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Pond, Leslie; Watts, Colin

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Functional Early Endosomes Are Required for Maturation of Major Histocompatibility Complex Class II Molecules in Human B Lymphoblastoid Cells*

Leslie Pond and Colin Watts‡

From the Department of Biochemistry, Wellcome Trust Building, University of Dundee, Dundee DD1 4HN, United Kingdom

Major histocompatibility complex (MHC) class II molecules are targeted together with their invariant chain (Ii) chaperone from the secretory pathway to the endocytic pathway. Within the endosome/lysosome system, Ii must be degraded to enable peptide capture by MHC class II molecules. It remains controversial exactly which route or routes MHC class II/Ii complexes take to reach the sites of Ii processing and peptide loading. We have asked whether early endosomes are required for successful maturation of MHC class II molecules by using an *in situ* peroxidase/diaminobenzidine compartment ablation technique. Cells whose early endosomes were selectively ablated using transferrin-horseradish peroxidase conjugates fail to mature their newly synthesized MHC class II molecules. We show that whereas transport of secretory Ig through the secretory pathway is virtually normal in the ablated cells, newly synthesized MHC class II/Ii complexes never reach compartments capable of processing Ii. These results strongly suggest that the transport of the bulk of newly synthesized MHC class II molecules through early endosomes is obligatory and that direct input into later endosomes/lysosomes does not take place.

MHC[1] class II molecules are peptide-binding glycoproteins that display a highly diverse set of peptides on the surface of B lymphocytes, dendritic cells, and macrophages. Specific peptide/MHC class II complexes are recognized by $CD4^+$ T cells, for example, to trigger protective immune responses. Extensive studies on the biosynthesis of MHC class II molecules have established that unlike MHC class I molecules, they intersect the endocytic pathway before their appearance on the surface (Refs. 1 and 2; reviewed in Refs. 3–5). This diversion is essential (*a*) to enable proteolytic removal of the invariant chain (Ii, a specialized chaperone), and (*b*) to ensure admixing of MHC class II molecules with processed peptide material from endocytosed exogenous antigens. It is well established that targeting motifs within Ii are responsible for the delivery of MHC class II/Ii complexes to the endocytic pathway (6–10), but it has been more difficult to resolve the precise route taken from the Golgi apparatus to the endosome system. Much of the data on this pathway has been obtained by studying human B lymphoblastoid cells that are potent antigen-presenting cells and express high levels of MHC class II molecules. Earlier studies in these cells have shown that most MHC class II molecules that have assembled with peptide are found in late endosomes or lysosomes before their expression on the cell surface (11–13). This observation suggested that transport might occur directly from the Golgi network to the late endosomes/MHC class II compartments, and several studies appear to support this possibility (14–16). However, reports that some Ii could be detected on the surface of antigen-presenting cells (17) were followed by a direct biochemical demonstration of the transient appearance of newly synthesized class II/Ii complexes on the cell surface (18–20). In addition, a recent reappraisal of the steady-state distribution of Ii, MHC class II molecules, and other markers in human B lymphoblastoid cells indicates the presence of class II/Ii complexes in early endosomes (21). In murine B cells, there is also evidence that newly synthesized MHC class II molecules are found in early endosomes (22, 23) as well as in later compartments (24, 25).

Thus, morphological and biochemical evidence indicates that in human B lymphoblastoid cells, at least a proportion of newly synthesized MHC class II/Ii complexes are found on the cell surface or in early endosomes before Ii processing and peptide loading. However, using these techniques, it is difficult to determine what fraction of molecules travel by this route. Morphology can only reveal steady-state MHC class II/Ii levels that may be low for rapidly traversed sectors of the pathway. Similarly, asynchronous kinetics of MHC class II molecule transport through the secretory and endocytic pathway make it difficult to establish exactly what proportion of molecules must pass through a particular sector. One way of resolving this issue would be to selectively block the early part of the endocytic pathway and to assess the ability of MHC class II molecules to mature in these cells. If traffic through the cell surface or early endosomes is obligatory, maturation should be blocked under these conditions. On the other hand, if MHC class II molecules can target from the Golgi to both early and late endocytic compartments, the ablation of the early part of the pathway should not disrupt trafficking to later elements, and successful MHC class II molecule maturation should occur. To date, this type of approach has given rise to somewhat contradictory results. Using concanamycin B to block transport from early to late endosomes, Benaroch *et al.* (14) showed that newly synthesized MHC class II molecules appeared only slowly on the cell surface and were not accessible to endocytosed neuraminidase. They concluded that the major route was direct transport from the Golgi to late endosomes and/or MHC class II compartments (14). In contrast, Wang *et al.* (26) recently analyzed MHC class II molecule maturation in transfected HeLa

cells expressing a dominant negative mutant of the GTPase dynamin, which is required for endocytosis through clathrin-coated pits. In these cells, MHC class II molecules failed to mature, indicating that transport via the cell surface was the major route followed by newly synthesized MHC class II molecules (26). Because of these contradictory results, and because of the possibility that trafficking of ectopically expressed MHC class II molecules in cells such as HeLa might be different from trafficking in *bona fide* antigen-presenting cells, we have reassessed the importance of the early endosome system in MHC class II maturation in human B lymphoblastoid cells. To do this, we have used a peroxidase-ablation technique that allows selected domains of the endocytic pathway to be inactivated in intact cells. Here we show that ablation of early endosome function in human EBV-B cells prevents detectable maturation of MHC class II molecules as measured by Ii processing and peptide binding. These results are consistent with a model that involves obligatory passage of MHC class II molecules through the early endosome system before transport to the sites of Ii processing and peptide loading.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human EBV-transformed cell lines FC4 and EDR have been described previously (12). Pala cells were a kind gift from P. Cresswell. All cells were maintained in RPMI 1640 medium or in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented as described previously (27).

Metabolic Labeling—Cells were pre-incubated in methionine and cysteine-free medium (MEM; Sigma) for 15–30 min at 37 °C before labeling. ^{35}S-Translabel (Amersham Pharmacia Biotech) was added to a final concentration of 0.5–1.0 mCi/ml for 15–30 min as indicated, and then the cells were washed in DHB. The cells were resuspended in DHB containing 2 mM methionine and, where indicated, 10 μg/ml transferrin-horseradish peroxidase (Tf-HRP) conjugate at 37 °C. In some experiments, leupeptin (Sigma) was included at a final concentration of 1 mM. After Tf-HRP loading, the cells were collected by centrifugation and resuspended in DHB for peroxidase-mediated ablation.

Transferrin peroxidase-mediated Ablation—Human transferrin was conjugated to HRP exactly as described previously (27). The Tf-HRP conjugate was used at 10 μg/ml and loaded at 37 °C in DHB medium for 20 min or for the times indicated. Peroxide and DAB-mediated ablation was performed essentially as described previously (27), except that the removal of surface-bound Tf-HRP by acid treatment was made unnecessary by the use of ascorbic acid to quench any extracellular peroxidase activity (28). Cells (10^7 cells/ml) were resuspended in 85 mM NaCl, 50 mM ascorbic acid, 20 mM Hepes, pH 7.4, and 0.01% H_2O_2, with or without 1.5 mg/ml DAB. The two aliquots were incubated in the dark for 30 min at 0 °C. After peroxidase-mediated ablation, the cells were washed extensively and either prepared for immunoprecipitation (see below) or, in some experiments, subjected to an additional chase at 37 °C in complete Iscove's medium supplemented with 2 mM methionine. The viability of the cells after the peroxidase-mediated ablation and subsequent chase steps was ~90%.

MHC Class II Immunoprecipitation and Capture of Secreted Ig—For precipitation of MHC class II complexes, cells were lysed at 2×10^7 cells/ml in lysis buffer as described previously (12). After centrifugation (14,000 rpm, 2 min, 4 °C) to remove nuclei and cell debris, the supernatants were subjected to pre-clearing with Pansorbin (Calbiochem). In some experiments, a second pre-clearing step was performed with protein G-Sepharose (Amersham Pharmacia Biotech). The pre-cleared lysates were incubated with mAb DA6.231 (29) for 1 h at 0 °C followed by protein G-Sepharose for 1 h. The washed immunoprecipitates were eluted in SDS sample buffer at 25 °C or at 95 °C, as indicated, and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (27). For reprecipitation of MHC class II-associated Ii molecules, washed DA6.231 immunoprecipitates were resuspended in 50 μl of phosphate-buffered saline/1% SDS and eluted at 95 °C for 5 min. The eluted proteins were transferred to 950 μl of lysis buffer, and Ii molecules were immunoprecipitated using mAb VIC-Y1 (30). To capture Ig secreted during chase incubations, cells were removed by centrifugation, and the supernatants (~1.0 ml) were incubated at 4 °C for 1 h with protein A-Sepharose or fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem). The beads or bacteria were washed several times in Tris-buffered saline containing 1% Triton X-100 and once in Tris-buffered

Chase time (min): 0 30 60 90 120 120 | 0 30 60 90 120
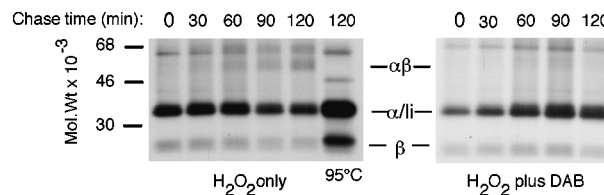
FIG. 1. **Inactivation of early endosomes blocks the appearance of SDS-stable MHC class II dimers.** EBV-B cells (clone FC 4) were labeled with ^{35}S-Translabel for 30 min, chased in the presence of Tf-HRP for an additional 30 min, and then incubated at 4 °C with H_2O_2 plus (*right panel*) or minus (*left panel*) DAB for 30 min (see "Materials and Methods" for further details). The cells were reincubated for the times shown before immunoprecipitation of MHC class II molecules.

saline before the elution of immunoglobulin into SDS sample buffer.

Microscopy—Fluorescence microscopy of peroxidase-ablated cells was performed essentially as described previously (27). After peroxidase-mediated ablation as described above, the cells (5×10^4 cells in 0.5 ml) were resuspended at 10^6 cells/ml, and aliquots (0.05 ml) were spun onto a microscope slide in a Cytospin centrifuge (800 rpm, 4 min; Shandon Scientific, Runcorn, United Kingdom). The cells were fixed in 3.7% HCHO, quenched, and permeabilized and pre-blocked with 0.2% saponin and 0.2% fish skin gelatin as described previously (27). Cells were incubated with primary antibodies diluted in saponin/fish skin gelatin/phosphate-buffered saline for 30 min and then washed extensively before incubation with fluorescein isothiocyanate-conjugated secondary antibodies. Antibodies were as follows: (*a*) transferrin receptor, mAb OKT9; (*b*) MHC class II, mAb DA6.231; and (*c*) TGN-46, sheep antiserum to human TGN-46 (final concentration, 10 μg/ml; a kind gift of S.Ponnambalam). Secondary antibodies were fluorescein isothiocyanate-coupled donkey anti-mouse or anti-sheep Ig (Jackson Laboratories). The cells were mounted in Citifluor and viewed on a Zeiss Axioplan microscope. Images were recorded using a Kodak DCS 420 digital camera and processed using Adobe Photoshop software.

RESULTS

Ablation of Early Endosomes Abrogates the Formation of SDS-stable Peptide-loaded MHC Class II Molecules—To assess whether or not functional early endosomes are required for newly synthesized MHC class II molecules to reach the sites of Ii processing and peptide loading, we used a peroxidase-mediated compartment ablation technique. As described previously, this technique allows selective ablation within intact cells of those compartments to which HRP has been targeted (12, 27, 31, 32). For example, Tf-HRP conjugates selectively ablate early endosomes and recycling endosomes. The DAB polymerization reaction is controlled in all experiments by the omission or inclusion of DAB monomer. We asked first whether newly synthesized MHC class II molecules could mature to yield SDS-stable αβ dimers in cells lacking functional early endosomes. The human EBV-transformed B cell line FC4 was pulse-labeled with [^{35}S]methionine/cysteine and then loaded with Tf-HRP for 30 min. Cell aliquots were exposed to DAB alone or to DAB plus H_2O_2 at 0 °C and then chased at 37 °C for different times. As shown in Fig. 1, cells chased in the presence of H_2O_2 alone showed a band of approximately 60 kDa that increased in intensity during the chase and disappeared upon heating the sample to 95 °C. However, in cells exposed to H_2O_2 and DAB to allow polymer formation, SDS-stable dimers failed to appear during the chase. This result suggested that functional early endosomes are required for successful MHC class II molecule maturation.

Selective Ablation of Early Endosomes by Tf-HRP Conjugates—To confirm that compartment ablation had been selective in these cells, we assessed the integrity of different compartments by fluorescence microscopy. Cells loaded with Tf-HRP and exposed to H_2O_2 alone showed staining for intracellular transferrin receptor, the trans-Golgi marker TGN-46, and MHC class II molecules (Fig. 2, *a*, *c*, and *e*). Cell surface staining for transferrin receptors and MHC class II
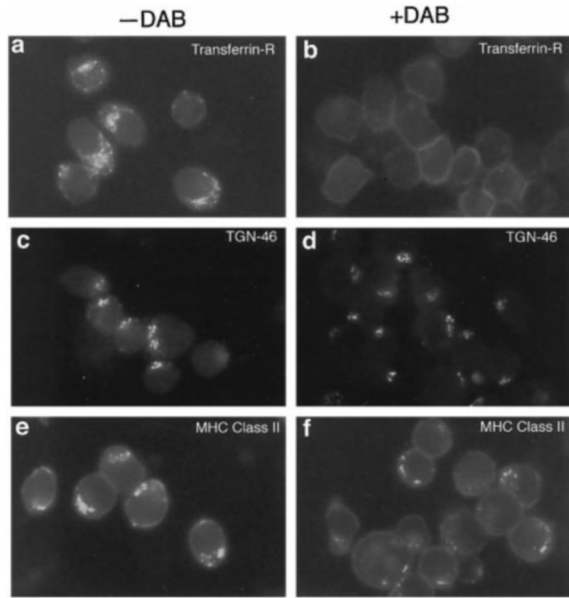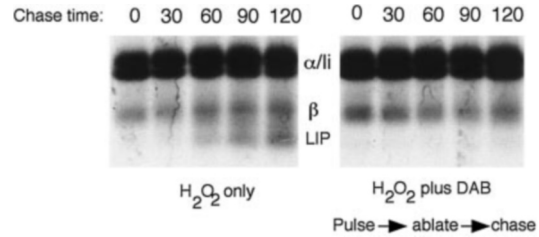
FIG. 2. **Transferrin peroxidase selectively ablates early endosome compartments.** Pala cells were incubated with Tf-HRP for 20 min at 37 °C, washed, and then treated with H_2O_2 with (*b, d,* and *f*) or without (*a, c,* and *e*) DAB. The cells were then processed for fluorescence microscopy as described under "Materials and Methods" and stained for transferrin receptor (*a* and *b*), TGN-46 (*c* and *d*), or MHC class II (*e* and *f*). Note the ablation of intracellular transferrin receptor staining (*b*). Intracellular TGN-46 staining and MHC class II staining remain unaffected.

FIG. 3. **The leupeptin-induced Ii peptide (*LIP*) does not appear in cells lacking functional early endosomes.** EBV-B cells (clone EDR) were labeled with ^{35}S-Translabel in the presence of 1 mM leupeptin. After 15 min, the cells were washed and resuspended in Tf-HRP plus leupeptin. After a 30-min loading at 37 °C, the cells were divided and incubated at 4 °C in H_2O_2 with or without DAB. After further incubation at 37 °C for the times shown, MHC class II molecules and associated Ii fragments were immunoprecipitated with the DA6.231 mAb.

molecules was also observed. In contrast, in cells incubated with H_2O_2 and DAB, intracellular staining of transferrin receptors could no longer be detected (Fig. 2*b*). Importantly, both intracellular MHC class II and TGN-46 labeling were unaffected by Tf-HRP-catalyzed DAB polymerization, demonstrating selective ablation of a defined sector of the endocytic pathway (Fig. 2, *d* and *f*) and making it unlikely that the failure of MHC class II molecules to mature was simply due to the ablation of the Golgi apparatus by low levels of transferrin receptor trafficking through this compartment (33). Quantitative immunoblotting for TGN-46 confirmed that there was no loss of this marker in ablated cells (data not shown).

Early Endosomes Are Required for Class II/Ii Complexes to Reach Proteolytically Active Compartments—A requirement for early endosomes in MHC class II molecule maturation could be explained either by a specific requirement for MHC class II molecule traffic through these compartments or by a requirement for the delivery of some other factor, for example, processed peptide material, to downstream sites of MHC class II molecule maturation. In the latter case, early endosomes would still be required even if the targeting of newly synthesized MHC class II molecules themselves was direct. To analyze the transport and maturation of MHC class II molecules in a way that did not depend on peptide availability and SDS-stable dimer formation, we asked whether Ii processing was taking place in cells lacking functional early endosomes. We took advantage of the fact that defined intermediates in Ii processing can be amplified in cells treated with leupeptin. In particular, an Ii-processing intermediate (leupeptin-induced peptide) of around 22 kDa accumulates in leupeptin-treated cells (34). As before, pulse-labeled EBV-B cells were loaded with Tf-HRP and then incubated with H_2O_2 in the presence or absence of DAB. To amplify Ii-processing intermediates, the cells were additionally incubated in the presence of leupeptin. As expected, under these conditions, we could not detect the accumulation of SDS-stable dimers (data not shown). Instead, after

60 min of chase, we observed the accumulation of the Ii-derived leupeptin-induced peptide fragment in Tf-HRP-loaded cells that had been treated with H_2O_2 alone after Tf-HRP loading but not in cells treated with DAB and H_2O_2. Thus, in cells lacking functional early endosomes, class II/Ii complexes cannot access the sites of Ii processing.

Passage through Early Endosomes Is Required for Normal Ii Processing in Pala Cells—To substantiate the above finding (and to rule out the possibility that leupeptin failed to reach the relevant compartments in the ablated cells), we analyzed the processing of Ii/MHC class II complexes in Pala cells in which Ii-processing intermediates can be readily detected even in the absence of protease inhibitors (for example, see Ref. 35). As shown in Fig. 4*a*, a particularly prominent fragment migrating with an apparent size of ~14 kDa was readily observed, was most prominent after 150 min of chase, and declined thereafter. To confirm that this fragment arose from the N terminus of Ii, we treated immunoprecipitates of MHC class II molecules at 95 °C and reprecipitated them with VIC-Y1 antibody, which is specific for the N-terminal cytosolic domain of Ii (30). As shown in Fig. 4*b*, the p14 fragment (as well as larger forms of Ii) was precipitated under these conditions, confirming that it is an N-terminal fragment of the Ii.

We next established the location of the MHC class II molecules associated with this p14 Ii fragment with respect to the early endosome system. Cells were pulse-labeled, chased for different times in the presence of Tf-HRP, and then incubated at 0 °C in the presence of H_2O_2 with or without DAB. As shown in Fig. 4*a*, ablation of early endosomes using this protocol did not result in any significant loss of the p14 Ii fragment, indicating that it was accumulating downstream of early endosomes, as defined by the itinerary of the recycling Tf-HRP conjugate (Fig. 4*a*). This is fully consistent with our earlier data that showed that the majority of both biochemically detectable SDS-stable MHC class II dimers and peptide/class II complexes detectable by T cells are found in late endosomes/lysosomes before expression on the cell surface (12, 27). However, when early endosomes were ablated after pulse labeling but before the chase (pulse-ablate-chase protocol), the appearance of both the Ii p14 fragment and an Ii fragment around 22 kDa was completely abolished (Fig. 5*a*). Quantitation of a second experiment clearly showed that neither of these Ii fragments is produced in cells lacking functional early endosomes (Fig. 5*b*). However, transport of class II/Ii complexes through the Golgi complex still occurred, as indicated by the conversion of oligosaccharides on the MHC class II β chain from their immature to mature state (Fig. 5, *b* and *c*).

To demonstrate unequivocally that selective ablation of early endosomes accounted for the failure of MHC class II molecules

Early Endosomes and MHC Class II Maturation

a

b
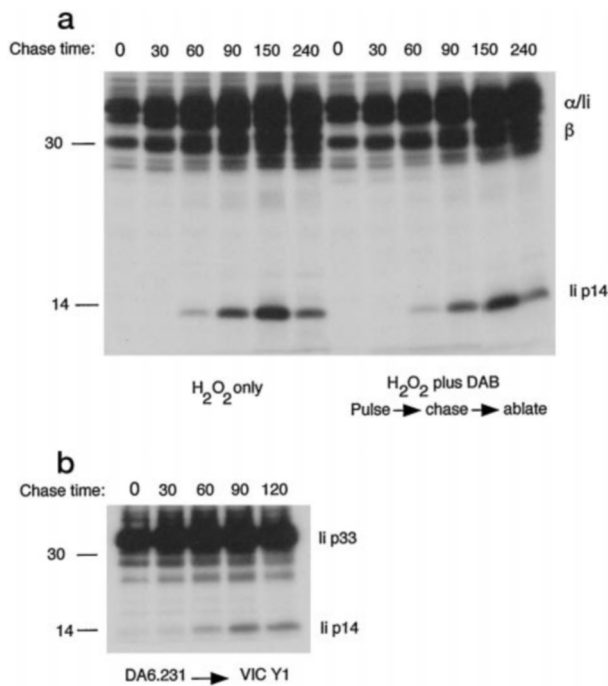
FIG. 4. **The natural Ii-processing intermediate p14 is not found within early endosomes**. *a,* Pala cells were labeled with [35S]methionine for 30 min and chased at 37 °C for the times shown. Tf-HRP was added for the last 30 min for each time point. Each sample was divided and incubated with H_2O_2 with or without DAB. MHC class II and associated Ii fragments were precipitated with the DA6.231 mAb. *b,* DA6.231 precipitates from non-ablated cells (no DAB) processed as described in *a* were reprecipitated with VIC-Y1 antibody. Intact and p14 fragments of Ii were recovered.

to mature, we asked whether secretory protein traffic could still occur in the HRP-ablated cells. After first establishing that Pala secretes immunoglobulin into the medium like other EBV-transformed cell lines, we pulse-labeled cells with [35S]methionine/cysteine, loaded them with Tf-HRP, and ablated the Tf-HRP-positive compartments as described above. After reincubation at 37 °C for different times, both the cells and the reincubation medium were retained for separate analysis. Any Ig secreted into the medium during the chase was captured on protein A and analyzed on a 10% SDS gel. As shown in Fig. 6*a*, radiolabeled Ig heavy and light chains appeared in the medium during the chase from cells incubated in H_2O_2 alone but also, importantly, from cells incubated in H_2O_2 and DAB. This demonstrates that compartment ablation under our conditions of Tf-HRP loading is confined to early endosomes and does not extend into the secretory pathway. Ablation of early endosomes in this experiment was successful because in the same cells, we observed, as before, a virtually complete blockade in Ii processing (Fig. 6*b*). Quantitation of Ig secretion in several experiments indicated that there was a lag in the ablated cells relative to the non-ablated cells (Fig. 6*b*). We do not know the reason for this, but one possibility is that the ablation of the early endosome system interferes with the recycling of soluble or membrane-bound proteins that may be needed for optimal secretory pathway traffic. In any case, it is clear that the secretory pathway is still functional in the ablated cells, as judged by both β chain maturation and by Ig secretion. Taken together, these data demonstrate that a functional early endosome system is required for the successful maturation of newly synthesized MHC class II molecules. This is true despite the fact that the products of maturation (Ii-processing intermediates and peptide/$\alpha\beta$ dimers) accumulate downstream of these compartments.

a

b

c
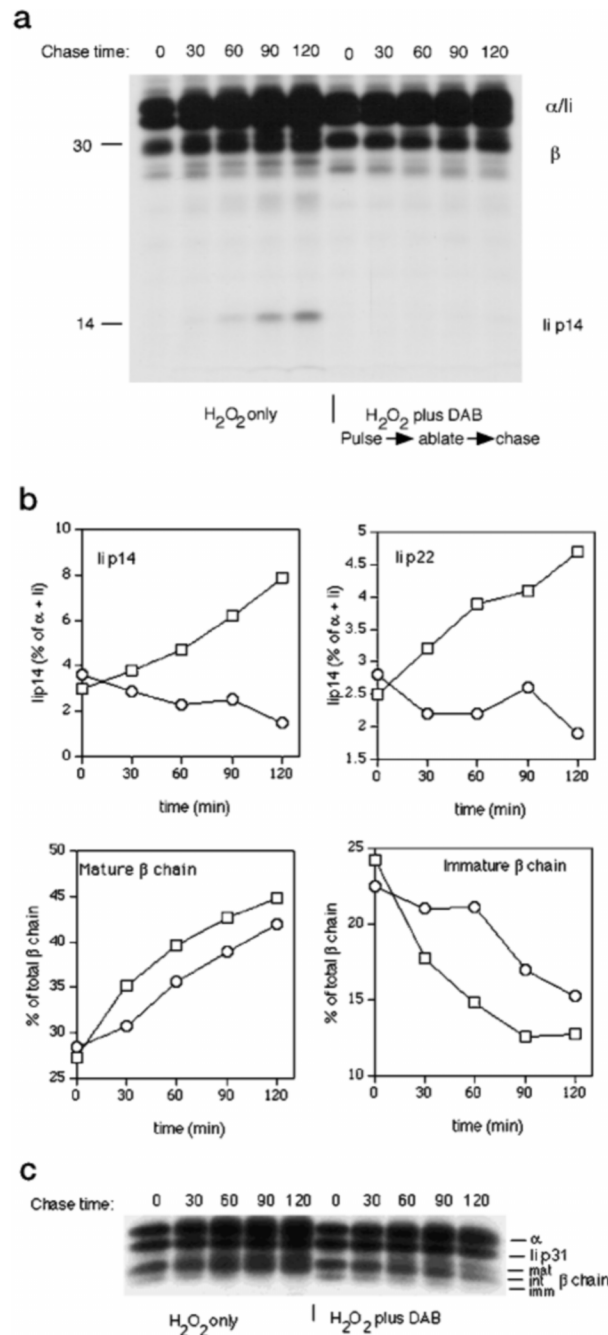
FIG. 5. **Evidence that early endosomes must be negotiated before Ii processing in later endosomes.** *a,* Pala cells were labeled with 35S-Translabel for 30 min, washed, and incubated for an additional 30 min in Tf-HRP. Each sample was divided and incubated with H_2O_2 with or without DAB. After further incubation at 37 °C for the times shown, MHC class II and associated Ii fragments were precipitated with mAb DA6.231. *b,* quantitation of an experiment performed identically to that shown in *a*. The bands corresponding to Ii p14, Ii p22, and immature and mature β chain were quantitated using a phosphorimager from cells that had been treated with H_2O_2 with ($\bigcirc$) or without ($\square$) DAB. *c,* portion of the gel showing β chain maturation used to obtain the quantitation shown in *b* above. Immature, intermediate, and mature forms of the β chain can be seen.

DISCUSSION

Several previous studies have investigated how newly synthesized MHC class II/Ii complexes are delivered to the endocytic pathway. As outlined under "Introduction," class II/Ii complexes have been detected not only in later endosomal and lysosomal compartments, but also at much lower levels in early endosomes and, indeed, on the cell surface (17–19, 22, 36, 37).
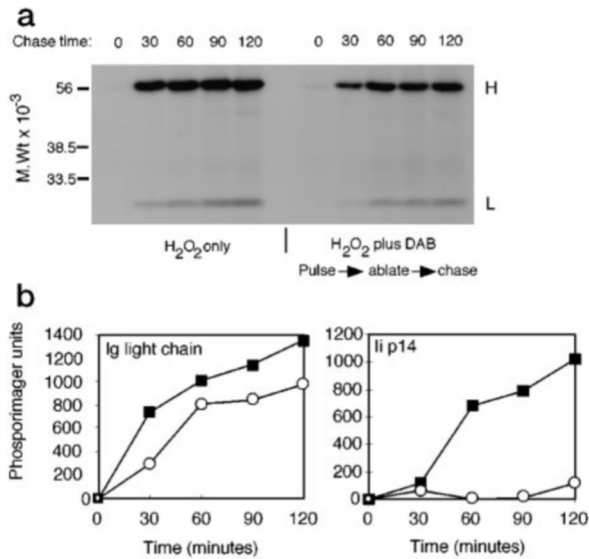
FIG. 6. **The secretory pathway is still functional in Tf-HRP loaded and ablated B cells.** *a,* Pala cells were labeled and processed as described under "Materials and Methods" and the Fig. 5 legend, except that each culture supernatant generated during the chase was retained. After the removal of cells and cellular debris, secreted Ig was captured on fixed *S. aureus* cells and analyzed on 10% SDS gels. *H* and *L* denote Ig heavy and light chains. *b,* quantitation of Ig light chain appearance in the medium of Tf-HRP-loaded cells treated with H_2O_2 with (○) or without (■) DAB. The appearance of Ii p14 in the same cells was also monitored as described in the Fig. 5 legend. Quantitation of gels was performed using a phosphorimager.

This has led to a model whereby at least some MHC class II/Ii complexes leaving the Golgi apparatus are transported initially to the earliest parts of the endocytic pathway and/or the cell surface. However, other evidence indicates that MHC class II/Ii complexes are directly targeted to later endosomal and lysosomal compartments (13–16). Here, we have used a new approach to address this important question. Selective inactivation or ablation of intracellular compartments can be achieved by targeting HRP to those compartments. This has been achieved on the secretory pathway by expression of a secreted form of HRP (38) and in the endocytic pathway by endocytosis of HRP conjugated to transferrin or other endocytosed ligands including antigens (12, 27, 31, 32). By functionally inactivating the earliest compartments of the endocytic pathway in this way, we have been able to ask whether or not it is required for successful MHC class II/Ii maturation. We find that the invariant chain is not processed and that SDS-stable class II/peptide dimers do not assemble in cells whose early endosomes have been inactivated. The possibility that our internalized Tf-HRP conjugate functionally inactivated later endosomes or lysosomes is rendered very unlikely by our earlier demonstration that this conjugate recycles efficiently (27), does not ablate the major cellular MHC class II compartments (Ref. 12 and Fig. 2), and does not affect compartments in which processed fragments of Ii accumulate (Fig. 4*a*). Moreover, whereas Tf-HRP can access the Golgi complex in some cell types (33), this did not occur under the conditions of loading we used because in Tf-HRP-loaded and ablated cells, immunodetection of the trans-Golgi marker TGN-46 was still observed, oligosaccharide maturation on MHC class II β chains still occurred, and immunoglobulin was still secreted into the culture medium.

Taken together with our own earlier data (12, 27), this result suggests a model for MHC class II maturation in human B cells (Fig. 7). Three principal points can be made. First, as shown previously (12) and in this study (Fig. 4*a*), the products of MHC class II maturation, namely, invariant chain processing inter-
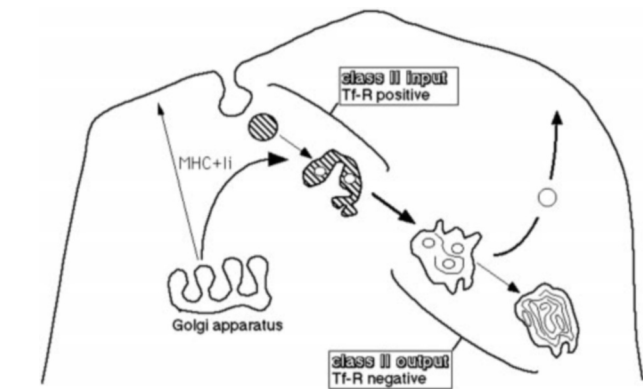
FIG. 7. **Route map for MHC class II maturation in human B cells.** The diagram is based on data obtained in the present study and data in Refs. 12 and 27 using the same Tf-HRP ablation technique. Endosomes that can be ablated are indicated by *cross-hatched bars*. Most MHC class II/peptide complexes accumulate downstream of early, transferrin receptor-positive endosomes and reach the surface without passing through early endosomes. However, delivery of newly synthesized MHC class II/Ii complexes occurs early in the pathway and, as shown by others, probably via the cell surface in some cases. The figure indicates the likely route taken by most newly synthesized molecules: the itinerary of recycling MHC class II molecules is omitted but likely involves passage primarily through the early transferrin receptor-positive endosomes.

mediates and SDS-stable $\alpha\beta$ dimers, accumulate, for the most part, downstream of transferrin-positive early endosomes, as judged by their resistance to Tf-HRP-mediated cross-linking. Second, these early endosomes were not necessary for the transport of assembled peptide/$\alpha\beta$ complexes to the cell surface, as measured by the biologically relevant assay of T-cell stimulation (27). Third, despite this lack of involvement of early endosomes in the later stages of MHC class II/peptide assembly and surface transport, they are nonetheless an essential gateway through which the majority of newly synthesized MHC class II molecules must pass to reach the sites of Ii processing and peptide loading. We therefore propose that the points of entry and exit of newly synthesized MHC class II molecules along the endocytic pathway are different. Newly synthesized MHC class II/Ii complexes are delivered primarily into early transferrin receptor-positive domains but leave, once peptide-loaded, primarily from later transferrin receptor-negative domains (Fig. 7). As also documented by others, transport of MHC class II molecules between these domains is accompanied by Ii processing and peptide loading (18–25).

Recently, Kleijmeer *et al.* (21) have reassessed the steady-state distribution of MHC class II molecules, Ii, and other markers of the endocytic pathway in both human and murine B-cell lines. In a human EBV-transformed B-cell line similar to those used in our study, they defined up to six different types of structures involved in MHC class II maturation. Their analysis revealed Ii, presumably associated with MHC class II, in the so called "early" MHC class II compartments and, to some extent, in conventional early endosomes (21). They proposed that MHC class II/Ii complexes leaving the Golgi apparatus target to a variety of endosomal structures. Our results are consistent with this study and with other studies that reported the presence of newly synthesized MHC class II molecules in low-density, early endosomes and/or on the cell surface (19–23, 37). The requirement that we observe for functional transferrin receptor-positive endosomes now suggests that the input of MHC class II/Ii does not occur later than type 3 structures as defined by Kleijmeer *et al.* (21). MHC/Ii complexes found in later compartments must therefore traffic there from earlier transferrin-positive endosomes.

Our results are more difficult to reconcile with studies that

indicate that direct transport of MHC class II/Ii complexes to later endocytic compartments occurs. In part, this might be explained by the difficulties noted above of detecting rapid passage through compartments in which MHC class II molecules do not accumulate. When traffic from early to late endosomes was disrupted by the vacuolar H^+-ATPase inhibitor concanamycin B, MHC class II molecules did not appear to accumulate in early endosomes, as might have been expected if early endosomes are the principal target of post-Golgi vesicles carrying class II molecules (14). Conceivably, concanamycin B interferes not only with normal early to late endosome traffic, but also with normal fusion of Golgi-derived vesicles with early endosomes. This could explain why Ii degradation was strongly inhibited in concanamycin B-treated cells and why the accumulated MHC class II/Ii complexes were not accessible to endocytosed tracers (14).

The compartment ablation technique we have used cannot easily determine what proportion of newly synthesized complexes reach early endosomes via the cell surface or, instead, directly from the Golgi apparatus. Using a mutant of dynamin that blocks clathrin-coated pit function, Wang *et al.* (26) recently provided convincing evidence that a majority of complexes in transfected HeLa cells reach early endosomes via the cell surface. It remains to be established whether this is also the case in *bona fide* antigen-presenting cells such as B lymphocytes or whether both routes are used to direct newly synthesized MHC class II molecules to early endosomes, as indicated by other studies (37, 41). Passage through the early endosome system via the cell surface or by direct transport from the Golgi apparatus to early endosomes should ensure exposure of the maturing population of MHC class II molecules to the full range of processed antigenic material. The fact that lysosomal hydrolases also appear to be targeted initially to the earliest parts of the endocytic pathway (39, 40) should facilitate such a scenario.

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