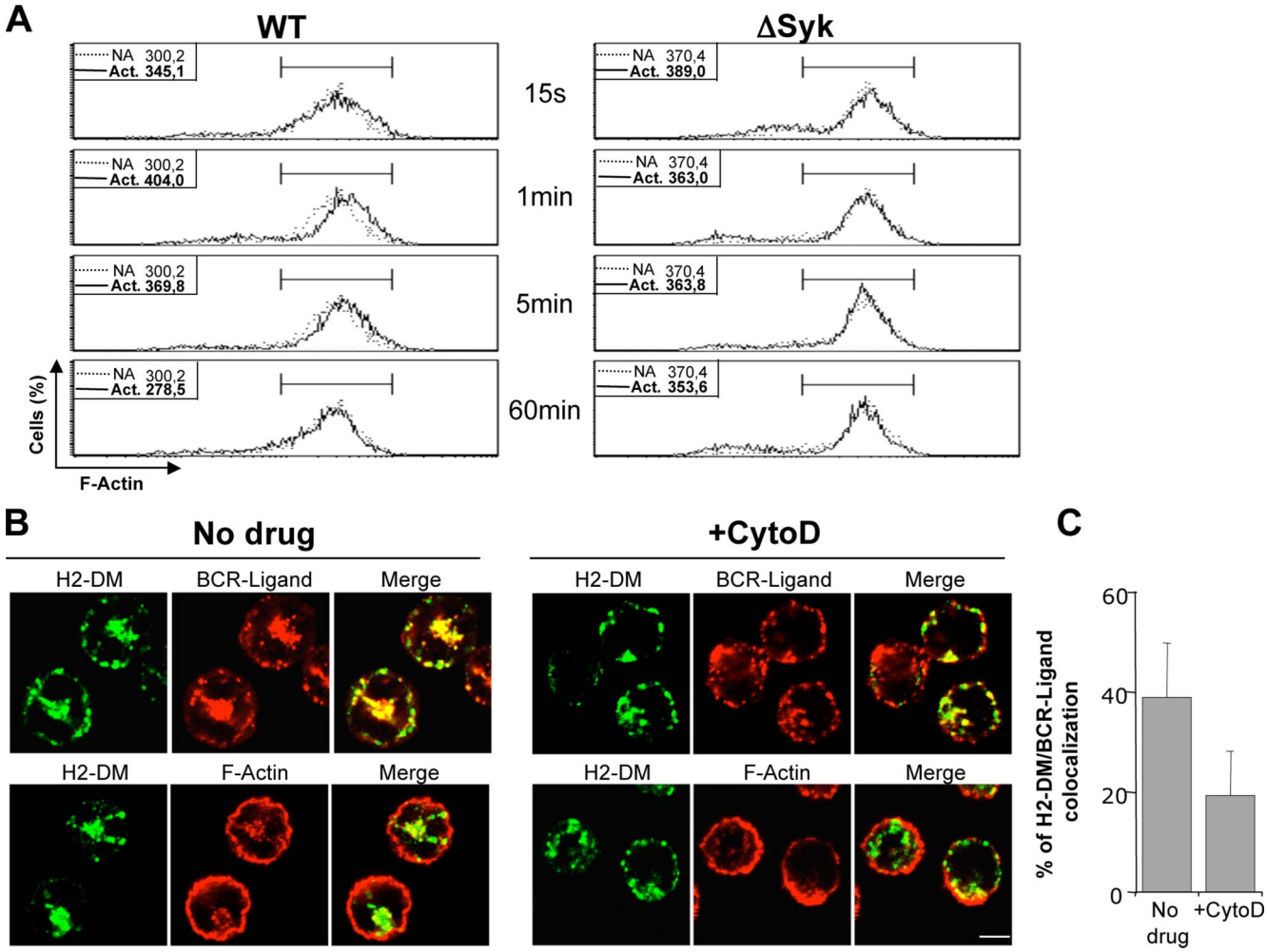

Figure 9. Inhibition of BCR-triggered actin dynamics alters endocytic trafficking. (A) WT and ΔSyk cells were activated by BCR cross-linking for different time periods, fixed, stained with phalloidin-FluoroProbe546 to detect actin filaments and analyzed by flow cytometry. BCR engagement stimulates actin polymerization in WT, but not in Syk-deficient cells, as shown by a transient and fast increase in the mean of fluorescence measured (NA, nonactivated cells, Act, BCR-activated cells). (B) Confocal images of WT cells activated by BCR cross-linking and immediately treated for 15 min with cytochalasin D (10 μg/ml). Cells were fixed and stained for the indicated markers. Bar, 5 μm. (C) Quantification of colocalization between H2-DM and BCR-internalized Ag from images obtained in two independent experiments as the one described in B. The Metamorph colocalization program was used (~100 cells in total, two independent experiments). Clustering of H2-DM lysosomes and convergence with Ag-carrying vesicles is impaired when preventing BCR-stimulated actin polymerization.

these results: although WT cells show a modest, but reproducible, fast and transient increase in the levels of F-actin upon BCR engagement, such an increase was not appreciated in Syk-deficient cells (Figure 9A). We conclude that Syk is required to repolymerize and reorganize the actin cortex in response to BCR engagement.

Having shown that Syk-deficient cells fail to polymerize actin upon BCR stimulation, we next aimed to assess whether altered endocytic trafficking in the absence of Syk does indeed result from this defect. For this, we used cytochalasin D, a drug that prevents actin polymerization. In order not to interfere with the early wave of actin depolymerization, we first stimulated the cells and then rapidly added the drug into the medium. Cells were fixed 15 min later and stained for H2-DM together with the Ag or F-actin. Clustering of H2-DM+ lysosomes toward the cell center was impaired in cytochalasin D–treated cells (Figure 9B). In addition, colocalization of the Ag with H2-DM was significantly reduced when actin polymerization was compromised (Figure 9, A and C, for quantification). Therefore, we conclude that the defect in Ag processing of Syk-deficient cells is likely to result from their inability to properly polymerize and reorganize their actin network upon BCR engagement, an event that is required to ensure proper convergence of BCR-Ag complexes with H2-DM–carrying lysosomes.

DISCUSSION

Cooperation between T and B lymphocytes is essential for production of high-affinity antibodies and generation of B-cell memory responses. It relies on the ability of B-cells to recognize an Ag through their specific BCR and present it to $CD4^+$ T-cells in the context of MHC II molecules (Mitchison, 2004; Bernard *et al.*, 2005). Here we show that Syk, a tyrosine kinase activated downstream of the BCR, regulates Ag processing and presentation. By using a mAb restricted to a specific MHC II-peptide complex, we demonstrate that Syk is required for efficient formation of MHC II-peptide complexes as well as for their subsequent transport to the cell

surface. Equivalent results were obtained when analyzing Syk mutant cells reconstituted with the kinase-dead form of the enzyme, indicating that Syk kinase activity is essential for Ag processing in B lymphocytes. The Ag processing defect of Syk-deficient cells is not due to impaired internalization, but most likely results from their failure to ensure convergence between the vesicles that carry the internalized Ag with the ones that transport the molecules required for its processing, in particular MHC II and H2-DM. This endocytic trafficking defect associates to a failure of Syk-deficient cells to properly remodel their actin cytoskeleton in response to BCR engagement. This suggests that actin filaments are essential for the transport and positioning of vesicles whose cargo is required for Ag processing and presentation.

It was recently shown that Ag binding to the BCR induces a fast and transient wave of actin depolymerization followed by an event of polarized actin repolymerization (Hao and August, 2005). This is believed to allow the coalescence of lipid-rafts in order to sustain BCR signaling. Both our immunofluorescence and time-lapse experiments showed that Syk-deficient cells display a nonpolarized actin cortex as compared with their WT counterpart, suggesting that Syk might control BCR-triggered polarization of the actin network. It is therefore tempting to propose that polarization of the actin cytoskeleton induced upon BCR stimulation regulates the trafficking and repositioning of the organelles involved in Ag processing and presentation. This would be an efficient way for B-cells to coordinate various convergent trafficking events. These events include 1) the endocytosis of BCR-Ag complexes and the transport of 2) MHC II-Ii complexes, which is likely to occur from the ER and Golgi, and of 3) lysosome-resident H2-DM molecules. Syk-dependent repolarization of the actin network may allow the association of MHC II– and H2-DM–carrying vesicles with motor proteins that transport organelles from the cell periphery toward the cell center, such as Myosin V or VI actin-based motors. Interestingly, we have recently shown that Myosin II is activated upon BCR engagement, associates to MHC II-Ii complexes, and allow their convergence toward the vesicles carrying BCR-uptaken Ag (Vascotto *et al.*, 2007a). Whether Syk controls Myosin II activation and association to MHC II-Ii molecules shall therefore now be addressed.

Which are the targets of Syk that account for BCR-induced remodeling of the actin cytoskeleton? The hematopoietic actin-related protein kinase Pyk2, which is homologous to focal adhesion kinase (FAK), may be a good candidate for such task. Pyk2 is activated downstream of Src and Syk kinases in both platelets and osteoclasts (Sada *et al.*, 1997; Blair *et al.*, 2005). Pyk2 interacts with gelsolin, an actin-binding, -severing, and -capping protein (Wang *et al.*, 2003). Both Pyk2 and gelsolin are essential for dynamic organization of the actin cytoskeleton in migrating osteoclasts (Chellaiah *et al.*, 2000; Duong *et al.*, 2001). In addition, Pyk2-deficient macrophages exhibit an unpolarized actin cytoskeleton, and similar to cells with a dominant negative form of Syk (Matsusaka *et al.*, 2005), they fail to directionally migrate in response to chemokine stimulation (Okigaki *et al.*, 2003). Interestingly, Pyk2-deficient mice display a defect in their B-cell compartment: marginal zone B-cells (MZB) do not develop in these animals, suggesting that Pyk2 indeed plays a key role in B lymphocyte development and homeostasis (Guinamard *et al.*, 2000). Whether a deregulation in Pyk2/Gelsolin activities in Syk-deficient cells could account for their inability to polymerize actin in response to BCR stimulation should next be investigated.

We observed that BCR engagement triggers a fast membrane spreading event that is followed by cell contraction. This observation is in good agreement with the recent report demonstrating that BCR stimulation induces inactivation of the ERM protein, Ezrin, subsequent detachment of the membrane from the actin cytoskeleton and repolarization of membrane microdomains (Gupta *et al.*, 2006). Syk-deficient cells do efficiently spread, but their membrane displays a filopodium- rather than a lamellipodium-like morphology upon spreading. Such morphology is further exacerbated at later time points upon stimulation, time at which WT cells have contracted back. This could reflect an abnormal balance between the activity of Rho, Rac, and CDC42 small-GTPases in BCR-stimulated Syk-deficient cells. These three GTPases are responsible for actin contraction, lamellipodium, and filopodium formation, respectively, and were all shown to be activated upon BCR engagement (Westerberg *et al.*, 2001; Saci and Carpenter, 2005). CDC42 was further described as being responsible for the formation of membrane spikes (Westerberg *et al.*, 2001). Whether Syk controls the respective activity of Rho, Rac, and CDC42 remains to be investigated.

Interestingly, a similar cell spreading/cell contraction two-phase response was recently shown to be essential for uptake of membrane-associated Ag by B lymphocytes (Fleire *et al.*, 2006), which is probably the most physiological way that B-cells use to acquire Ag in lymphoid organs (Batista and Neuberger, 2000; Carrasco and Batista, 2006). It was proposed that this response allows B-cells to spread over Ag-bearing membranes and then to collect and extract the Ag upon cell contraction (Fleire *et al.*, 2006). Although we show here that Syk-deficient B-cells efficiently internalize soluble BCR ligands, they may be unable to extract membrane-bound Ag because of a failure to reorganize their actin network in response to Ag stimulation. Accordingly, actin-dependent phagocytosis of immune complexes was shown to rely on the activity of Syk in both macrophages and dendritic cells (Crowley *et al.*, 1997; Sedlik *et al.*, 2003). Syk therefore emerges as a key regulator of the interactions between endocytic vesicles and the actin cytoskeleton that are triggered upon Ag recognition and which are required for its processing and presentation.

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