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MHC Class II Antigen Processing in B Cells: Accelerated Intracellular Targeting of Antigens

Paul C. Cheng,* Carrie R. Steele,* Lin Gu,* Wenxia Song, † Susan K. Pierce²*

Processing and presentation by Ag-specific B cells is initiated by Ag binding to the B cell Ag receptor (BCR). Cross-linking of the BCR by Ag results in a rapid targeting of the BCR and bound Ag to the MHC class II peptide loading compartment (IIPLC). This accelerated delivery of Ag may be essential in vivo during periods of rapid Ag-driven B cell expansion and T cell-dependent selection. Here, we use both immunoelectron microscopy and a nondisruptive protein chemical polymerization method to define the intracellular pathway of the targeting of Ags by the BCR. We show that following cross-linking, the BCR is rapidly transported through transferrin receptor-containing early endosomes to a LAMP-1⁺, β-hexosaminidase⁺, multivesicular compartment that is an active site of peptide-class II complex assembly, containing both class II-invariant chain complexes in the process of invariant chain proteolytic removal as well as mature peptide-class II complexes. The BCR enters the class II-containing compartment as an intact mIg/Igα/Igβ complex bound to Ag. The pathway by which the BCR targets Ag to the IIPLC appears not to be identical to that by which Ags taken up by fluid phase pinocytosis traffic, suggesting that the accelerated BCR pathway may be specialized and potentially independently regulated. The Journal of Immunology, 1999, 162: 7171–7180.

The processing and presentation of Ag by Ag-specific B cells following the binding of the Ag to the B cell Ag receptor (BCR) is a highly efficient process (1). The BCR serves at least two functions to bind and transport Ag to the class II peptide loading compartment (IIPLC) (2) and to initiate signal transduction cascades (3–5). Recent results indicate that the transport and signaling functions of the BCR are interrelated and that signaling through the BCR influences the Ag-transport function of the BCR (6). In the absence of Ag, the BCR is constitutively internalized to dense vesicles. Cross-linking the BCR, however, results in increased internalization of the BCR and accelerated transport of the BCR and bound Ag to the IIPLC. The accelerated targeting of the BCR is reflected in the time required for processing and presentation of Ag initially bound to the BCR, which is approximately half that required for processing of Ags taken up by fluid phase pinocytosis (7). Thus, the accelerated targeting of Ag by the BCR may play a critical role in vivo, ensuring that Ag bound to the BCR is preferentially presented by Ag-specific B cells during periods of rapid Ag-driven expansion and helper T cell-dependent selection.

The accelerated transport of the BCR following cross-linking is dependent on signal cascades initiated by BCR cross-linking and is not due to simple aggregation of the BCR, as shown by the ability of kinase inhibitors that block BCR signaling to block accelerated trafficking (8). In addition, recent studies by Aluvihare et al. (9) using chimeric receptors provided evidence that the accelerated intracellular targeting of Ag by the BCR is dependent on the cytoplasmic domains of the Igo and Igβ components of the BCR. The acceleration in BCR trafficking induced by BCR cross-linking is accompanied by biochemical changes associated with the IIPLC, including changes in the profiles of phosphoproteins and low m.w. GTPases (10), and we speculate that these biochemical changes may be important in mediating the accelerated trafficking of the BCR.

At present, relatively little is known about the intracellular route of accelerated transport of the BCR or about the contents of the compartment into which the BCR delivers Ag. Several laboratories, including our own, have isolated and characterized the subcellular compartments in which peptide-class II complexes are formed (11–17). These studies provided evidence that a principal site of Ag processing is a late endocytic multivesicular or multilamellar compartment that has access to pinocytosed material and contains peptide-class II complexes, proteases, and the catalyst for class II peptide loading, DM. The relationship between these compartments and the compartment into which the BCR delivers Ag is not well established. Moreover, it is not known if the BCR complex, mIg and Igα/Igβ, remains intact during Ag targeting. Indeed, Vilen et al. (18) recently provided evidence that Ag stimulation leads to destabilization of the BCR reflected in the inability to coimmunoprecipitate mIg with Igα/Igβ. Thus, it is possible that the BCR does not traffic intact to the IIPLC. Previously, using subcellular fractionation and Ag-specific T cells to detect antigenic peptide-class II complexes, we showed that, within 15 min after binding of an Ag to the BCR, functional peptide-class II complexes derived from the BCR-bound Ag were present exclusively in dense compartments (13). Functional antigenic peptide-class II complexes continued to form in the dense compartment for 2 h, at which time the complexes were observed in subcellular fractions containing early endosomes presumably enroute to the plasma membrane, and on the plasma membrane where they accumulated for several hours. In subsequent studies, both biotinylated surface BCR and bound radiolabeled Ag were shown to be targeted to the

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3 Abbreviations used in this paper: BCR, B cell Ag receptor; CM, complete medium; DAB, 3,3'-diaminobenzidine; IEM, immunoelectron microscopy; Ii, invariant chain; IIPLC, class II peptide loading compartment; LAMP-1, lysosomal associated membrane glycoprotein-1; mtHSP70, mitochondrial heat shock protein 70; mIg, membrane Ig; SLIP, small leupeptin-induced invariant chain peptide; TIR, transferrin receptor.
same subcellular fractions in which the functional peptide-class II complexes were first observed to form. In addition, West et al. (16) used subcellular fractionation to show that newly assembled peptide-class II complexes resided in compartments accessible to Ag taken up by the BCR but not to transferrin receptors. Similar results were provided by Rudensky et al. (19), showing that Ag bound to the BCR trafficked through early endosomes to a peptide loading compartment within 1 h after binding to the BCR.

Here, we use a combination of immunoelectron microscopy (IEM) and a nondisruptive chemical polymerization technique mediated by HRP to describe the pathway of accelerated targeting of the BCR and bound Ag to the peptide loading compartments, and to further define the components of this compartment.

Materials and Methods
Cell lines and Abs
CH27 is a mouse B cell lymphoma line that is H-2a, IgM- 

\[ \text{Fc} \gamma R I B^{-} \]

\] (20). The cells were grown at 37°C in a 5% CO₂ atmosphere in DMEM, supplemented as previously described (21) and containing 15% FCS (15% complete medium (CM)). HRP-conjugated goat Abs specific for mouse IgG plus IgM (anti-Ig-HRP) were purchased from Jackson ImmunoResearch (West Grove, PA). For electron microscopy, 12-nm gold-labeled F(ab\')₂ goat Abs specific for mouse IgM (F(ab\')₂-anti-Ig) were prepared as previously described by Slot and Geuze (22). The mouse hybridoma, 17.3.3.s, producing an F-E-specific mouse IgG2a mAb (23), was obtained from the American Type Culture Collection (Manassas, VA), as was the rat hybridoma, RI7 217.1.3, producing an IgG2aa mAb specific for the mouse transferrin receptor (TIR). The rat hybridoma, D14B, producing an IgG2a mAb specific for mouse lysosomal associated membrane glycoprotein-1 (LAMP-1) was obtained from the Development Studies Hybridoma Bank (Iowa City, IA). The rat IgG2a hybridoma IN-1 specific for invariant chain (Ii) was kindly provided by Dr. N. Koch (Immunobiologie Zoologisches Institut Universität, Bonn, Germany) (24). The mouse hybridoma JG-1, producing an IgG3 mAb specific for mouse mitochondrial heat shock protein 70 (mHSP70) was generated and characterized in this laboratory by Green et al. (25). The rat IgG2a hybridoma 79a3, producing an IgG1 mAb specific for the cytosolic domain of mouse Igα was generated and characterized in this laboratory. Briefly, mice were immunized with a recombinant protein containing the entire cytoplasmic domain of Igα (residues 160–220) and GST (gift from Dr. M. Clark, University of Chicago, Chicago, IL). The spleens of immunized mice were fused with the B cell myeloma cell line SP20, and hybrids were screened for Abs that bound to the recombinant Igα/GST protein. Subsequent characterization showed that 79a3 bound to Igα in immunoblots and that the binding was inhibitable by the GST-Igα fusion protein. A rabbit polyclonal antiserum, specific for the cytosolic domain of Igα, was generated by immunizing rabbits with a synthetic peptide containing the cytoplasmic domain of Igα (residues 199–219) and a potent T cell epitope derived from tetanus toxoid (residue 582–599). All hybridomas were maintained in our laboratory, and mAbs were purified by protein A affinity chromatography. Rat IgG2a, mouse IgG2a, IgG2b, and IgG3 isotype control Abs were obtained from PharMingen (San Diego, CA). Rabbit γ-globulin was obtained from Jackson ImmunoResearch.

IEM
Cells were pulsed for 15 min at 37°C with 12-nm gold-labeled F(ab\')₂ anti-Ig (10 μg/ml) and chased for various times. Cells were washed and resuspended in 0.1 M phosphate buffer and fixed by incubation in a solution of 2% paraformaldehyde/1% acrolein for 2 h at 25°C. After washing with 0.1 M phosphate buffer, the samples were infused with 10% gelatin with 0.1 M phosphate buffer, the samples were infused with 10% gelatin

\[ \text{1.05 g/ml Percoll density gradient (Pharmacia, Piscataway, NJ) and centrifuged for 21 min at 10,000} \times g \text{ at 4°C for 15 min.} \]

The pellets were resuspended in 1 ml 0.1 M sodium citrate (pH 4.5) and 0.1 M 4-methylumbelliferyl-N-acetyl-b-D-glucosaminidase) was added. Samples were incubated at 25°C for 20 min, the reaction quenched with 2 ml 0.2 M glycine (pH 10.8), and fluorescence measured on a spectrofluorometer at  λex 365 nm and λem 448 nm.

Subcellular fractionation and HRP assay
CH27 cells (2 × 10⁸) were exposed to anti-Ig-HRP (20 μg/ml) for 15 min at 37°C, washed, and incubated for 0 or 60 min in 15% CM. At the end of each time point, cells were washed in PBS containing 1 mM EDTA. Cells were resuspended in 1 ml homogenization buffer (HB; 10 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA) in the presence of CLAP, homogenized gently, and centrifuged at 900 × g for 10 min to remove the nuclei. The pellet was resuspended in 1 ml HB, rehomogenized, and spun down. Supernatants were combined and centrifuged at 12,000 × g for 16 min to remove mitochondria. Supernatant (2.1 ml) was layered onto 9.1 ml of 1.05 g/ml Percoll density gradient (Pharmacia, Piscataway, NJ) and centrifuged for 21 min at 34,809 × g. Fractions of 1 ml were removed from the top of the gradient, and each fraction was tested for HRP activity. A 100-μl sample of each fraction was incubated with 100 μl of substrate solution (5 mg/ml 2, 2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid in 0.1 M sodium citrate buffer (pH 4.0) with 0.015% H₂O₂) for 5–10 min, after which absorbance was measured at 405 nm on an ELISA plate reader.

Results
IEM analysis of the compartments to which the BCR targets Ags
Using subcellular fractionation, we previously showed that upon cross-linking by anti-Ig, the membrane Ig (mlg) and bound Ag are internalized and targeted to a dense, class II-containing subcellular
cells were fixed and thin sections prepared for staining with mAbs specific for class II I-Ek molecules, Ii, and the TfR. The results showed that, within 15 min of cross-linking, anti-Ig was already detectable in subcellular compartments that contain anti-Ig-HRP will be polymerized in the presence of DAB and H2O2 into detergent insoluble polymers that can be removed from a cell lysate by centrifugation (16).

The trafficking of the anti-Ig-HRP bound to mlg was first characterized by subcellular fractionation to determine when anti-Ig-HRP entered the dense compartments previously shown to be sites of assembly of functional peptide-class II complexes. CH27 cells were incubated with anti-Ig-HRP for 15 min at 37°C, washed, and chased for 0 and 60 min. The cells were subjected to subcellular fractionation, and the HRP activity was measured in each of the fractions. Based on assays for a variety of enzymatic and serological markers, it has been established that fractions 1–3 contain early endosomes and Golgi, fractions 4–6 contain the plasma membrane and endoplasmic reticulum, fractions 7–8 are rab9+ vesicles through which class II complexes trafficking enroute to the IIPLC, and fractions 10–11 contain lysosomes, late endosomes, and the IIEplC (13, 27). At the 0-min chase point, after a 15-min pulse at 37°C, HRP activity was detected in the fractions that contain the plasma membrane and the early and late endosomes (Fig. 3). After 60 min of chase, the HRP activity was concentrated in the densest fractions.

To determine whether the compartments to which mlg is targeted are active sites of assembly of peptide-class II complexes, DAB polymerization analyses were performed using 35S metabolically labeled cells. CH27 cells were simultaneously pulsed with 35S-Met/Cys and anti-Ig-HRP for 15 min and chased for 60–180 min. Results of previous subcellular fractionation/pulse chase analysis showed that SDS-unstable class II molecules reach the IIPLC, where they acquire peptide and an SDS-stable conformation by 120 min (27). Thus, the time course of the present experiment allows anti-Ig-HRP to be concentrated in dense compartments at the time SDS-stable peptide-class II complexes first form. At the end of each time point, the cells were placed on ice, washed, and treated with DAB and H2O2 or DAB alone. The cells were lysed, and the lysates were centrifuged to remove large insoluble DAB-cross-linked protein polymers. The lysates were immunoprecipitated using an I-Ek-specific mAb, and the immunoprecipitates were subjected to SDS-PAGE without reducing or boiling the sample, conditions under which class II αβ heterodimers that have bound peptide are stable. After 60 min, the majority of class II molecules had not yet bound peptide and were SDS-unstable, migrating as free α- and β-chains (Fig. 4A). A portion of the SDS-unstable class II molecules were polymerized in the presence of DAB and H2O2, as seen by the reduction of free α- and β-chains, suggesting that they were present in anti-Ig-HRP-containing compartments. By 90 min, SDS-stable αβ dimers were first detected, and the number of SDS-stable dimers continued to increase over the 180-min chase.

The subcellular compartments to which sIg is targeted are active sites of peptide-class II complex assembly

A nondisruptive method of chemical polymerization that allows the identification of proteins that reside within the same subcellular compartment was used to follow the intracellular targeting of mlg and bound Ag. This method is based on the ability of HRP to catalyze the polymerization of proteins by DAB in the presence of H2O2. Thus, following a pulse with anti-Ig-HRP, those proteins in subcellular compartments that contain anti-Ig-HRP will be polymerized in the presence of DAB and H2O2, into detergent insoluble polymers that can be removed from a cell lysate by centrifugation (16).

The BCR is rapidly targeted to compartments containing class II molecules and Ii following BCR cross-linking. CH27 cells were incubated for 15 min at 37°C with 12-nm gold-labeled F(ab')2 anti-Ig. The cells were fixed and thin sections prepared for staining with mAbs specific for class II I-Ea (a) or Ii (b). The primary Abs were detected using 5-nm labeled protein G. The arrows indicate the 5-nm gold particles, and the bar indicates 0.1 μm.

**FIGURE 1.** The BCR is rapidly targeted to compartments containing class II molecules and Ii following BCR cross-linking. CH27 cells were incubated for 15 min at 37°C with 12-nm gold-labeled F(ab')2 anti-Ig. The cells were fixed and thin sections prepared for staining with mAbs specific for class II I-Ea (a) or Ii (b). The primary Abs were detected using 5-nm labeled protein G. The arrows indicate the 5-nm gold particles, and the bar indicates 0.1 μm.

compartment and that the movement of the mlg to the class II-containing compartment is accelerated, as compared with that of uncross-linked mlg (6). Here, we describe the compartments to which the mlg is targeted following cross-linking, using IEM. CH27 cells were incubated for 15 min with 12-nm gold-labeled F(ab')2 anti-Ig at 37°C, chased for 0 or 120 min, and processed for IEM. Cryosections were prepared and stained with Abs specific for the class II I-Ea molecules, Ii, and the TIR. The results showed that, within 15 min of cross-linking, anti-Ig was already detectable in multivesicular compartments that contain class II molecules and Ii (Fig. 1). The presence of the anti-Ig in class II-containing compartments after 15 min is consistent with our previous observation from subcellular fractionation that antigenic peptide-class II complexes derived from BCR-bound Ag are found in dense compartments as early as 15 min following Ag binding to the BCR (13). The IN-1 Ab used to detect Ii in these compartments recognizes a determinant in the cytoplasmic domain of Ii, thus it was not possible to know if these compartments contained intact Ii or whether the luminal domain of Ii had been proteolytically cleaved. The anti-Ig continues to concentrate in the multivesicular class II- and Ii-containing compartments up to 120 min of chase (Fig. 2). These compartments appeared to be deep in the cell and did not contain the TIR. Thus, following cross-linking the mlg and bound Ag appear to be targeted to multivesicular class II- and Ii-containing compartments that lack TIR. Transport is rapid, and a portion of the mlg reaches such compartments within minutes after cross-linking.
Significantly, as soon as the SDS-stable dimers were detected at 90 min, their levels were reduced by treatment with DAB and H₂O₂. These data indicate that the class II dimers were forming in a compartment that contained anti-Ig-HRP. The maximal reduction in the number of SDS-stable class II molecules by HRP-catalyzed DAB polymerization occurred after 90 min of chase with a 57% reduction and decreased thereafter, such that, after 180 min of chase, only 26% of SDS-stable class II molecules were still within anti-Ig-HRP-containing compartments (Fig. 4B). After 180 min, a portion of the SDS-stable class II molecules presumably were trafficking to the plasma membrane through anti-Ig-HRP-negative vesicles.

The BCR is targeted to compartments containing small leupeptin-induced invariant chain peptide (SLIP)-class II complexes

Having provided evidence that internalized anti-Ig-HRP was targeted to compartments in which newly synthesized class II molecules bound peptide, it was of interest to determine whether class II intermediates in Ag processing were also present in these compartments. After synthesis, the class II αβ heterodimers associate with the Ii, which directs the class II molecules to the endocytic system and blocks the peptide binding groove of the molecule until it reaches the endocytic system (28). There, Ii is cleaved sequentially, yielding several distinct fragments, including the 12-kDa fragment, known as SLIP. The SLIP-class II complex is highly transient and, under normal conditions, difficult to observe. The cysteine protease inhibitor leupeptin blocks the complete proteolysis of Ii, which our previous studies showed lead to an accumulation of SLIP-class II complexes in the IIPLC (29). Brachet et al. (30) recently provided evidence that leupeptin treatment of the B cell line A20 resulted in a shift in the SLIP-class II complexes from an early endocytic class II-containing compartment, referred to as CIIV, to a late endocytic compartment equivalent to the IIPLC. However, the CIIV-like compartments have not been observed in CH27 cells, and previously published pulse chase analysis coupled with subcellular fractionation provided no evidence.
of shift of the Ii-class II complexes from the IIPLC in CH27 cells in the presence of leupeptin (27). To determine whether the SLIP intermediate in Ii degradation is present in the compartment to which anti-Ig-HRP is targeted, CH27 cells were labeled with 35S-Met/Cys in the presence of leupeptin for 4 h, pulsed with anti-Ig-HRP for 15 min, and chased for 0–180 min. The DAB reaction was performed, and the samples were immunoprecipitated using the I-Ek-specific mAb 17.3.3s. After 4 h of leupeptin treatment, the class II immunoprecipitates showed intact Ii and SLIP associated with class II. With no chase of the HRP-anti-Ig there was no reduction in amount of SLIP following addition of DAB and H2O2 (Fig. 5, A and B). After a 60-min chase of the HRP-anti-Ig, there was detectable DAB polymerization of SLIP, indicating that the anti-Ig-HRP had entered the SLIP-class II-containing compartment. From 120 to 180 min there was significant DAB polymerization of SLIP in the presence of DAB and H2O2, indicating that the anti-Ig-HRP was concentrated in the compartment in which SLIP is formed.

The BCR trafficks through TfR-containing compartments following internalization

To characterize the route of anti-Ig from the plasma membrane to the IIPLC, the DAB reaction was used to follow the intersection of anti-Ig-HRP with the TfR. The TfR cycles between the plasma membrane and early endosomes (31, 32) and our earlier studies provided evidence that the TfR does not enter compartments in which peptide-class II complexes are assembled (33). CH27 cells were surface-biotinylated at 4°C to label the TfR. The cells were warmed to 37°C, pulsed for 3 min with anti-Ig-HRP, and chased for 0–180 min. At the end of each time point, the cells were placed on ice and the DAB reaction performed. The cells were lysed, and the lysate was cleared of insoluble protein polymers and immunoprecipitated using a mAb specific for TfR. The immunoprecipitates were subjected to SDS-PAGE and immunoblotting probing with streptavidin to detect biotinylated proteins. At 0 and 5 min of chase time, there was a dramatic reduction in TfR levels in the DAB and H2O2-treated cells, indicating that the anti-Ig-HRP was present in TfR-containing compartments (Fig. 6, A and B). After 30 min of chase, there was still a reduction in the TfR but considerably less than that observed at 0 and 5 min. At 60 and 120 min of chase, there was essentially no reduction. These results indicate that the mIg trafficks through the TfR1 early endosomes in 30 min enroute to the TfR2 class II-containing compartments. These results also indicate that the anti-Ig-HRP does not cycle from the peptide-loading compartment to the plasma membrane through the early endosomes during the 120-min chase, at least not in an enzymatically active form. These observations are consistent with the
H2O2 or DAB alone, lysed, and the lysates cleared of insoluble aggregates for 0–120 min. The samples were treated with DAB and were biotinylated at 4°C, pulsed with anti-Ig-HRP for 3 min, and treated with DAB in the absence of H2O2 (represented as the amount of TfR relative to that present at time 0 in samples containing the TfR was quantified by densitometry for each sample and represented as the amount of TfR relative to that present at time 0 in samples treated with DAB in the absence of H2O2 (B). Shown are the averages for three independent experiments.

Results from the IEM analysis, which show that TfR was absent from the late multivesicular compartments to which the BCR is targeted.

**Anti-Ig-HRP remains associated with an intact BCR en route to the IIPLC**

Anti-Ig-HRP was used to determine whether anti-Ig-HRP remains associated with the mlg of the BCR complex en route to the IIPLC and if the BCR mlg/Igα/Igβ complex remains intact during transport, CH27 cells were biotinylated at 4°C, pulsed with anti-Ig-HRP for 3 min, and chased for 0–120 min. The samples were treated with DAB and H2O2 or DAB alone, lysed, and the lysates cleared of insoluble protein polymers and immunoprecipitated using polyclonal Abs specific for Igα (Fig. 7) or with mAb79a3 (data not shown). Igα was nearly completely cross-linked at all chase times from 0 to 120 min (Fig. 7, A and B). Thus, anti-Ig-HRP appears to remain associated with the mlg during internalization, and trafficking to the IIPLC and the mlg remains associated with the Igα/Igβ complex following cross-linking and targeting.

**The BCR is targeted to compartments containing LAMP-1 and β-hexosaminidase**

The 110-kDa mature form of LAMP-1 is localized to late endosomes and lysosomes, cofractionating with the IIPLC in subcellular fractions 10–11 (13, 34). The DAB polymerization method was used to determine whether the BCR enters LAMP-1-containing compartments. CH27 cells were 35S-labeled for 15 min in the presence of anti-Ig-HRP for 15 min, washed, and chased for 60–180 min to allow adequate time for anti-Ig-HRP to concentrate in the IIPLC. At the end of each time point, the cells were exposed to either DAB and H2O2 or DAB alone, lysed, and the lysate cleared of protein polymers and immunoprecipitated using the LAMP-1-specific mAb, 1D4B. The anti-Ig-HRP enters LAMP-1-containing compartments, cross-linking a significant portion of LAMP-1 in the presence of DAB and H2O2 at 30 min (Fig. 8, A–C).

Having observed that mature LAMP-1 is present in the compartment to which anti-Ig-HRP is targeted, it was of interest to determine when during its biosynthesis mature LAMP-1 enters anti-Ig-HRP-containing compartments. To do so, CH27 cells were 35S-labeled for 15 min in the presence of anti-Ig-HRP for 15 min, washed, and chased for 60–180 min to allow adequate time for anti-Ig-HRP to concentrate in the IIPLC. At the end of each time point, the DAB reaction was performed, and LAMP-1 was immunoprecipitated from cell lysates. Mature LAMP-1 was first detected following 60 min of chase and increased over the 180-min chase (Fig. 8, D–F). Beginning at 60 min and continuing through 180 min, a significant reduction in LAMP-1 was observed in DAB- and H2O2-treated samples, indicating that, soon after maturation, LAMP-1 enters anti-Ig-HRP-containing compartments.

β-Hexosaminidase is an enzyme found in the endocytic system, including early endosomes, late endosomes, and lysosomes (35). Previously, by subcellular fractionation, we showed β-hexosaminidase activity in CH27 cells both in fractions containing early and late endosomes as well as fractions containing the IIPLC (6). To determine when BCR enters β-hexosaminidase-containing vesicles, CH27 cells were pulsed with anti-Ig-HRP for 15 min, chased for 0–180
min, treated with DAB and H2O2, lysed, cleared of protein polymers, and analyzed for β-hexosaminidase activity by a fluorographic assay. β-Hexosaminidase activity was unaffected by the DAB and H2O2 treatment during the first 60 min of chase (Fig. 9). After 90 min and continuing until 180 min, β-hexosaminidase activity was decreased significantly in DAB and H2O2-treated samples, indicating that the anti-Ig-HRP entered β-hexosaminidase-containing compartments. Taken together with the results presented above, these results indicate that the early TIR1+ endosomes into which the BCR is first internalized do not contain β-hexosaminidase, whereas the late, LAMP-1+, class II-containing compartments are β-hexosaminidase+.

**Anti-Ig-HRP does not catalyze the polymerization of proteins outside the endocytic system**

To verify that anti-Ig-HRP-catalyzed DAB polymerization occurred only within mIg-containing vesicles in the endocytic system, the effect of DAB polymerization on mtHSP70 was investigated. As for most mitochondrial proteins, mtHSP70 is encoded in a nuclear gene, which is translated in the cytosol and subsequently transported into mitochondria (36). CH27 cells were incubated with 35S-Met/Cys and anti-Ig-HRP for 15 min, washed, and chased for 60–180 min. The cells were treated with DAB plus H2O2 or DAB alone, cleared of insoluble protein aggregates, and immunoprecipitated for LAMP-1 with the mAb 1D4B. Immunoprecipitates were subjected to SDS-PAGE and radiography. A and D, Representative autoradiographs. The region of the gel containing the mature LAMP-1 was quantified for each sample and is given as the amount of LAMP-1 relative to that present at the earliest time point treated with DAB in the absence of H2O2 (B) or as the percent of total LAMP-1 (E). The percent reduction at each time point is also plotted in C and F. The values represent an average of the results of two (A–C) or three (D–F) independent experiments.

**FIGURE 8.** The BCR is targeted to LAMP-1+ compartments. A–C, CH27 cells were pulsed with 35S-Met/Cys for 15 min, washed, and chased for 4 h in 15% CM. Then, cells were pulsed with anti-Ig-HRP for 15 min at 37°C, washed, and chased for 0–120 min. D–F, Cells were pulsed with 35S-Met/Cys and anti-Ig-HRP for 15 min, washed, and chased for 60–120 min. The cells were treated with DAB plus H2O2 or DAB alone, cleared of insoluble protein aggregates, and immunoprecipitated for LAMP-1 with the mAb 1D4B. Immunoprecipitates were subjected to SDS-PAGE and radiography. A and D, Representative autoradiographs. The region of the gel containing the mature LAMP-1 was quantified for each sample and is given as the amount of LAMP-1 relative to that present at the earliest time point treated with DAB in the absence of H2O2 (B) or as the percent of total LAMP-1 (E). The percent reduction at each time point is also plotted in C and F. The values represent an average of the results of two (A–C) or three (D–F) independent experiments.

To verify that anti-Ig-HRP-catalyzed DAB polymerization occurred only within mIg-containing vesicles in the endocytic system, the effect of DAB polymerization on mtHSP70 was investigated. As for most mitochondrial proteins, mtHSP70 is encoded in a nuclear gene, which is translated in the cytosol and subsequently transported into mitochondria (36). CH27 cells were incubated with 35S-Met/Cys and anti-Ig-HRP for 15 min, washed, and chased for 60–180 min. The cells were treated with DAB in the presence or absence of H2O2, lysed, and the lysates cleared of protein polymers and immunoprecipitated using the mAb JG-1, specific for mtHSP70. No reduction in the level of mtHSP70 was observed at any time following the internalization of anti-Ig-HRP (Fig. 10). Thus, there appears to be little, if any, unrestricted polymerization of proteins in the cell preparations outside of the compartments in which mlg is targeted.

**The trafficking of HRP taken up by fluid phase pinocytosis**

Previous studies showed that CH27 cells process and present Ags taken up by fluid phase pinocytosis, although less efficiently than Ags internalized bound to the BCR (37). Thus, Ags taken up by
fluid phase pinocytosis presumably reach the IiPLC. Consequently, it was of interest to determine whether the intracellular trafficking of Ag taken up by fluid phase pinocytosis was the same as that of Ag targeted by the BCR. To do so, CH27 cells were pulsed with HRP for 15 min and chased for 0–180 min. Because the processing and presentation of Ags taken up by the BCR is known to be more efficient than that of Ags internalized by fluid phase pinocytosis, CH27 cells were incubated with >300 times the molar amount of HRP than the anti-Ig-HRP used in the studies described above. The ability of HRP to catalyze the DAB polymerization of TIR, β-hexosaminidase, LAMP-1, class II, SLIP, and Igα was tested as described above. A summary of the results obtained is presented and compared with the results obtained above for anti-Ig-HRP-catalyzed DAB polymerization. Both HRP and anti-Ig-HRP show the same time course of encounter with the TIR, contacting the TIR early during the first 30 min after internalization and losing contact thereafter (Fig. 11). The overall shape of the curve with time was similar for HRP and anti-Ig-HRP, although HRP resulted in somewhat less cross-linking of the TIR. Similarly, both HRP and anti-Ig-HRP enter β-hexosaminidase-containing compartments by 90–120 min after internalization (Fig. 11). Although the fluid phase HRP results in a somewhat lower maximal level of reduction of β-hexosaminidase as compared with anti-Ig-HRP, this result indicates that there is sufficient enzymatically active HRP in the cells after 180 min of chase to catalyze the cross-linking of a late endosomal/lysosomal protein. Significantly, HRP never reaches compartments that contain LAMP-1, class II molecules, or SLIP. Thus, there was no measurable reduction in the amounts of these proteins at any time after internalization of HRP up to 180 min of chase. These results indicate that HRP does not reach the class II-containing compartment to which anti-Ig-HRP is targeted, at least not in an enzymatically active form. Significantly, this finding suggests that the trafficking of pinocytosed Ag is not identical to that of Ag bound to the BCR.

To determine directly if HRP and the BCR traffic in the same vesicles, the ability of HRP to catalyze the DAB cross-linking of Igα was tested. The results showed that HRP contacted surface biotinylated Igα early after pulsing resulting in a 30–40% reduction but lost contact with Igα after 30 min, resulting in background levels of reduction (Fig. 11). Similar results were obtained in cells in which the BCR was cross-linked using anti-Ig during the HRP pulse (data not shown). Taken together, these results indicate that HRP and anti-Ig-HRP enter into TIR+ compartments together but diverge in their pathways thereafter. This result also indicates that the β-hexosaminidase+ compartment to which HRP is trafficked is not the same β-hexosaminidase+ compartment to which the BCR is targeted.

**Discussion**

In this report, we described the pathway of accelerated intracellular targeting of Ag by the BCR. Taken together, results of IEM and biochemical analysis using a nondestructive chemical polymerization procedure show that, following cross-linking, the BCR is rapidly targeted through early TIR+ endosomes to multivesicular, LAMP-1+, β-hexosaminidase+, class II-containing compartments. Moreover, the class II-containing compartment to which the BCR is targeted appears to be an active site of the assembly of peptide class II complexes and of β2-microglobulin.

Siemasko et al. (38) recently reported that signaling by BCR cross-linking induced redistribution, fusion and, acidification of LAMP-1+ endosomal vesicles to form a single large (>1 μm) vesicular complex in which the majority of intracellular class II molecules resided. The authors suggested that these vesicular complexes were sites of assembly of peptide-MHC complexes. However, we did not observe LAMP-1+ or class II+ multivesicular bodies that approached 1 μm in diameter in either CH27 and
A comparison of the trafficking of anti-Ig-HRP and HRP. The intracellular trafficking of HRP in CH27 cells was analyzed as described above in Figs. 4–9 to follow the intersection of HRP with the TfR (A), β-hexosaminidase (B), LAMP-1 (C), class II (D), SLIP (E), and Igα (F). In each experiment, the percent reduction in the marker proteins in samples treated with DAB and H2O2 as compared with DAB alone was determined and shown (●) as compared with the percent reduction achieved by anti-Ig-HRP (▴) calculated from the results given in Figs. 4–9.

FIGURE 11. A comparison of the trafficking of anti-Ig-HRP and HRP. The intracellular trafficking of HRP in CH27 cells was analyzed as described above in Figs. 4–9 to follow the intersection of HRP with the TfR (A), β-hexosaminidase (B), LAMP-1 (C), class II (D), SLIP (E), and Igα (F). In each experiment, the percent reduction in the marker proteins in samples treated with DAB and H2O2 as compared with DAB alone was determined and shown (●) as compared with the percent reduction achieved by anti-Ig-HRP (▴) calculated from the results given in Figs. 4–9.

splenic B cells (data not shown), suggesting that fusion of endocytic compartments into a giant processing compartment is not a prerequisite for efficient BCR-mediated Ag processing.

In this report, we also provide evidence that the Ag remains bound to the BCR as it trafficks from the plasma membrane to the IIPLC and that both the Ag and the BCR enter the Ag-processing compartments. Previous analysis of the role of the BCR in determining which regions of a protein Ag are ultimately presented by class II molecules predicted a close association of the BCR with the class II molecules in the process of peptide binding (39). The results presented here provide evidence of an opportunity for such contact. The observation that Ag and the BCR traffick together to the IIPLC also suggests that the Ag is not released for processing in earlier endocytic compartments. Although the dense late endocytic compartments are major sites of assembly of peptide-MHC complexes, for a subset of Ags, processing in early endosomes has been clearly documented (40). However, Ag processing in early endosomes appears to involve recycling class II molecules and is II-independent. Zimmerman et al. (41) recently provided evidence that Ag bound to the BCR is not processed in early endosomes by II-independent pathways. The results presented here show that the mIg is shuttled into another pathway to degradation the cell by fluid phase pinocytosis traffick to the class II containing compartments when BCR bound Ag is being targeted for processing. Indeed, Aluvihare et al. (9) recently provided evidence that accelerated trafficking of Ags by the BCR is essential for the presentation of low-affinity, but not high-affinity, Ags. In these studies, the presentation of low-affinity Ags was precluded unless delivery of the Ags was rapid. Thus, the ability of the BCR to rapidly target Ag and attract T cell help may provide a means of monitoring the affinity of the interaction of the BCR and Ag in vivo. Such monitoring may play a crucial role in selection of high-affinity B cells during Ag-driven expansion.

The results presented here also provide evidence that the BCR complex, mIg/Igα/Igβ, remains intact following cross-linking and targeting to the IIPLC. Vilen et al. (18) recently provided evidence that Ag stimulation of B cells resulted in the destabilization of the BCR complex. This finding raises the possibility that the mIg and Igα/Igβ dissociate following Ag binding and that only mIg is targeted to the IIPLC. The results presented here indicate that Igα is associated with mIg in the IIPLC. However, because Igα was identified by directly immunoprecipitating it from cell lysates, we do not know if the BCR is in a destabilized conformation.

Because BCR-mediated Ag processing and presentation is so highly efficient, it is likely the predominant means of Ag processing in vivo. Nonetheless, B cells are clearly able to present some Ags taken up by fluid phase pinocytosis, indicating that Ags entering the cell by fluid phase pinocytosis traffick to the class II peptide-loading compartments. This raises the issue of whether the pathway described here for BCR-targeted Ag delivery is the same for delivery of Ag taken up by fluid phase pinocytosis. One could imagine that it might be beneficial for B cells to exclude, either temporally or spatially, fluid phase proteins from the peptide-loading compartments when BCR bound Ag is being targeted for processing. That the BCR pathway to the IIPLC may be uniquely specified was suggested earlier by the results of Mitchell et al. (42) who showed that mIg that contained mutations in the transmembran domain were internalized following Ag binding and the Ag was degraded but not presented with class II molecules. Thus, mIg appears to contain information in its transmembrane domain necessary for the delivery to the IIPLC and, in the absence of this information, the mIg is shuttled into another pathway to degradative compartments. As stated above, Zimmerman et al. (41) recently provided evidence that the BCR targets Ag to II-class II-containing compartments and bypasses the early endosome previously characterized as an II-independent pathway (40) to which Ags that enter by fluid phase pinocytosis are targeted. The
results presented here show that both the anti-Ig-HRP, which enters the B cell bound to the BCR, and HRP, which enters the cell by fluid phase pinocytosis, are initially internalized into TIR⁺ early endosomes. Similarly, both anti-Ig-HRP and HRP subsequently enter β-hexosaminidase⁺ vesicles marking late endosomes/lysosomes. However, unlike anti-Ig-HRP, which rapidly moves to class II-containing compartments, HRP never reaches class II-containing compartments, at least not in an enzymatically active form, even after chase periods of up to 24 h (data not shown). Moreover, by chemical cross-linking, HRP was shown to lose contact with the BCR 30 min following internalization. This loss of contact and the failure of HRP to cross-link class II molecules would not appear to be simply attributable to the loss of total HRP from the cell with time because there is sufficient HRP activity to cross-link β-hexosaminidase up to 180 min of chase time. However, loss of HRP from the cell could attribute to the failure to detect HRP entry into the IILP. IEM studies by others following the trafficking of gold-labeled proteins provide ample evidence that Ags taken up by fluid phase pinocytosis enter class II-containing, late endocytic Ag-processing compartments (11, 43). However, IEM studies follow the trafficking of gold particles, while, here, we followed the trafficking of the enzymatic activity of the HRP. Thus, if HRP is degraded before entering the IILP, it would not be detected. Taken together, these results suggest that there is a yet undefined mechanism to transport degraded Ag from the site of proteolysis in the endocytic system to the IILP.

In summary, the results presented here describe the intracellular pathway by which the BCR rapidly targets bound Ags for processing and provides evidence that this pathway may not be identical to the pathway by which Ag taken up in receptor-independent mechanisms traffic. Future studies defining the molecular mechanism underlying the accelerated targeting of the BCR to the class II peptide loading compartment may provide significant new insights as to how this process is controlled.

References