Phosphoinositide 3-Kinase Activation by Igβ Controls de Novo Formation of an Antigen-presenting Compartment*

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Marie Granboulan‡§, Danielle Lankar‡, Graça Raposo¶, Christian Bonnerot‡, and Claire Hivroz‡‡

From the INSERM U520, UMR 144, Institut Curie, 26 rue d'Ulm, 75248 Paris Cedex 05, France

Antigens that bind B cell antigen receptor (BCR) are preferentially and rapidly processed for antigen presentation. The BCR is a multimeric complex containing a signaling module composed of Igα and Igβ. Signaling pathways implicated in antigen presentation through the BCR are ill defined. Here we demonstrate that phosphoinositide 3-kinase (PI3K) inhibitors preclude antigen presentation induced by BCR or Igβ but not Igα. Unraveling the mechanisms responsible for this inhibition, we show that PI3K inhibitors block neither antigen internalization nor degradation. Rather PI3K inhibitors block de novo formation of a multivesicular antigen processing compartment, which is induced by triggering of the BCR or IgG. Strikingly, we found using fluorescent probes binding specifically to PI3K products that BCR and Igβ but not Igα induce PI3K activation in endocytic compartments wherein antigen is transported. Altogether, these results strongly suggest that Igβ couples the BCR to PI3K activation that is instrumental for de novo formation of the antigen processing compartment and efficient antigen presentation.

Binding of antigens (Ag)† to the B cell receptor (BCR) not only triggers B cell activation but also facilitates Ag presentation. BCR-mediated Ag uptake allows presentation at much lower Ag concentrations than fluid phase endocytosis (1). This is because of targeting of BCR-bound Ag to specialized endocytic compartments wherein Ag processing can take place (for review see Ref. 2). BCR-Ag complexes first enter early endosomes and are targeted to late endocytic compartments where they are degraded in peptides (3, 4). These peptides are then loaded onto MHC class II and the complexes are displayed at the surface of Ag presenting cells (2, 5, 6). Peptide loading occurs mostly in late endocytic compartments, called the MHC class II-enriched compartment, an endocytic multivesicular and/or multilaminar structure, which bears markers of late endosome (7–9). We have recently shown in murine B cells that BCR engagement dynamically induces the formation of an Ag-processing compartment presenting with these characteristics (10).

Several studies have shown that BCR-dependent Ag presentation is signaling dependent (11, 12). BCR is a multimeric receptor complex in which an Ag-recognition subunit, membrane-bound Ig, is noncovalently associated with one heterodimer of Igα and Igβ (13), which assures its signaling capacity (14). Accelerated Ag targeting to Ag-processing compartments by the BCR seems to be dependent on Igα/Igβ because mutants of Ig that do not bind to Igα/Igβ were unable to target Ag for MHC class II presentation (15, 16). Moreover we have shown that Igα and Igβ are both able to increase the efficiency of Ag presentation (17) although they differ in their endocytic traffic (18).

Signaling pathways induced after BCR triggering have been widely investigated, however, the distinct contribution of these pathways to BCR-induced Ag presentation are still poorly characterized. One of the pathways activated after BCR activation involves phosphoinositide 3-kinase (PI3K) (19). PI3K activation by the BCR leads to activation of the serine/threonine kinase Akt (20, 21) and regulates membrane association and function of the Bruton's tyrosine kinase (22). Recent studies have shown the key role of PI3K in B cells, disruption of its expression by genetic deletion results in impaired B cell development (23). In other cellular models, PI3K has been shown to control vesicular transport of several proteins in early and late endocytic compartments (24, 25), endosomes fusion (26), and morphogenesis of multivesicular bodies (MVB) (27, 28).

We herein report that PI3K inhibitors block Ag presentation induced by the BCR, Igβ but not Igα and that this blockage is linked to inhibition of de novo formation of a multivesicular Ag processing compartment induced by BCR and Igβ triggering. Furthermore, using fluorescent probes for PI3K products, we demonstrate that PI3K is activated along the Ag endocytic journey and that Igβ is the chain that couples BCR to this PI3K activation. Altogether these data strongly suggest that Igβ-dependent PI3K activation creates the lipidic environment necessary for neogenesis of an endocytic compartment where Ag processing can efficiently take place.

MATERIALS AND METHODS
Chemicals and Antibodies—RPMI 1640 with glutamax, PBS, penicillin, streptomycin, sodium pyruvate, 2-mercaptoethanol, Tris, glycine, and G418 were purchased from Invitrogen; LY 294002 from Calbiochem; and Pronase from Roche Molecular Biochemicals. Other chemicals were from Sigma.

The following Abs were used: 2.4G2 (rat IgG) an anti-mouse FcR mAb; a polyclonal rabbit anti-phospho-Ser-AKT Ab and a polyclonal mouse anti-phospho-mitogen-activated protein kinase Ab (Cell Signal- ing); a polyclonal goat anti-Erk2 (Santa Cruz Biotechnology); a polyclonal rabbit anti-HRP Ab (ICN); 1D4B an anti-mouse Lamp1 mAb,
native and biotinylated rat anti-mouse IL-2 mAb (BD Pharmingen). F(ab')2 fragments of fluorescein isothiocyanate-coupled donkey anti-rat IgG Ab and donkey anti-rabbit (ImmunoTech); a biotinylated rat anti-H2-M (a kind gift from L. Karlsson); a polyclonal rabbit anti-mouse IgG F(ab')2 (ICN) was biotinylated following the manufacturer's protocol (Biotin, Pierce); and streptavidin Alexa 594 was from Molecular Probes.

**Cell Culture**—The following cell lines have been used and previously described (11). IIA1.6, a FcγR-defective variant of the A20 B lymphoma cells has been transfected with constructs encoding the cytoplasmic domain of the Igα or Igβ subunits linked to the extracellular and transmembrane domains of the mouse FcγRII, respectively, FcγR-Igα and FcγR-Igβ, as described in Ref. 29. Anti-TNP A20 cells were obtained by transfection of genomic clones encoding the light and heavy chain of the mouse FcγRII and the 12/26 peptide derived from the α C1 repressor complexed to I-A\(^d\) class II molecules (11). All cell lines were grown in RPMI glutamax, 10% fetal calf serum (Dutchel), 5 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin.

**Internalization and Degradation**—Anti-TNP A20 cells (2 x 10\(^5\) cells/well) were washed in internalization buffer (RPMI, 5% fetal calf serum, 20 mM Hepes, pH 7.4), treated 20 min at 37 °C with wortmannin or Me\(_2\)SO (vehicle), incubated on ice for 1 h with 4 μg/ml iodinated TNP-BSA, washed, and incubated for various time at 37 °C in the presence of wortmannin. Cells were either submitted to trichloroacetic acid precipitation (20% final) to study degradation, or incubated in internalization buffer containing 10 μg/ml Pronase at 4 °C overnight to remove surface bound radioactivity. Radioactivity present in the supernatant was counted and percentage of internalized or degraded Ag was calculated. Degradation assay by FcγR-Igβ-expressing cells was performed with the same protocol, except that cells were incubated on ice 2 h with 10 μg/ml iodinated TNP-BSA precomplexed with 20 μg/ml anti-DNP Ab.

**Internalization assay by chimera** was performed as previously described (17). FcγR-Igβ cells were washed in internalization buffer, treated 20 min at 37 °C with wortmannin or Me\(_2\)SO, incubated on ice for 1 h with preformed immune complexes against HRP (IC-HRP, 10 μg/ml HRP and 400 μg/ml anti-HRP) rabbit polyclonal), washed, and incubated for various times at 37 °C. Internalization was stopped by addition of cold PBS. After washing, half of the cells were lysed with PBS, 1% Triton X-100 for 5 min at room temperature, and transferred on ice, the other half was kept on ice in PBS. Cell surface and total HRP activity were measured by addition of cold OPD substrate buffer.

**Antigen Presentation**—Ag presentation was assessed as previously described (11) with the following modifications. A fragment of the recombinant C1 \(\lambda\) repressor (from amino acid 1 to 102) was produced, it was either complexed with mAbs anti-C1 \(\lambda\) repressor 22D and 51F for studies on FcγR-chimera, or TNP-coupled for studies on BCR, as previously described (11). B cells were incubated 20 min at 37 °C with 200 nM wortmannin, 10 μg/ml LY294002 or Me\(_2\)SO before addition of Ags. After 20 min incubation times of incubation with the Ags, B cells were fixed 5 min on ice with 0.05% glutaraldehyde. T cell hybridoma were then incubated for 20 h with the Ag-pulsed B lymphoma and IL-2 production in the supernatant was measured by enzyme-linked immunosorbent assay, according to the manufacturer's protocol (BD Pharmingen). TMB substrate (BD Pharmingen) was used for revelation. All experiments were performed in triplicates.

**Western Blot Analysis**—Cells were washed in culture medium without fetal calf serum, and incubated with wortmannin, LY294002, or Me\(_2\)SO for 20 min prior to Ag stimulation. Ag used for stimulation was either TNF coupled to ovalbumin or immune complexes against C1 (IC-C1) preformed at 37 °C. Cells were harvested and lysed in lysis buffer (20 mM Tris, pH 7.4, 140 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM Na~3~VO\(_4\), 1% aprotinin, 2 μg/ml antipain, leupeptin) for 20 min at 4 °C. Equal amounts of postnuclear cell lysates were analyzed under reducing conditions by SDS-PAGE and electrobolted on Immobilon P membrane (Millipore). The antibody-Ag complexes were visualized by an enhanced chemiluminescence detection system according to the manufacturer's instructions (ECL, Amersham Biosciences).

**Immunofluorescence and Confocal Microscopy**—To analyze PI3K, cells were transiently transfected with a plasmid encoding a chimera of the Akt PH domain coupled to GFP (kind gift from T. Balla) and used for immunofluorescence 24 h after. Cells were washed in PBS and 0.2% poly-L-lysine-coated glass slides, after washing in internalization buffer, cells were pretreated with PI3K inhibitors for 20 min. Anti-TNF A20 B cells were incubated on ice for 1 h with OVA coupled to Cy3 and TNP. FcγR-chimera were incubated on ice for 1 h with preformed IC containing HRP-rodhamine (10 μg/ml) and anti-

**FIG. 1.** BCR-mediated Ag presentation depends on PI3K. Anti-TNF A20 B cells were preincubated for 20 min at 37 °C with 0.1% Me\(_2\)SO (dmso) (control), 10 μg/ml LY294002, or 200 nM wortmannin. **A**, Western blot analysis showing phospho-Akt in total lysates from B cells stimulated with 10 μg/ml TNP-OVA. Equal amounts of proteins were present in each lane as shown with anti-ERK2 Abs. **B** and **C**, anti-TNF A20 B cells were incubated with various concentrations of TNP-C1 (B) 6 μg/ml (C), or 10 μg/ml C1 peptide 12–26. Kinetic studies (C) were performed by adding the Ag sequentially every 30 min. After 2 h at 37 °C, cells were fixed and incubated with the T cell hybridoma 24.4. Secreted IL-2 was measured by enzyme-linked immunosorbent assay. **D**, Ag presentation was assessed using the same protocol with uncoupled C1. Data are representative of three independent experiments.

HRP rabbit polyclonal Ig (400 μg/ml) or biotinylated rabbit anti-mouse IgG F(ab')2 (ICN) precomplexed with streptavidin Alexa 594 (Molecular Probes). Cells were washed, incubated at 37 °C for 30 min, washed in cold PBS, fixed in 3% paraformaldehyde for 30 min, and washed with PBS, 20 μg/ml. After permeabilization with 0.05% saponin in PBS supplemented with 0.2% BSA, the cells were incubated with 2.5 μg/ml anti-CD107a Ab for 45 min, washed, and incubated with 2.5 μg/ml F(ab')2 fragments of fluorescein isothiocyanate-coupled donkey anti-rat IgG Ab for 45 min. Double immunofluorescence analysis were performed using a Leica TCS SP2 confocal laser scanning microscope.

**Immunoelectron Microscopy**—Cells were pretreated with wortmannin or Me\(_2\)SO, counted 1 h on ice with ×10 preformed IC-HRP, washed,
and then incubated for 30 min at 37 °C. Cells were fixed for 2 h at room temperature with 2% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4. Fixed cells were processed for ultrathin cryosectioning and immunogold labeling as described previously (30). For single MHC II labeling, sections were indirectly immunogold-labeled with the rat mAb anti-I-A M5114, followed by a rabbit anti-rat Ab (Dako, Denmark), for double MHC II and Ii labeling, sections were then labeled with a rabbit serum anti-Ii chain (IiNH2, a kind gift from J. Davoust). Double labeling for Lamp1 and MHC II was performed by incubating ultrathin cryosections with a rat monoclonal anti-Lamp1 Ab and a biotinylated-M5114. These Abs were followed by a rabbit anti-rat Ab and a rabbit anti-biotin Ab (Biovalley, Marne la Vallée, France), respectively. Bound antibodies were detected with protein A coupled to 10- and 15-nm gold particles (purchased from J. W. Slot, Utrecht University, Utrecht, The Netherlands). The sections were contrasted, embedded in a mixture of 2% methylcellulose and 4% uranyl acetate, and viewed with a CM120 Twin Phillips electron microscope.

RESULTS

Inhibitors of PI3K Block BCR-mediated Ag Presentation—We first verified that BCR triggering induces PI3K activation in our model. Anti-TNP A20 B lymphoma cells were treated with two PI3K inhibitors, wortmannin and LY294002, for 20 min, and activated with TNP-coupled ovalbumin. BCR stimulation induced PI3K activation, as shown by the phosphorylation of Akt on serine 473 (Fig. 1A). Both inhibitors abolished Akt phosphorylation (31), they also impaired BCR-induced mitogen-activated protein kinase activation as previously shown (32). The inhibitors used in this study were carefully titrated to ensure that they neither affected the surface expression of BCR and MHC class II molecules, nor induced apoptosis of A20 B cells (as shown by annexin V labeling, data not shown). For this reason the concentrations of wortmannin and LY294002 used did not completely inhibit Akt phosphorylation after 2 h of treatment.

We then studied the effect of PI3K inhibitors on BCR-induced Ag presentation by TNP A20 B cells. Cells were pre-treated for 20 min with 200 nM wortmannin, 10 μM LY294002, or vehicle (Me2SO) alone and incubated for 2 h with different concentrations of the 1-102 fragment of the \( \lambda c1 \) repressor coupled to TNP. B cells were then fixed, washed thoroughly, and incubated with the 24.4 T cell hybridoma, which recognizes peptide 12–26 from the \( \lambda c1 \) repressor. IL-2 production by the T
cell hybridoma was quantified in the supernatants. As shown in Fig. 1B, TNP A20 B cells incubated in the presence of either wortmannin or LY294002 displayed a 50% reduction in IL-2 production at all concentrations of Ag tested. A kinetic analysis of BCR-mediated Ag presentation confirmed that PI3K inhibitors diminished Ag presentation at all time points tested (Fig. 1C).

Remarkably, this inhibition of Ag presentation by PI3K inhibitors was selective for BCR-internalized Ag. Indeed, PI3K inhibitors did not inhibit presentation of synthetic peptides 12–26 of the λ c1 repressor (Fig. 1, B and C). Moreover LY294002 (Fig. 1D) and wortmannin (data not shown) did not inhibit the fluid phase-mediated Ag presentation of the 1-102 fragment of a λ c1 repressor when not coupled to TNP, notably this presentation required 10-fold more λ c1 repressor than the BCR-mediated Ag presentation. We conclude that PI3K is specifically required for presentation of Ag internalized via the BCR.

**PI3K Controls Igβ- and Not Iga-mediated Ag Presentation**

Our results suggest that PI3K activity controls BCR-mediated Ag processing. We and others have demonstrated that Igo and Igβ have distinct signaling functions inside the BCR complex (29, 33). However, both are able to induce Ag presentation (17). We therefore decided to examine the role of PI3K in Iga- and Igβ-mediated Ag presentation. To address this question we used an A20 IIA1.6-derived B cell line that expresses chimeric receptors consisting of the extracellular and transmembrane domains of the mouse FcγRII coupled to the intracytoplasmic domain of Igo or Igβ (29). We first assessed PI3K activation of both chimeras by measuring Akt phosphorylation on serine 473. Chimera-expressing cells were incubated with ICs consisting of a λ c1 repressor precomplexed with two mAbs against the λ c1 repressor. As shown in Fig. 2A, Akt was constitutively phosphorylated in cells expressing FcγR-Igo, this basal phosphorylation was inhibited by pretreatment of the cells with LY294002 or wortmannin. Cross-linking of Igo did not induce any increase of Akt phosphorylation, however, it induced mi-

togen-activated protein kinase phosphorylation (Fig. 2A) showing the functionality of this chimera. In contrast, cross-linking of the FcγR-Igβ chimera induced PI3K activity, as reflected by an increased phosphorylation of Akt, which was inhibited by pretreatment with LY294002 or wortmannin (Fig. 2B). This is the first demonstration that PI3K is specifically activated by the Igβ chain.

We then studied the effect of PI3K inhibitors on Iga- and Igβ-induced Ag presentation. B cells expressing FcγR-Igo and Igβ chimeras were pretreated for 20 min with 10 μM LY294002, 200 nM wortmannin, or vehicle (Me2SO) alone. Ag presentation was measured by incubating the chimera-expressing cells with several doses of λ c1 repressor precomplexed with two mAbs directed against the λ c1 repressor. B cells were then fixed, washed, incubated with the 24.4 T cell hybridoma, and IL-2 production quantified in the supernatants. As shown in Fig. 2C, PI3K inhibitors did not inhibit FcγR-Igα-induced Ag presentation. A kinetic analysis of the Igα-mediated Ag presentation confirmed that PI3K did not play a significant role in Igα-mediated Ag presentation (Fig. 2E). In contrast, both PI3K inhibitors inhibited by 50% FcγR-Igβ-mediated Ag presentation (Fig. 2D). The role of PI3K was confirmed in a kinetic analysis of Igβ-mediated Ag presentation (Fig. 2F for LY294002, and data not shown for wortmannin). Presentation of synthetic peptides 12–26 of the λ c1 repressor by this B cell line was not significantly modified by incubation with either LY294002 or wortmannin (Figs. 2, D and F). We also verified that both inhibitors did not affect the surface expression of chimera and MHC class II molecules (data not shown). Altogether, these results demonstrate that PI3K is activated specifically by Igβ and that Igβ-mediated Ag processing depends on this PI3K activation.

**PI3K Is Not Required for BCR-mediated Ag Internalization or Degradation**—At which step of antigen presentation is PI3K required? To address this question we monitored the ability of B cells to internalize Ag in the presence or absence of PI3K

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**Fig. 3. PI3K activity is not required for Ag internalization or degradation.** Cells were pretreated with 0.01% Me2SO (dmsa) (control) or 100 nM wortmannin for 20 min. A, anti-TNP A20 cells were incubated with 125I-labeled TNP-BSA for 1 h at 4 °C, washed, and incubated at 37 °C. The internalized fraction was calculated after overnight Pronase treatment of the cells. B, FcγR-Igβ chimera-expressing cells were coated with IC-HRP Abs for 1 h on ice, washed, and incubated at 37 °C. Total and surface HRP activity was measured. C and D, anti-TNP A20 B cells were treated with 125I-labeled TNP-BSA as in A, and FcγR-Igβ chimera-expressing cells with 125I-labeled TNP-BSA precomplexed with anti-DNP Abs. Undegraded Ag was precipitated with trichloroacetic acid. Radioactivity of degraded Ag in the supernatant and trichloroacetic acid-precipitable undegraded Ag in the pellet was determined. Data are representative of two or more independent experiments.
accumulation of MHC class II molecules in newly formed Lamp1+ MVBS that contain the Ag, and to which H2-M is recruited (10). Is PI3K activity required for formation of such compartments? To address this question Ag-stimulated cells were analyzed by immunofluorescence in the presence and absence of PI3K inhibitors. Unstimulated TNP A20 cells and FcγR-Igβ expressing cells showed multiple small Lamp1+ vesicles (Fig. 4A, green) distributed throughout the cytoplasm. Following ligation of the BCR with TNP-OVA these vesicles were remarkably redistributed and by 30 min formed a single large perinuclear aggregate in most of the cells. Equivalent results were obtained after Igβ ligation, using preformed ICs of rhodamine-coupled-HRP/anti-HRP Abs (Fig. 4). Endocytosed Ag fully co-distributed with Lamp1 in these aggregates in 82% of TNP A20 cells and 70% of FcγR-Igβ-expressing cells. As shown in Fig. 4C, Igβ-internalized Ags were also found in H2-M positive compartments. Of note, redistribution of Lamp1+ vesicles and targeting of Ag to the Lamp1 compartment were observed only when activating FcγR-Igβ-expressing cells (Ag fully co-distributed with Lamp1 only in 15% cells, data not shown).

Pretreatment of B cells with 200 nM wortmannin prevented both BCR and FcγR-Igβ-induced redistribution of Lamp1+ vesicles in large perinuclear aggregates. This pretreatment also reduced the co-distribution of BCR or FcγR-Igβ internalized Ag with Lamp1 (Fig. 4, panels A and B) and H2-M (data not shown and panel C). After 30 min, the co-distribution of Ag with Lamp1 was observed only in 24% of TNP A20 cells and was not observed in FcγR-Igβ-expressing cells. As expected, no effect of wortmannin was detected when analyzing FcγR-Igβ-expressing cells (data not shown).

Wortmannin Modifies the Formation of Ag Processing Compartment—Our data obtained by confocal microscopy strongly suggest that BCR ligation through the Igβ chain generates a compartment wherein Ag loading onto MHC class II molecules takes place and that this is dependent on PI3K activity. To confirm this hypothesis, we performed immunogold labeling on ultrathin cryosections, to analyze the morphology of MHC class II-enriched compartments and the distribution of MHC class II molecules. As shown in Fig. 5A, FcγR-Igβ-expressing B cells stimulated for 30 min with pre-formed ICs of HRP/anti-HRP Abs displayed endosomal structures with multivesicular morphology, which have been previously characterized upon BCR cross-linking (10). These structures were scarcely observed in nonactivated cells (data not shown). In these compartments, labeling for MHC class II was observed in the internal membrane vesicles, which also contained Lamp1 (Fig. 5A) and Ag (data not shown) witnessing their Ag processing function. In wortmannin-treated cells, pre-formed ICs did not induce the formation of MVBS, instead endocytic compartments mostly devoid of internal vesicles were seen (Fig. 5B). In a small proportion of cells, wortmannin had an intermediate effect, classical multivesicular Ag processing compartments were observed closely apposed to electron lucent compartments (data not shown). This may explain the partial inhibition of Ag presentation by wortmannin. However, in most of the cells, only the electron lucent endocytic compartments devoid of internal membranes were observed, their limiting membranes were labeled with anti-class II and anti-Lamp1 antibodies (Fig. 5D), and they also contain Ag (data not shown). In addition, in wortmannin-pretreated cells, labeling for MHC II was observed also in tubulovesicular structures closely apposed to the enlarged compartments (Fig. 5B). These endocytic compartments also contained the invariant chain Ii (Fig. 5C). We previously showed that BCR stimulation increases the intracellular MHC class II molecules by a factor 3 after 30 min (10), a quantitative...
analysis of the class II present in the electron lucent compart-
ment and the tubulovesicular network demonstrated that a
similar increase was observed in wortmannin-treated cells
(data not shown).

Interestingly, in a large proportion of the wortmannin-
treated cells we observed juxtaposed electron lucent compart-
ments differentially enriched, at their limiting membranes, in
MHC class II molecules or Lamp1 (Fig. 5D). Altogether, these
results strongly suggest that PI3K activity is implicated in
denovo formation of a multivesicular Ag processing compartment,
which is induced by BCR activation in an Igβ/H9252
dependent
fashion.

PI3K Is Activated Along the Ag Endocytic Pathway—we
have shown that PI3K is implicated in denovo formation of a
multivesicular antigen-processing compartment and that PI3K
is activated by BCR and Igβ but not by Igα (Fig. 2A). We thus
wondered if PI3K is activated in the endocytic compartments
wherein the Ag is transported. The PH domain of Akt has been
shown to bind to phosphatidylinositol 3,4-bisphosphate and
phosphatidylinositol 3,4,5-trisphosphate (36–38) and can be
used as a probe to detect these PI3K products. We thus trans-
fected FcyR-Igα and -Igβ chimera-expressing B cells with a
PH-Akt domain coupled to GFP and performed confocal micro-
scopy experiments. In resting cells, PH-Akt-GFP labeling was
mainly observed at the plasma membrane, with some diffuse
labeling in the cytoplasm and the nucleus. Five minutes after
ligation of Igβ chimera with pre-formed IC-HRP, MHC class II protein
A gold, 15 nm (PAG 15) are present in multivesicular endosomes that also label
for Lamp1 protein A gold, 10 nm (PAG 10). MHC class II molecules are also de-
tected in small vesicles. B, cells pretreated with 200 nm wortmannin MHC
class II (PAG 10) are visualized in enlarged compartments mostly devoid of in-
ternal membranes. Occasionally small membrane vesicles are observed in close
apposition to the lumenal side of limiting membrane. Note the small tubular vesic-
ular structures highly labeled for MHC class II surrounding the enlarged com-
partments. C, in wortmannin-treated cells Igβ (PAG 15) codistributed with MHC
class II (PAG 10) at the limiting mem-
brane of electron lucent compartments. D, in wortmannin-treated cells some elec-
tron lucent compartments contained mostly class II (PAG 15) and others con-
tained class II and Lamp1 (PAG 10). Bars = 100 nm.

Fig. 5. Wortmannin modifies the formation of the Ag processing com-
partment. Ultrathin cryosections of FcyR-Igβ-expressing cells were single or
double immunogold labeled for MHC class II (M5114) (B), MHC class II and Igβ
(C), or MHC class II and Lamp1 (A and
D). A, in cells stimulated for 30 min with pre-formed IC-HRP. MHC class II protein
A gold, 15 nm (PAG 15) are present in
multivesicular endosomes that also label
for Lamp1 protein A gold, 10 nm (PAG
10). MHC class II molecules are also de-
tected in small vesicles. B, cells pretreated with 200 nm wortmannin MHC
class II (PAG 10) are visualized in enlarged compartments mostly devoid of in-
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tron lucent compartments contained mostly class II (PAG 15) and others con-
tained class II and Lamp1 (PAG 10). Bars = 100 nm.
FIG. 6. PI3K is activated along the Ag endocytic pathway. A, FcγR-Igβ-expressing cells were transiently transfected with a plasmid encoding the PH-Akt domain fused to GFP. Cells on polylysine-coated coverslips were incubated 1 h on ice with IC-HRP-rhodamine. After washing cells were incubated for 5 (upper panel) or 30 min (lower panel) at 37 °C, and fixed. Left panel shows the distribution of PH-Akt-GFP in resting cells. B and C, FcγR-Igα-expressing cells were treated as in A, except that they were incubated with biotinylated anti-IgG F(ab')2 precomplexed with streptavidin-Alexa 594 to trigger the BCR (C). Results are typical from three independent experiments.

2). Strikingly, in these very cells that express both FcγR-Igα chimera and IgG, BCR cross-linking did induce PH-Akt-GFP concentration at the site of contact (Fig. 6C, upper panel), and on the Ag-containing endocytic vesicles (Fig. 6C, lower panel). These results showed that the absence of PI3K activation by Igα is not because of a defect intrinsic of the FcγR-Igα chimera-expressing cells.

BCR cross-linking with TNP-OVA-Cy3 in anti-TNP A20 B cells induced PH-Akt-GFP translocation to Ag containing vesicle as well (data not shown). Altogether, these results demonstrate that PI3K is activated in the intracellular compartments wherein the Ag has been endocytosed by the BCR and that this activation is mediated by Igβ.

DISCUSSION

BCR is a multimeric receptor complex composed of an Ig noncovalently associated with an heterodimer of Igα and Igβ, which mediates BCR-induced signaling. BCR-mediated Ag uptake increases its processing efficiency by targeting the Ag to compartments competent for Ag degradation and peptide loading onto MHC class II molecules (39, 40). We and others have previously shown that signaling through the BCR regulates Ag presentation (11, 12), however, the precise mechanisms involved are still unknown. Here, we show that BCR-mediated PI3K activation is instrumental for Ag presentation because it controls the neogenesis of the multivesicular Ag processing compartment, which contains MHC class II, Ii, H2-M, Lamp1, and the Ag. Moreover, we demonstrate for the first time that PI3K activation accompanies the Ag endocytic journey in an Igβ-dependent manner. These data strongly suggest that modification of PI3K of the lipidic environment of the endosomes wherein the BCR-bound Ag is targeted is required for the dynamic formation of the Ag processing compartment.

Several groups have already suggested a role for PI3K in Ag presentation (41, 42). However, the precise mechanisms implied were not unraveled. In one of these studies, the authors showed that FcoRc-induced Ag presentation was inhibited by LY294002 and wortmannin and that cross-linking of FcoRcs induced activation of PI3K in peptide loading endocytic compartments (42). These results obtained with another immunoreceptor are in accordance with ours and probably reflects the fact that activation of FcoRc also induces de novo formation of an Ag processing compartments.

Although we demonstrate that BCR-induced PI3K activation plays a crucial role in Ag presentation, we do not know which PI3K species is implicated. There is increasing evidence for an important role for class IA PI3Ks in the regulation of the immune system (43). It has recently been shown that p110δ, which is highly expressed in lymphocytes, is required for BCR signaling (23), it may thus be a key player of BCR-induced Ag presentation. Alternatively, the murine analogue of Vps34, which phosphorylates phosphatidylinositol at the 3' position of the inositol ring, may be implicated. Indeed, Vps34 is required for internal vesicle formation within multivesicular endosomes (28). A previous study has shown in a melanoma cell line that PI3K is implicated in MHC class II-enriched compartment formation (27). Our study shows unambiguously that PI3K is required for neogenesis of an Ag processing compartment with important consequences on Ag presentation.

Igα and Igβ intracytoplasmic tails induce differential Ag presentation (11) and differ in their signaling abilities (29, 33). Herein, we show for the first time that Igβ triggering induces PI3K activation whereas Igα does not and that PI3K is activated along the endocytic pathway of BCR- and Igβ-transported Ag. We have previously shown that Igα and Igβ differ both in their endocytic traffic (18) and in their ability to present Ag (17). Our results strongly suggest that PI3K activation by Igβ is causal to these differences. A recent study showed that Ag presentation requires the recruitment of B cell linker protein by the Igα BCR subunit (44). Thus Igα and Igβ by activating, respectively, a B cell linker protein or PI3K-dependent pathways may differentially contribute to Ag presentation. We indeed observed that activation of FcγR-Igα expressing B cells by ICs, which induces Ag presentation, does not induce de novo formation of MVBs in contrast to the activation of FcγR-Igβ expressing B cells.2

Ligation of many tyrosine kinase receptors induces activation of their kinase activity, autophosphorylation of their intracytoplasmic domains, and recruitment of signaling proteins; this has been thoroughly documented for the epidermal growth factor receptor. Signaling is accompanied by enhanced endocytosis of receptor-ligand complexes. Ligated receptors are associated with signaling proteins along the endocytic pathway suggesting that intracellular signaling can take place in endocytic compartments. By analogy, recruitment and/or transport of signaling proteins by the BCR toward endosomal compartments may modify their composition. Herein, we point to one of these effectors: PI3K. BCR triggering induces activation of PI3K, which may then be transported together with the BCR along the endocytic pathway. Alternatively PI3K could be spe-

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2 M. Granboulan, D. Lankar, G. Raposo, C. Bonnerot, and C. Hivroz, unpublished data.
Igβ-dependent PI3K Activation Controls Ag Presentation

Specifically recruited to specific intracellular compartments as suggested by the fact that membranes of the Ag containing endocytic compartment are enriched in PI3K products (Fig. 6). There, products generated by PI3K could recruit and/or activate the molecular machinery necessary for the formation of the multivesicular Ag processing bodies. In other models, it has been shown that MVBS are enriched in phosphatidylinositol 3-phosphate (45), a PI3K product and that inward vesiculation within MVBS is inhibited by wortmannin (27, 28).

In this study we show that PI3K inhibition does not preclude internalization of BCR-bound Ag and strikingly does not prevent degradation of BCR-internalized Ags despite inhibition of MVBS formation. Alternative interpretations of this result can be proposed (1). In the absence of PI3K activation, Ag degradation takes place in different endocytic compartments, implying that the generated antigenic peptides will not be loaded onto MHC II molecules (2). Ag presentation does not occur because Ag are not being degraded in a productive fashion. They could be either degraded by a different set of proteases, or completely destroyed. Our assay for Ag degradation does not allow us to distinguish between these hypothesis. A definitive conclusion will need further investigation.

Absence of Ag presentation may be because of inhibition of fusion of endocytic compartments by wortmannin, indeed we observed a non-negligible number of electron lucent compartments expressing only MHC class II or only Lamp1 at their limiting membranes (Fig. 5D). This inhibition of fusion could preclude gathering of several molecules that are essential for Ag processing. For example, H2-M, which has been shown to play an important role in loading of peptide on MHC class II molecules (46) and to be targeted to MVBS by BCR triggering (10), is not observed in Ag containing compartments in wortmannin-treated cells (Fig. 4).

A lot remains to learn about the intracellular compartments responsible for Ag processing and peptide loading as well as about the control of their formation. Signaling induced by Ag binding to its receptor will directly influence the environment of endosomes and thereby control formation of Ag processing compartments. This will ultimately lead to presentation of differently processed Ags. Identification of the cytosolic effectors used by Ag receptors will allow better understand the molecular mechanisms that control Ag processing.

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