Ubiquitynylation of Igβ Dictates the Endocytic Fate of the B Cell Antigen Receptor


*J Immunol* 2007; 179:4435-4443; doi: 10.4049/jimmunol.179.7.4435 http://www.jimmunol.org/content/179/7/4435

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Ubiquitinylation of Igβ Dictates the Endocytic Fate of the B Cell Antigen Receptor


In both infection and autoimmunity, the development of high-affinity Abs and memory requires B cells to efficiently capture and process Ags for presentation to cognate T cells. Although a great deal is known about how Ags are processed, the molecular mechanisms by which the BCR captures Ag for processing are still obscure. In this study, we demonstrate that the Igβ component of the BCR is diubiquitinylated and that this is dependent on the E3 ligase Itch. In contrast, analysis of ubiquitin-defective receptors demonstrated that the attachment of ubiquitins to Igβ is required for endosomal sorting and for the presentation of Ag to T cells, yet, ubiquitinylation is dispensable for receptor internalization. Membrane-bound Igμ was not detectably ubiquitinylated nor were the conserved lysines in the mu cytosolic tail required for trafficking to late endosomes. These results demonstrate that ubiquitinylation of a singular substrate, Igβ, is required for a specific receptor trafficking event. However, they also reveal that E3 ligases play a broader role in multiple processes that determine the fate of Ag-engaged BCR complexes.

The Journal of Immunology, 2007, 179: 4435–4443.

The ability of B lymphocytes to capture, process, and present Ags to T cells is requisite for normal humoral immune responses (1) and contributes to the pathogenesis of both B and T cell-mediated autoimmune diseases (2). B lymphocytes preferentially capture polyvalent Ags which, by aggregating the BCR and initiating signaling cascades, elicit a coordinated series of cellular responses which ensure that even low-affinity Ags are productively captured (3). Among these responses is the accelerated endocytosis and delivery of engaged receptors to MHC class II-enriched late endosomes (3). Most commonly, the MHC class II containing compartment resembles late endosomes with a complex structure consisting of a limiting membrane studded with Lamp-1 and a lumen containing MHC class II-bearing intraluminal multivesicular bodies (IMBs) (4) derived from BCR-laden transport vesicles (5). Following Ag processing and the loading of MHC class II with peptide, the IMBs are either transported to the cell surface, where they define the interface with cognate T cells (6), or secreted as exosomes (7). Despite the importance of delivering Ag to the late endosomal-processing compartments, little is known about how this occurs.

IMBs are not unique to lymphocytes but are a characteristic feature of late endosomes/lysosomes in both yeast and select mammalian cells. Recent advances in these systems have identified how proteins are targeted for inclusion in IMBs (8, 9). Receptors labeled with single ubiquitin (Ub) molecules are recognized in early endosomes by Hrs/Vps27p and ESCRT I complexes which sort receptors toward late endosomes (10). At the limiting membrane of targeted vesicles, components of the ESCRT II/III complex (11, 12) segregate ubiquitinylated molecules into IMBs (13, 14). In some cases ubiquitinylation of receptors, or of components of the endocytic machinery, can initiate sorting into clathrin-coated pits and receptor endocytosis (15, 16).

Labeling receptors with Ub requires the successive activities of three classes of enzymes. Ub-activating enzyme (E1) binds Ub and mediates transfer to a Ub-conjugating enzyme (E2). Final substrate specificity is determined by an Ub protein ligase (E3) which transfers Ub to the e-amino group of a lysine in the targeted protein. Of the over 100 E3 ligases that have been identified (17), most can be classified into two families based on shared catalytic domains: the homologous to the E6-associating protein C terminus (HECT) and really interesting new gene (RING) families. RING-type E3 ligases such as C-cbl and cbl-b function as adaptor proteins between Ub-E2 conjugates and substrates, whereas HECT-type E3 ligases such as Itch, Nedd4, and AIP4 accept activated Ub from an E2 and mediate transfer to the targeted substrate (18). Final substrate specificity is determined by an E3 ligase (E3) which transfers Ub to the e-amino group of a lysine in the targeted protein. Of the over 100 E3 ligases that have been identified (17), most can be classified into two families based on shared catalytic domains: the homologous to the E6-associating protein C terminus (HECT) and really interesting new gene (RING) families. RING-type E3 ligases such as C-cbl and cbl-b function as adaptor proteins between Ub-E2 conjugates and substrates, whereas HECT-type E3 ligases such as Itch, Nedd4, and AIP4 accept activated Ub from an E2 and transfer it to the targeted substrate (18).

In this report, we demonstrate that the HECT family E3 ligase Itch mediates the constitutive ubiquitinylation of the BCR Igβ.

Received for publication January 24, 2007. Accepted for publication July 26, 2007.

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1 This work was supported by grants from the National Institutes of Health (to M.R.C.: GM52736, GM067772; to H.B.: CA87986, CA76119, CA99900, CA99163) and the Arthritis Foundation (to M.R.C.). H.B. acknowledges support from the Jean Ruggles-Romoser Chair for Cancer Research.

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3 Abbreviations used in this paper: IMB, intraluminal multivesicular body; Ub, ubiquitin; HECT, homologous to the E6-associated protein C terminus; RING, really interesting new gene; STAM, signal-transducing adaptor molecule; WT, wild type; PDGF, platelet-derived growth factor; BS3, Bis(sulfosuccinimidyl) suberate; OGP, N-octyl-β-D-glucopyranoside; mlg, membrane-bound Ig; VHS, Vps27-h-STAM; UIM, Ub-interacting motif; LMP, latent membrane protein; Tf, transferrin; SLAP, Src-like adaptor protein.

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chain and that this is required for normal sorting through the endocytic pathway. In the absence of either Itch or Igβ ubiquitylation, internalized BCRs arrest in early endosomes and do not traffic to late endosomal Ag-processing compartments. In contrast, comparison of Itch T°/− B lymphocytes and ubiquitylation-defective BCR complexes revealed that Itch plays an additional role in mediating BCR internalization. These results began to define the molecular events mediating the capture of Ag by B cells for processing and presentation to cognate T cells.

Materials and Methods

Cell lines

The murine B cell lymphoma A20IIA1.6 (IgG2a, FcRn, I-Ad<sup>−</sup>), and I-E<sup>−</sup>) was cultured in DMEM (Invitrogen Life Technologies) containing 10% FBS (Sigma-Aldrich), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 7.5% CO₂.

Signal-transducing adaptor molecule (STAM) fusion proteins

The tandem Vps27-h-STAM (VHS)-Ub-interacting motif (UIM) domains from adaptor proteins STAM1 and STAM2, corresponding to amino acid positions 1–213 and 1–192, respectively, were cloned into the pGEX-2TK bacterial expression vector (Amersham Biosciences). GST-VHS-UIM fusion proteins were expressed in Escherichia coli strain BL21 and purified on glutathione-conjugated agarose beads (Pierce). The ability of eluted solubile GST-VHS-UIM protein fragments to bindUb was confirmed in binding assays with Ub-conjugated agarose beads (Boston Biochem) (data not shown).

Derivation of 3-8μ-transfected cell lines

RNA was isolated from 3-8μ-transgenic splenic B cells (D. Nemazee, The Scripps Research Institute, La Jolla, CA) with TRZol (Invitrogen Life Technologies). RT-PCR was conducted using the SuperScript II Reverse Transcriptase kit (Invitrogen Life Technologies) followed by PCR (Pfu Turbo; Stratagen) with primers to amplify the open reading frame encoding wild-type (WT) 3-8μ (mu WT, forward: CGGAGG ACCACATGGAATGGCTGGAATCTTG, reverse: ATAAAGAATG GCCGCGCTATTTCCATTGGAAGGTTGAC) or 3-8μm in which the two cytoplasmic lysines had been mutated to arginines (μ<sup>KA</sup>; reverse: ATAAAGAATGCAGCCGGCCTACCTACCTGGAACAGGTTGACGG TG). The resulting PCR products were sequenced and then subcloned into pcDNAneo (Invitrogen Life Technologies). A20IIA1.6 cells were transfected with the plasmid (Amaxa Nucleofection) and stable cell lines were derived by limiting dilution and G418 selection (1 mg/ml; Invitrogen Life Technologies). Cell lines expressing similar levels of surface mu, as ascertained by flow cytometry, were selected for further analysis.

Derivation and analysis of chimeras

The methods for construction, expression, and activation of the platelet-derived growth factor receptor (PDGFR) chimeras have been previously described (19). The three lysines of the cytoplasmic tail of IgG were mutated to arginines (μ<sup>KA</sup>, reverse: ATAAAGAATGCGGGCCGCTACCTACCTGGAACAGGTTGACGG TG). The resulting PCR products were sequenced and then subcloned into pcDNAneo (Invitrogen Life Technologies). A20IIA1.6 cells were transfected with pcDNAneo (Amaxa Nucleofection) and stable cell lines were derived by limiting dilution and G418 selection (1 mg/ml; Invitrogen Life Technologies). Cell lines expressing similar levels of surface μ, as ascertained by flow cytometry, were selected for further analysis.

Receptor internalization

Purified splenic B cell or A20IIA1.6 cells were stained for either the BCR or chimeric receptor as described above. Cells were then incubated at 37°C for the indicated intervals and then placed on ice. To remove surface-bound Abs, 100 μg/ml of stripping buffer (100 mM glycine, pH 2.5) was added to each sample at room temperature for 2 min. Cells were then washed twice with ice-cold FACS buffer (3% FBS in PBS) and examined by flow cytometry (FACSscan; BD Biosciences). The percentage of internalized receptors was calculated using the following formula: percent SR<sub>T</sub> = 100 × ([ARF<sub>T</sub> − AF<sub>T</sub>]/[AF<sub>R</sub> − AF<sub>T</sub>]) where SR<sub>T</sub> is the percent of surface receptor internalized at time “T”. AF<sub>T</sub> is the acid-resistant fluorescence at time “T”. ARF<sub>T</sub> indicates cellular autofluorescence of cells (median fluorescence of unstained cells or cells that were stained and then acid-stripped), and AF<sub>R</sub> refers to the median fluorescence of cells that were stained for 30 min at 4°C.

Immunoprecipitation and immunoblotting

Purified splenocytes or A20IIA1.6 cells were stimulated via the BCR or chimeric receptor as described above. Following stimulation, cells were lysed with 1% Nonidet P-40 lysis buffer on ice, precleared with protein G-Sepharose (Amersham Biosciences) and immunoprecipitated with the murine mAb PDI1 (Santa Cruz Biotechnology) at 4°C overnight. Samples were resolved by SDS-PAGE (10%) and transferred to nitrocellulose filters (VWR Scientific). The filters were then blocked with 3% BSA-TBS (pH 8.0) with 1% Triton X-100. Filters were then incubated with either affinity purified anti-Ig or anti-Igβ rabbit antisera (19). As these Abs were raised to GST fusion proteins, antisera were extensively preabsorbed with GST-agarose before use. Filters were then incubated with HRP-conjugated secondary Abs. Washed filters were developed using ECL (Amersham Biosciences).

Ag presentation to T cells

To assess Ag presentation by the PDGFR chimeras, the receptors on aliquots of cells (10⁵/sample) were first aggregated as described above and then targeted with serial dilutions of rabbit anti-rat IgG1 (BD Biosciences) for 15 min. Labeled cells were stimulated by warming to 37°C for the indicated times. Samples were then fixed in 100% methanol for 5 min at −20°C and then permeabilized with PBS containing 0.1 mM Bis-(sulfosuccinimidyld) suberate (BS<sub>3</sub>; Pierce) and 0.1% n-octyl-β-D-glucopyranoside (OGS; Sigma-Aldrich) at 25°C for 15 min. Excess BS<sub>3</sub> was quenched with 100 mM ethylenediamine (pH 7.5; Sigma-Aldrich) at 25°C for 15 min. Samples were then blocked in 1% nonfat powdered milk/PBS/0.1% OGP and stained with IFN-γ (BD Calibio) as described previously (20). For transferrin receptor staining, cells were incubated in serum-starved medium (0.5% FCS) for 48 h, loaded with 10 μg/ml Alexa 594-transferrin (Molecular Probes) at 37°C for 10 min, then stimulated with Alexa 647-conjugated Abs (Molecular Probes) for 30 min and fixed as described above. Images were collected using a Leica TCS SP2 confocal microscope (Leica). Where indicated, strong colocalization was defined as >50% overlap in the distribution of two different markers in a single cell while weak colocalization was defined as between 1 and 10% overlap. For each experimental time point, 50 cells were counted and all experiments were done in triplicate.

B splenocyte purification

The studies described in this article have been reviewed and approved by the University of Chicago Institutional Review Board. Spleens were isolated from BALB/c WT mice, Itch<sup>T°/−</sup>C57BL/6 mice or Itch<sup>T°/−</sup> littermate controls. All mice were 6–10 wk old. Following hypotonic lysis, splenocytes were incubated with biotinylated anti-CD11b, anti-CD11c, anti-Gr1, anti-CD3, anti-CD4, anti-CD8, anti-NK1.1, and anti-TER 119 (BD Biosciences) on ice for 30 min, washed, then incubated with streptavidin microparticles (Miltenyi Biotec) at 30 min at 4°C. Following separation by MACS column (Miltenyi Biotec), purity of B cells was assayed by flow cytometry. Only populations of >90% purity were used in experiments.
Results

Igβ is diubiquitylated

Studies in both mammalian and yeast cells have demonstrated that ubiquitylation is a signal for receptor internalization, intracellular trafficking, and lysosome-mediated degradation (9). To investigate whether ubiquitylation contributes to BCR endocytosis and endocytic trafficking, we first determined whether endogenous IgG and/or Igβ were ubiquitylated. Lysates from resting splenic B lymphocytes from 6- to 10-wk-old BALB/c WT mice were precleared with protein A-Sepharose and then immunoprecipitated with rabbit anti-Igβ Abs. As a control, aliquots of each lysate were resolved by SDS-PAGE and immunoblotted with anti-actin Abs. As a control, aliquots of each lysate were resolved by SDS-PAGE and immunoblotted with anti-actin Abs. As a control, aliquots of each lysate were resolved by SDS-PAGE and immunoblotted with anti-actin Abs.

We next examined whether Igβ was ubiquitylated in the B cell lymphoma cell line A20IIA1.6 (IgG2a⁺, FcR⁻, I-A^d⁻, I-E^d⁻) (25). Aliquots of cells either were left unstimulated or were stimulated with rabbit anti-mouse IgG Abs for the indicated times (Fig. 2A). Samples were immunoprecipitated with anti-Ub Abs and immunoblotted with anti-Igβ Abs as described above. As was observed in splenic B cells, a single immunoreactive band with a relative molecular mass of 54 kDa was observed. In contrast to splenic B lymphocytes, the amount of detectable material rapidly decreased following BCR stimulation (n = 5).

To examine whether IgG was ubiquitylated, anti-IgG immunoprecipitations were immunoblotted with anti-IgG Abs. As demonstrated in Fig. 2B, prolonged exposures (~20 times greater than those used in the previous experiments) revealed an ubiquitylated protein with a relative molecular mass of 50 kDa, consistent with that of Igβ.

To confirm that Igβ was ubiquitylated, we next examined whether Igβ could be ubiquitylated with monoubiquitin-binding domains. The STAM1 is involved in recognizing and sorting ubiquitylated receptor complexes (9, 23, 24). For both STAM1 and the family member STAM2, Ub binding is mediated by tandem Vps27-h-STAM (VHS) and Ub-interacting motif (UIM) domains. We constructed a GST fusion protein containing the STAM1 or STAM2 VHS and UIM domains and used these in pull-down assays with splenic B cell lysates. As seen in Fig. 1C, the STAM1 fusion protein precipitated a 54-kDa species that was reactive with anti-Igβ Abs. Interestingly, the interaction with STAM1 was specific, as the corresponding STAM2 fusion protein did not precipitate ubiquitylated Igβ (Fig. 1D). From these data, we conclude that Igβ is ubiquitylated.

FIGURE 1. Ubiquitylation of Igβ in splenic B lymphocytes. A, Lysates from resting splenic B lymphocytes (10⁷/sample) were precleared with protein A-Sepharose and then immunoprecipitated with mouse anti-Ub or isotype control Abs. Precipitations were resolved by SDS-PAGE and immunoblotted with rabbit anti-Igβ Abs. As a control, aliquots of each lysate were resolved by SDS-PAGE and immunoblotted with anti-actin Abs. B, Splenic B cells (10⁷/sample) were stimulated with anti-IgM plus IgG F(ab)₂ Ab (10 µg/ml) for the indicated times (minutes), precleared, and then immunoprecipitated with anti-Ub Abs. Precipitations were then analyzed as in A. C, Lysates from aliquots of splenic B cells (10⁷/sample) were stimulated with anti-IgM plus IgG F(ab)₂ Ab for the indicated times (minutes) then precipitated with the indicated GST-STAM1 fusion proteins bound to glutathione-agarose. Precipitations were resolved by SDS-PAGE and then immunoblotted with rabbit anti-Igβ Abs. D, Lysates from aliquots of splenic B cells (10⁷/sample) were precipitated with indicated GST-STAM1 or GST-STAM2 fusion proteins bound to glutathione-agarose and immunoblotted as in C. The GST-STAM1 and GST-STAM2 fusion proteins are faintly visible with relative molecular masses of 48 and 40 kDa, respectively.

FIGURE 2. Igβ is ubiquitylated at the cell surface. A, Aliquots of A20IIA1.6 cells (10⁷/sample) were stimulated with anti-IgG Abs (10 µg/ml) for the indicated times (minutes). Cell lysates were immunoprecipitated with anti-Ub Abs, resolved by SDS-PAGE, transferred to nylon membrane, and immunoblotted with anti-Igβ Abs. As a control, aliquots of each lysate were resolved by SDS-PAGE and immunoblotted with anti-actin Abs. B, A20IIA1.6 cells were stimulated as in A. Anti-Ub immunoprecipitates were Western blotted with anti-IgG Abs.
Itch is required for Igβ ubiquitinylation

We next sought to identify the E3 ligase(s) required for ubiquitinylating Igβ. A hint of which ligase might be involved was provided by studies demonstrating that Itch ubiquitinylated the EBV protein latent membrane protein 2A (LMP 2A) (26). As LMP 2A has structural and functional homology with the BCR, we examined whether Itch ubiquitinylated Igβ.

Itch−/− mice and WT littermate controls were sacrificed and aliquots of purified B splenocytes either were left unstimulated or were stimulated with F(ab)2 anti-IgM plus IgG Abs for 30 min. Cells were immunoprecipitated with anti-Ub Abs, and immunoblotted with anti-Ig Abs (upper panel). Corresponding total cell lysates were immunoblotted with anti-Igα (middle panel) and anti-actin Abs (lower panel). Below each anti-Igβ and -Igα immunoreactive species is shown the relative density of that band divided by the relative density of the corresponding actin band. Below each anti-Igβ and anti-Igα immunoreactive species is shown the relative density of that band divided by the relative density of the corresponding actin band.

We next determined whether Itch played a role in BCR internalization. The BCR complexes on splenic B cells from Itch−/− or WT littermate controls were lysed, immunoprecipitated with anti-Ub Abs, and immunoblotted with anti-Igβ Abs (upper panel). Corresponding total cell lysates were immunoblotted with anti-Igα (middle panel) and anti-actin Abs (lower panel). Below each anti-Igβ and -Igα immunoreactive species is shown the relative density of that band divided by the relative density of the corresponding actin band.

With diubiquitinylated Igα (22). The intensity of this band increased after 15 min and a higher molecular mass species consistent with triubiquitinylated Igα was detected at 30 min. The delayed kinetics of Igα ubiquitinylation indicates that it occurs after receptor endocytosis and sorting.

**Itch is required for Igβ ubiquitinylation**

We next examined whether sorting of the ligated BCR out of early endosomes was dependent upon Itch. Cell aliquots were first loaded with Alexa 594-conjugated transferrin (red) for 10 min at 37°C, fixed, and then visualized by confocal microscopy. C, Splenic Itch−/− or littermate control splenic B cells were stimulated with FITC-conjugated F(ab)2 anti-IgM plus IgG Abs (green) for 20 min, loaded with Alexa 594-conjugated transferrin (red) for 10 min at 37°C, fixed, and then visualized by confocal microscopy. C, Splenic Itch−/− or littermate control splenic B cells were stimulated with FITC-conjugated F(ab)2 anti-IgM plus IgG Abs (green) for 30 min. Cells were then fixed and counterstained with PE-conjugated ID4B (red) and visualized by confocal microscopy.

We next examined whether aggregation-induced BCR internalization on Itch−/− splenocytes. A, Ligand-induced BCR internalization on Itch−/− (▲, lower curve) or littermate control (●) splenocytes. Cells were labeled with PE-conjugated F(ab)2 anti-IgM plus IgG Abs on ice, washed, and warmed to 37°C for the indicated times. Samples were then stripped with glycin buffer to remove surface retained Abs and assayed by flow cytometry. B, Splenic Itch−/− or littermate control splenic B cells were stimulated with FITC-conjugated F(ab)2 anti-IgM plus IgG Abs (green) for 20 min, loaded with Alexa 594-conjugated transferrin (red) for 10 min at 37°C, fixed, and then visualized by confocal microscopy. C, Splenic Itch−/− or littermate control splenic B cells were stimulated with FITC-conjugated F(ab)2 anti-IgM plus IgG Abs (green) for 30 min. Cells were then fixed and counterstained with PE-conjugated ID4B (red) and visualized by confocal microscopy.

**Itch is required for normal BCR internalization and endocytic trafficking**

We next determined whether Itch played a role in BCR internalization. The BCR complexes on splenic B cells from Itch−/− or WT littermate controls were labeled with PE-conjugated anti-IgM/ IgG F(ab)2 Abs on ice for 15 min. Cells were then warmed to 37°C for the indicated times after which surface-retained Abs were stripped from the cell surface (27). Samples were then examined by flow cytometry and the percentage of internalized receptors was calculated as described in Materials and Methods. As demonstrated in Fig. 4A, aggregation-induced BCR internalization was attenuated in Itch−/− B splenocytes (n = 4).

We next examined whether sorting of the ligated BCR out of early endosomes was dependent upon Itch. Cell aliquots were first loaded with Alexa 594-conjugated transferrin (TT) to label early endosomes and then stimulated with FITC-conjugated anti-IgM plus IgG F(ab)2 Abs for 30 min. Samples were then fixed and visualized by confocal microscopy. In WT splenic B cells, there was essentially no detectable colocalization of the internalized.
BCR with Tf+ early endosomes (2 of 50 cells per experiment ± 2, n = 3) (Fig. 4B). In contrast, in the Itch−/− B splenocytes, the internalized BCR strongly colocalized with Tf+ early endosomes (44/50 ± 4, n = 3).

It is possible that the observed differences in BCR trafficking between WT and Itch−/− splenic B cells were due to changes in the kinetics of endocytic transport rather than absolute differences in endocytic trafficking. Internalized BCR complexes normally rapidly transit through early endosomes. Indeed, after 15 min of BCR stimulation, internalized BCRs strongly colocalized with Tf+ early endosomes in WT cells (60% of cells, data not shown). However, even after 60 min of stimulation, in Itch−/− B lymphocytes, endocytosed BCRs were still retained in Tf+ early endosomes (data not shown). These data indicate that mutation of the Igβ cytosolic lysines imposes a block in sorting between early and late endosomes.

It would be anticipated that BCR complexes arrested in early endosomes could not gain access to late endosomes. To assess this prediction directly, the BCR on splenic B cells from Itch−/− or littermate controls was ligated with FITC-conjugated anti-IgM plus IgG F(ab)2 Abs for 30 min and then cells were fixed, permeabilized, and counterstained with the anti-LAMP-1 Ab ID4B. As seen in Fig. 4C, in WT splenic B cells, most internalized BCR complexes colocalized with Lamp-1+ late endosomes (41/50 ± 2, n = 3). In contrast, in Itch−/− cells none of the internalized BCRs strongly colocalized with Lamp-1+ late endosomes while a few demonstrated weak colocalization (5/50 ± 3, n = 4). Similar results were obtained if cells were stimulated for 60 min (data not shown). From these data, we conclude that Itch is required for both normal BCR internalization and for normal sorting through early and late endosomes.

**Igβ ubiquitinylation is not required for receptor internalization**

We next sought to determine which Itch-dependent BCR functions could be ascribed to Igβ ubiquitinylation. There are three lysines in the cytoplasmic tail of Igβ that could serve as ubiquitinylation sites. As ubiquitinylation is often promiscuous, a cDNA was constructed encoding an Igβ cytosolic tail in which all three lysines were mutated to alanines. This DNA fragment was assembled with one encoding the extracellular and transmembrane domains of PDGFRαs (IgβKAA) in the retroviral vector MIGRI (20). Following packaging, viral supernatants were used to infect A20IIA1.6 cells already expressing PDGFRβIgα (20). GFP+ infectants were isolated using FACS and expression of each chimera was confirmed by flow cytometry and immunoblotting (Fig. 5, A and B, n = 3). Immunoblotting anti-Igβ immunoprecipitations with anti-Ub Abs demonstrated that ubiquitinylation-defective Igα/Igβ heterodimers were formed by adding PDGF-BB. These heterodimers can then be aggregated with anti-receptor Abs (19). Previous experiments have demonstrated that this system accurately recapitulates the signaling and trafficking properties of the endogenous BCR (19).

We first assessed whether mutating the lysines in Igβ altered global receptor signaling. A20IIA1.6 cells expressing either Igα/Igβ or Igα/IgβKAA were stimulated as indicated and total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine Abs (4G10). As can be seen in Fig. 5D, there was no significant difference between the two populations (n = 3).

We next determined whether Igβ ubiquitinylation contributed to receptor internalization. Chimeric receptors were stained on the cell surface (20) with PE-conjugated Abs on ice for 15 min. Cells were then warmed to 37°C for indicated times after which surface retained Abs were stripped from the cell surface, analyzed by flow cytometry, and the fraction of internalized receptors was determined. As demonstrated in Fig. 6A, the kinetics and magnitude of Igα/Igβ or Igα/IgβKAA internalization were nearly identical (n = 3). These results indicate that Igβ ubiquitinylation is not required for receptor endocytosis.

**Igβ ubiquitinylation is required for sorting from early endosomes to late endosomes**

We next examined whether Igβ ubiquitinylation played a role in endocytic sorting through Tf+ early endosomes (20). Cells expressing Igα/Igβ or Igα/IgβKAA were preloaded with Alexa 594-conjugated Tf and then stimulated with PE-conjugated anti-receptor Abs for 30 min. As demonstrated in Fig. 6B, visualization...
After incubation for 45 min at 37°C, cells were washed and then cocultured.

**FIGURE 6.** Igβ ubiquitinylation is dispensable for receptor endocytosis but is required for normal endocytic trafficking and Ag presentation. A. Internalization of Igβ<sup>−/−</sup> (●) or Igβ<sup>KΔA</sup> (■) containing complexes assayed as in Fig. 4A. B. Indicated receptor complexes were stimulated with Alexa 647-conjugated Abs (red) for 30 min, following loading with Alexa 594-conjugated transferrin (green) for the last 10 min of stimulation at 37°C. After fixation, samples were visualized by confocal microscopy. C. Indicated receptor complexes were stimulated with Cy5-conjugated Abs (green) for 30 min, counterstained with anti-Lamp-1 Abs (ID4B, red) and then visualized by confocal microscopy. D. Receptor complexes containing either Igβ<sup>−/−</sup> (●) or Igβ<sup>KΔA</sup> (■) were aggregated as described in Materials and Methods and then targeted with serial dilutions of rabbit anti-rat IgG1. After incubation for 45 min at 37°C, cells were washed and then cocultured with the T cell clone 2R50 for ~36 h. ELISA was used to measure IL-2 production. Each graph shows the mean values of samples done in triplicate from a representative single experiment (n = 4).

by confocal microscopy revealed that Igα/Igβ<sup>KΔA</sup> strongly colocalized with Tf in 90% of cells (45/50 ± 2, n = 3). In contrast, Igα/Igβ was excluded from Tf<sup>−</sup> early endosomes (1/50 ± 1 with any colocalization, n = 3). Similar results were obtained if cells were stimulated for 60 min (data not shown).

We next compared Igα/Igβ and Igα/Igβ<sup>KΔA</sup> in their ability to colocalize with Lamp-1<sup>−</sup> vesicles (20). For these experiments, chimeric receptors were stimulated with PE-conjugated Abs as described (20) (pseudocolor green) for 30 min at 37°C. Samples were then fixed, permeabilized, and counterstained with ID4B. Photomicrographs of a typical result are shown in Fig. 6C. Compared with Igα/Igβ, in which 80% cells (40/50 ± 2, n = 3) demonstrated strong colocalization with Lamp-1<sup>−</sup> vesicles, in cells expressing Igα/Igβ<sup>KΔA</sup>, 14% of cells demonstrated weak colocalization with Lamp-1<sup>−</sup> vesicles (7/50 ± 3, n = 3) and no cells demonstrated strong colocalization. The pattern of colocalization observed with the Igα/Igβ<sup>KΔA</sup> mutant is similar to that observed with receptors containing only Igα which arrest in early endosomes (Fig. 5C, lower panel) (22).

**Igβ ubiquitinylation is required for receptor-facilitated Ag presentation to T cells**

Ag is usually processed and loaded onto newly synthesized MHC class II in specialized Lamp-1<sup>−</sup> late endosomes (28). As receptor ubiquitinylation appeared to be necessary for entry into late endosomes, we next examined whether it was also required for the processing and presentation of receptor-targeted Ags to T cells. Chimeric receptors were stimulated as above and then pulsed with serial dilutions of rabbit anti-rat IgG1 Abs at 37°C for 45 min. Cells were then washed and used as APCs in assays with the processed rabbit Ig-specific T cell clone 2R50 (21). After 36 h, IL-2 production was assayed by ELISA. As demonstrated in Fig. 6D (n = 4), mutation of the Igβ ubiquitinylation sites completely abrogated the ability of the receptor to facilitate the processing and presentation of Ags to cognate T cells.

**Membrane-bound Igμ (mIgμ) as a potential ubiquitinylation substrate**

Recently, it has been reported that mIgμ is ubiquitinylated and that this may be involved in BCR trafficking (29). However, in several experiments using either anti-Ub Abs or STAM 1/2 fusion proteins, we could not detect either mIgμ or mIgγ ubiquitinylaton (n = 6, data not shown). As this negative result did not preclude a potential role for mIgμ ubiquitinylation in endocytic trafficking, we derived A20IIA1.6 cell lines expressing similar surface levels of either WT 3-83 mIgμ or a 3-83 mIgμ molecule in which the two cytosolic lysines were mutated (mIgμ<sup>KΔK</sup>) (Fig. 7A and data not shown). Using these transfected cell lines, we examined whether the conserved lysines in the cytosolic tail of mIgμ were involved in BCR internalization and targeting to late endosomes. As can be seen in Fig. 7B, receptor complexes containing either mIgμ or mIgμ<sup>KΔK</sup> were efficiently internalized from the cell surface following aggregation by PE-conjugated anti-Igμ F(ab)2 Abs (n = 3).

We next examined whether the mIgμ cytosolic lysines were required for BCR endocytic sorting. Either mIgμ<sup>−/−</sup> or mIgμ<sup>KΔK</sup>-expressing cells were stimulated with Alexa 594-conjugated anti-Igμ F(ab)2 Abs for the indicated times and then fixed and counterstained with anti-Lamp-1 Abs. As demonstrated in Fig. 7C, the mIgμ<sup>KΔK</sup> mutant receptor could efficiently traffic to late endosomes by 30 min. However, examination of the kinetics of BCR trafficking revealed that the bulk endocytic transit of mIgμ<sup>KΔK</sup> was delayed with ~50% less cells demonstrating strong colocalization of mIgμ<sup>KΔK</sup> receptors with Lamp-1<sup>−</sup> endosomes at 15 min (Fig. 7,
and D). At later time points, colocalization of mIg/H9262 and mIg/H9262K/H9004 receptors was comparable. These data indicate that while the lysines in the mIg/H9262 cytosolic tail are not absolutely required for BCR endocytic trafficking, they may enhance the rapidity of endocytic transit.

We next examined whether the mIg/H9262 cytosolic lysines were required for receptor facilitated Ag presentation to T cells. Either mIg/H9262 or mIg/H9262K/H9004 receptors were incubated with serial dilutions of rabbit anti-mouse mu-specific Abs. Cells were then cocultured with the T cell clone 2R50 and IL-2 production was measured by ELISA \( (n = 3) \).

**FIGURE 7.** Role of Igμ cytosolic lysines in BCR trafficking and Ag presentation. A, Transmembrane and intracellular sequence of WT mIgμ and mIgμ in which cytosolic lysines have been mutated to arginines. B, Internalization assay of A20IIA1.6 3–83 WT or 3–83K/H9004. Cells were assayed as in Fig. 4A. C, Indicated IgM receptor complexes were stimulated with Alexa 594-conjugated Abs (red) for the indicated times, then counterstained with anti-Lamp1 Abs (ID4B, green) and then visualized by confocal microscopy. D, Samples represented in C were scored for the percent of cells demonstrating strong BCR colocalization with Lamp-1 \(^{-1}\) late endosomes \((\square, 3\text{-}83 \text{ WT}; \blacksquare, 3\text{-}83K/H9004)\). E, IgM receptor containing either 3-83 WT (■) or 3-83K/H9004 (○) were targeted with serial dilutions of rabbit anti-mouse IgM, mu-specific Abs then cocultured with the T cell hybridoma 2R50. IL-2 production was assayed by ELISA as described \((n = 3)\).

**Discussion**

In the periphery, recognition of polyvalent Ags by the BCR initiates two processes requisite for normal humoral responses. One is the activation of signaling cascades leading to clonal expansion and differentiation. The other is the capture of ligating Ags for processing and presentation to cognate T cells (30). In this study, we demonstrate that Itch-mediated Igμ ubiquitinylation is required for sorting of endocytosed BCR complexes to the late endosomal Ag-processing compartments. These studies reveal a fundamental mechanism governing the fate of endocytosed BCR complexes. Furthermore, they demonstrate that the molecular events determining endocytic trafficking in both yeast and mammalian cells are conserved in lymphocytes and contribute to processes central to normal immune responses.

Although Igβ ubiquitinylation is required for endocytotic sorting, it is dispensable for receptor endocytosis. In contrast, the E3 ligase responsible for Igβ ubiquitinylation is required for both processes. A similar relationship between receptor ubiquitinylation and the responsible E3 ligase has been reported for the epidermal growth factor receptor (31, 32). However, there are also clear examples where receptor ubiquitinylation is required for internalization (33),
indicating that ubiquitinylation can mediate different receptor-specific functions.

In the case of the BCR, we and others have demonstrated receptor endocytosis is dependent upon conserved tyrosine-based signaling motifs within the cytosolic tail of Igα/Igβ (34, 35). Different motifs for endocytosis and trafficking are consistent with the known biological functions of the BCR. Successful signaling induces the phosphorylation of the tyrosine-based Igα/Igβ internalization motifs preventing internalization (35). Selective retention of phosphorylated BCRs on the cell surface is predicted to prolong signaling responses and enhance cellular responses to low-avidity ligands.

In contrast to the inductive phosphorylation that characterizes BCR signaling and determines receptor endocytosis, resting BCRs are constitutively ubiquitylated. This premarking for endocytic sorting is consistent with previous observations that, once internalized, monomeric and multimerized receptor complexes traverse a common endocytic route to late endosomes (36). Although receptor ligation is not required for Igβ ubiquitylation, BCR signaling might be required as mutation of the Igβ cytosolic tyrosines diminishes Igβ ubiquitylation (34).

Although receptor complexes share a common endocytic route, it is well-known that receptors bound to polyvalent Ags target to late endosomes much more rapidly (28). Such targeting has been demonstrated to ensure the productive and preferential capture of low-affinity Ags encountered in polyvalent arrays (3). As the mechanisms of endocytic sorting are similar, we postulate that polyvalent Ags are preferentially captured because they are more rapidly internalized (37).

It has recently been reported that mlgμ is ubiquitylated and this was correlated with the effects of proteasome inhibition on BCR trafficking and degradation (29). However, under conditions in which we readily detected Igβ ubiquitylation, we never detected mlgμ ubiquitylation. The reasons for this are not clear. However, subsequent mutagenesis demonstrated that even if mlgμ is ubiquitylated, it was not the primary determiner of BCR intracellular trafficking.

Mutating the mlgμ cytosolic lysines did diminish receptor-facilitated Ag presentation and this correlated with a modest delay in BCR trafficking to late endosomes. This could reflect undetectable changes in BCR ubiquitylation. However, preliminary evidence indicates that the mlgμ cytosolic lysines regulate the stability of the surface BCR complex (data not shown). This would also be predicted to alter the efficiency of receptor-facilitated Ag presentation.

Both our data and recently published observations (38) indicate that Igα is a substrate for ubiquitylation by Cbl. However, the delayed kinetics and low level of Igα ubiquitylation suggest that this ubiquitylation event does not play a role in BCR endocytic trafficking. In support of this conclusion, mutation of the potential Igα cytosolic ubiquitylation sites did not affect BCR endocytic trafficking (Dr. A.-M. Lennon-Dumenil, unpublished observation). Furthermore, Igβ, but not Igα, is required for sorting out of early endosomes and for targeting to late endosomes/lysosomes (20).

It is unclear how Itch mediates Igβ ubiquitylation. In EBV-infected B cells, LMP 2A interacts with Itch through proline-rich PY motifs (39, 40). However, no such motifs are present in the BCR. Furthermore, the constitutive ubiquitylation of Igβ makes it unlikely that molecules recruited to the activated BCR are furnishing Itch recruitment domains. Itch constitutively colocalizes with endophilin A1 and clathrin H chain (41). As internalization of the BCR is dependent upon clathrin (42), residence on these membrane-associated structures may be sufficient for Itch to ubiquitylate the BCR.

There are several mechanisms by which Itch could contribute to endocytosis independent of receptor ubiquitylation. Itch could function to ubiquitylate and regulate components of the endocytic machinery (43), such as endophilin A1, which controls local membrane curvature (16, 44) and constitutively colocalizes with Itch (41). It is possible that Itch clusters endocytic adaptor proteins, as has been demonstrated for Cbl (45), and recruits receptors into clathrin-coated pits (46). In addition to the molecules that directly mediate endocytosis, genome-wide functional analyses have revealed that several interrelated signaling pathways regulate receptor endocytosis (47). Significantly, among these are the JNK/MAPK pathways, components of which regulate Itch (48, 49).

Although it is clear that Itch is required for ubiquitylating Igβ, our results indicate that other E3 ligases can inefficiently mediate this function. Other members of the HECT family of E3 ligases, including Nedd4, are expressed in lymphocytes (39, 40). These ligases share several conserved domains and it is therefore likely that they can ubiquitylate similar substrates. However, the presence of these E3 ligases was not sufficient to functionally compensate for Itch as Itch −/− splenocytes manifested a severe block in endocytic sorting (Fig. 4).

The constitutive ubiquitylation of the BCR contrasts with the inducible ubiquitylation of the TCR. In the case of the TCR, C-Cbl is recruited to the phosphorylated TCR through an intermediate, the Src-like adaptor protein (SLAP) (50). Ubiquitylation likely occurs in early endosomes thereby preferentially targeting internalized phosphorylated receptors for degradation. Although SLAP expression is primarily limited to thymocytes, low levels of expression in B lymphocytes may also modulate BCR-dependent responses (51). However, the expression of C-Cbl is primarily restricted to developing lymphocytes (52) (M. Zhang and M. R. Clark, unpublished observations) and therefore it is unlikely that SLAP/C-Cbl play a major role in regulating peripheral AgR expression. Another Cbl family member, Cbl-b, is expressed in peripheral B cells. However, examination of Cbl-b −/− splenocytes reveals that Cbl-b has no role in either Igβ ubiquitylation or in sorting of the BCR through early endosomes (M. Zhang and M. R. Clark, unpublished observations).

In summary, our findings reveal molecular mechanisms that control the endocytic fate of the Ag-engaged BCR and the presentation of ligand-derived peptides to cognate T cells. Central to these processes is the ubiquitylation of Igβ and the sorting of internalized BCR complexes through early endosomes. The functional specificity of Igβ ubiquitylation contrasts with the multiple roles played by the requisite E3 ligase Itch. Understanding the biological importance of Igβ ubiquitylation will provide insights into how BCR endocytic transit both determines peripheral B cell fate and contributes to B cell-mediated immune responses.

**Acknowledgments**

We thank Lorrie Elliott (Northwestern University, Evanston, IL) and Sarah Powers (University of Chicago, Chicago, IL) for their editorial assistance and helpful discussions.

**Disclosures**

The authors have no financial conflict of interest.