# Trafficking of spontaneously endocytosed MHC proteins

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**Class I MHC protein primarily presents endogenous antigen but also may present exogenous antigen. Here, we investigated the intracellular pathway of spontaneously internalized class I MHC protein by confocal microscopy. $\beta_2$-microglobulin ($\beta_2$m), labeled with a single fluorophore, was exchanged at the surface of B cell transfectants to specifically mark cell surface and endocytosed class I MHC protein. Intracellular $\beta_2$m colocalized with fluorophore-conjugated transferrin, implying that class I MHC protein endocytosed into early endosomes. These endosomes containing fluorescent $\beta_2$m were found close to or within the Golgi apparatus, marked by fluorescent ceramide. Even after 24 hr of incubation, very little fluorescent $\beta_2$m was found in intracellular organelles stained by $DiOC_6$, marking the endoplasmic reticulum, or fluorophore-conjugated low density lipoprotein, marking late endosomes and lysosomes. Fluorophore-conjugated superantigens (staphylococcal enterotoxin A and B), presumed to enter cells bound to class II MHC protein, also were found to endocytose into $\beta_2$m-containing early endosomes. Staining with mAb and use of transfectants expressing MHC protein attached to green fluorescent protein confirmed the presence of intracellular compartments rich in both class I and II MHC protein and demonstrated that class I and II MHC protein also colocalize in discrete microdomains at the cell surface. These cell surface microdomains also contained transferrin receptor and often were juxtaposed to cholesterol-rich lipid rafts. Thus, class I and II MHC protein meet in microdomains of the plasma membrane and endocytose into early endosomes, where both may acquire and present exogenous antigen.**

Class I MHC protein is expressed on most mammalian cell types and presents peptides for T cell immunosurveillance. Class I MHC protein principally presents peptides derived from endogenous protein to cytotoxic T cells (1). However, in certain antigen-presenting cells, peptides derived from exogenous antigen also are presented by class I MHC protein (2–4). At least three independent pathways of protein processing and subsequent peptide presentation by class I MHC protein have been described (ref. 5 and references therein).

The dominant pathway uses endogenously synthesized proteins that have been processed in the cytosol by proteasomes. Peptides are transferred by TAP (transporter associated with antigen processing) to the endoplasmic reticulum (ER) where they bind in the grooves of nascent class I MHC protein. The peptide/class I MHC heavy chain/$\beta_2$-microglobulin ($\beta_2$m) complex then is transported via the Golgi apparatus to the cell surface. TAP-deficient cell lines are severely impaired in class I MHC-mediated antigen presentation (6, 7), providing evidence that this pathway is indeed dominant for class I MHC presentation. In a second pathway, exogenous antigen is internalized and processed into peptides that are transported to the ER to bind class I MHC protein (8, 9). However, at least some exogenous peptide appears to reach class I MHC protein through a pathway completely independent of the ER (10).

In this third pathway, endosomal processing and endocytosed class I MHC protein may be involved. Class I MHC protein has been shown to internalize from the cell surface in T cells, B cells, fibroblasts, and macrophages (11–16), despite earlier studies suggesting that MHC molecules are not internalized at all (17, 18). Endocytosis is cell-specific in that only subsets of some cell types endocytose class I MHC protein efficiently (19). Recycling of endocytosed class I MHC protein back to the cell surface also has been observed (13).

The endocytosis and recycling of class I MHC protein may facilitate peptide exchange, allowing class I MHC protein to bind multiple peptides in one lifetime. Indeed, at the acidic pH of endosomes, peptide exchange occurs readily (20). Also, recycling class II MHC protein has been shown to bind peptide in endosomes (21, 22). Endocytosis of class I MHC protein has been followed by gold-conjugated antibody (14, 15), antibody-coated liposomes (23), and fluorescent antibody (24). mAbs to class I and class II MHC proteins also have been shown to be in close proximity at the cell surface by using fluorescence resonance energy transfer techniques and scanning force microscopy (25–28). However, artifacts may arise because of antibody crosslinking and intracellular dissociation of antibody from substrate MHC protein.

During the preparation of this manuscript, green fluorescent protein (GFP)-linked-class I MHC protein was reported to accumulate in HLA-DR+ and CD63(tetraspan protein)+ endosomes, termed MIIC, in IFN-$\gamma$-stimulated melanoma cells (29). Importantly, exogenous antigen presentation could be inhibited by raising endosomal pH. The class I MHC protein in the MIIC compartment was presumed to be endocytosed from the cell surface as accumulation in compartments was insensitive to brefeldin A (an inhibitor of ER-Golgi transport).

Here, spontaneously endocytosed class I MHC protein has been directly labeled with exogenously exchanged fluorescent $\beta_2$m (20, 30, 31). In particular, a mutant of $\beta_2$m was prepared in which a cysteine was substituted for a serine, distal to the region where $\beta_2$m associates with class I MHC heavy chain, that was then labeled with a single Texas red (TR) or Alexa 488 fluorophore. This $\beta_2$m was exchanged into class I MHC protein at the B cell surface and imaged by confocal microscopy. In addition, using fluorophore-conjugated superantigen, mAb, and transferrin, we show that class II MHC protein internalizes together with class I MHC protein. Furthermore, both class I and class II MHC proteins were found to accumulate in specific membrane microdomains at the cell surface that contain transferrin receptor and are juxtaposed to cholesterol-rich lipid rafts.

## Materials and Methods

**Mutation and Fluorescent Labeling of $\beta_2$m.** cDNA encoding $\beta_2$m was mutated by PCR so that serine at residue 88 was replaced by cysteine and expressed in *Escherichia coli* as described (31).

Abbreviations: $\beta_2$m, $\beta_2$-microglobulin; ER, endoplasmic reticulum; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; GFP, green fluorescent protein; TR, Texas red; LDL, low density lipoprotein; 221, 721.221 cells.

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Briefly, after purifying the inclusion body from *E. coli* in 8 M urea, the protein was refolded by dilution to 2 mM in 0.4 M L-arginine, 2 mM EDTA, 100 mM Tris (pH 8), 1 mM pepstatin, 1 mM leupeptin, 750 mg/liter reduced glutathione, and 300 mg/liter oxidized glutathione. Refolded mutant $\beta_2$m was purified by FPLC using a Superdex S-200 column (Amersham Pharmacia).

Mutant $\beta_2$m was labeled at Cys-88 by mixing for 2 hr with 2 mM TR maleimide (Molecular Probes) or with Alexa 488 maleimide (20 mol of dye per mol of protein). Unbound dye was removed by FPLC. Labeled $\beta_2$m (2.5 mg/ml) was stored at 4°C in PBS. TR-labeled-$\beta_2$m (TR-$\beta_2$m) efficiently bound MHC heavy chains *in vitro* (32, 33).

**Fluorescent Labeling of Superantigen and mAb.** One hundred micrograms of staphylococcal enterotoxin A (SEA) (1 $\mu$g/$\mu$l of 0.1 M sodium bicarbonate buffer, pH 8) was incubated with 5 $\mu$l FITC succinimidyl ester (Molecular Probes, dissolved to 20 mg/ml in dimethylformamide) for 1 hr at room temperature. Unbound dye was removed by gel filtration (PD10, Amersham Pharmacia). A total of 0.1 mg anti-CD59 mAb (p282, PharMingen) was labeled with Alexa 568 according to the manufacturer's instructions (Molecular Probes), and 1 mg staphylococcal enterotoxin B (SEB) and 0.1 mg murine anti-class II MHC protein mAb (TÜ39, PharMingen) were labeled with Cy5 according to the manufacturer's instructions (Amersham Pharmacia).

**Fluorescent Staining of Cell Lines.** 721.221 (221) is a B cell derived by mutagenesis to abrogate expression of class I MHC protein at the cell surface (34). Transfectants of 221 were prepared as described to express HLA-Cw6 (221/Cw6) (35) and HLA-Cw6 linked at the C terminus to GFP (221/Cw6-GFP) (36). Cells were incubated with 100 nM Alexa 488- or TR-$\beta_2$m in serum-free hybridoma medium (Life Technologies, Grand Island, NY) and costained, as appropriate, with 100 nM FITC-SEA, 9 $\mu$g/ml Cy5-SEB, 10 $\mu$g/ml Bodipy-FL low density lipoprotein (LDL) (Molecular Probes) and 50 $\mu$g/ml FITC- or TR-transferrin (Molecular Probes). Cells were removed from incubation with the fluorescent molecules at the times given.

To characterize the perinuclear location of internalized $\beta_2$m, the ER was marked with $DiOC_6$ (Molecular Probes). After incubation with TR-$\beta_2$m for 2 hr, cells were stained in 0.25 $\mu$g/ml $DiOC_6$ for 10 sec. To stain the Golgi apparatus, Bodipy-FL $C_5$-ceramide [*N*-(4,4-difluoro-5, 7-dimethyl-4-bora-3a-4a-diaza-s-indacene-3-pentanoyl)sphingosine; Molecular Probes] and Bodipy-TR ceramide [*N*-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)sphingosine; Molecular Probes] were used. After incubation with 100 nM TR-$\beta_2$m or 9 $\mu$g/ml Cy5-SEB for 90 min, cells were incubated in 5 $\mu$M Bodipy-FL $C_5$-ceramide for an additional 30 min. 221/Cw6-GFP cells also were incubated with 5 $\mu$M Bodipy-TR ceramide for 30 min.

To mark intracellular class II MHC protein, cells were fixed and permeabilized according to the manufacturer's instructions (Cytofix/Cytoperm kit, PharMingen), and then incubated with 3 $\mu$g/ml Cy5-labeled mAb TÜ39 on ice.

**Confocal Microscopy.** After staining, cells were washed twice and resuspended in PBS/1% BSA/0.1% azide. Six microliters of cells suspended in PBS was pipetted onto micro slides (Corning). Fluorescence was imaged by laser scanning confocal microscopy, using the 488-nm (green), 568-nm (red), and 647-nm (blue) laser lines for excitation of appropriate fluorophores (LSM 410, Zeiss). For each experiment, cells stained with each fluorophore alone were imaged to ensure that fluorescence emission did not bleed between channels. Cells displayed for each experiment are representative of many cells imaged over at least three independent experiments.

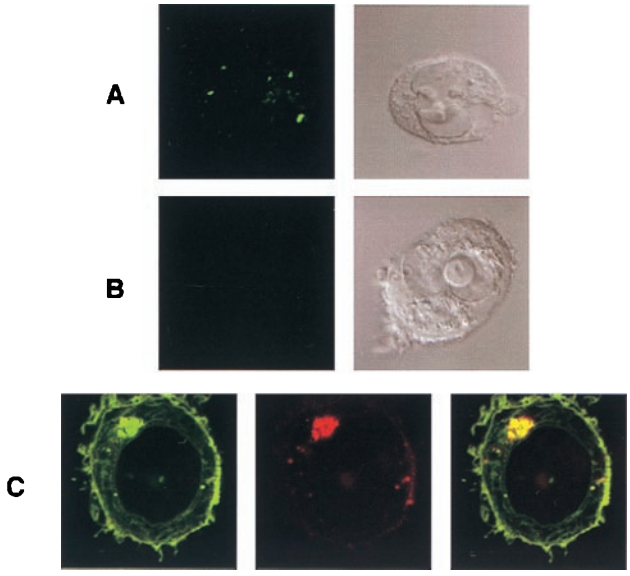


**Fig. 1.** Fluorophore-conjugated $\beta_2$m specifically marks internalized class I MHC protein. Untransfected 221 cells incubated with (*A*) TR-$\beta_2$m (red) and with FITC-conjugated transferrin (green) or (*B*) with TR-$\beta_2$m (red) alone, for 24 hr. (*Left*) An overlay of red and green fluorescence, and the corresponding Nomarski image (*Right*). (*C*) Transfected 221/Cw6-GFP cells incubated with TR-$\beta_2$m (red) for 2 hr. (*Left*) Green staining of Cw6-GFP, (*Middle*) red staining of $\beta_2$m, and (*Right*) an overlay of fluorescence colors is displayed. (*Right*) Surface labeling by TR-$\beta_2$m is time dependent; compare Fig. 2*A*. Yellow indicates colocalization of green and red staining. Magnification: ×6,000.

## Results

**Exogenously Exchanged Fluorophore-Conjugated $\beta_2$m Specifically Stains Cells Expressing Class I MHC Protein and Internalizes into the Same Intracellular Vesicles as GFP-Linked HLA-C Heavy Chain.** First, to confirm whether $\beta_2$m specifically associated with cell surface class I MHC protein upon incubation with cells, untransfected 721.221 B cells (221 cells) were incubated with both TR-$\beta$2m and FITC-transferrin. Because untransfected 221 cells do not express class I MHC protein at the cell surface (34), $\beta_2$m should not accumulate in these cells (the concentration of $\beta_2$m added was below that at which significant amounts of protein would be internalized by pinocytosis; ref. 20). Indeed, no staining of 221 cells by $\beta_2$m (red) was seen while staining by transferrin (green) was clearly evident (Fig. 1*A*). Similarly, when 221 cells were incubated with TR-$\beta_2$m alone, no staining was seen either intracellularly or at the cell surface (Fig. 1*B*). In contrast, incubation of 221/Cw6-GFP transfectants with TR-$\beta_2$m showed both red and green staining (or yellow in the overlap) in every intracellular compartment that contained $\beta_2$m (Fig. 1*C*), indicating that the $\beta_2$m colocalized with endocytosed GFP-linked HLA-Cw6 heavy chain. Exogenously exchanged fluorescent $\beta_2$m also internalized to the same intracellular location as class I MHC heavy chain marked by mAb HC10 (20). Notably, intracellular vesicles containing endocytosed MHC protein accumulated in one perinuclear region of the cell.

**Class I MHC Protein Colocalizes with Class II MHC Protein.** 221/Cw6 cells were incubated with TR-$\beta_2$m and FITC-conjugated superantigen SEA to mark surface and endocytosed class I MHC and class II MHC protein respectively, for 30 min, 2 hr, and 24 hr. At all three time points, TR-$\beta_2$m (red) and FITC-SEA (green) fluorescence were found to accumulate in the same intracellular compartments about 1 $\mu$m in diameter (Fig. 2*A*). Similarly, Alexa 488 (green)-conjugated $\beta_2$m and Cy5 (blue)-conjugated SEB colocalized in 221/Cw6 cells (Fig. 2*B*). Nearly all compart-

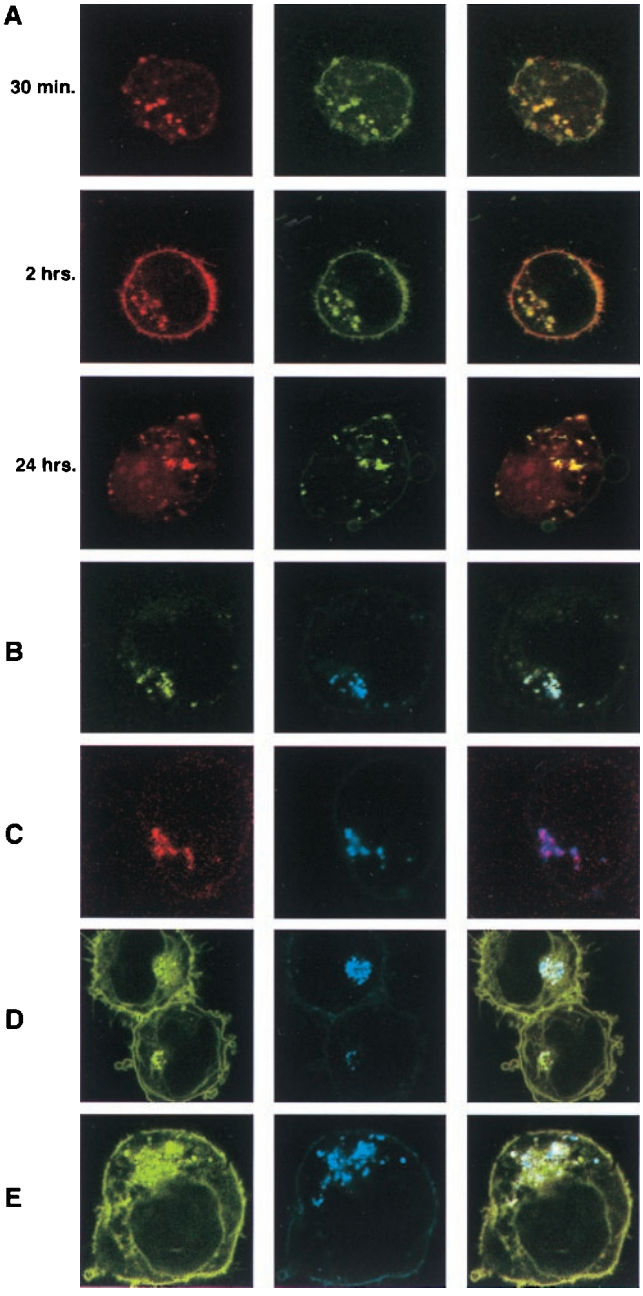


**Fig. 2.** Class I MHC protein cotraffics with class II MHC protein. (*A*) Intracellular trafficking of exogenously added $\beta_2$m and superantigen. 221/Cw6 cells incubated with TR-$\beta_2$m (red) and FITC-conjugated SEA (green) for 30 min, 2 hr, and 24 hr. (*B*) 221/Cw6 cells incubated with Alexa 488-$\beta_2$m (green) and Cy5-conjugated SEB (blue) for 2 hr. (*C*) 221/Cw6 cells incubated with TR-$\beta_2$m (red) for 2 hr, then fixed, permeabilized, and stained with Cy5-conjugated mAb TÜ39 (blue). (*D*) 221/Cw6-GFP cells incubated with Cy5-SEB (blue) for 2 hr. HLA-Cw6-GFP (green), Cy5-SEB (blue), and an overlay of both is shown. (*E*) 221/Cw6-GFP cells fixed, permeabilized, and stained with Cy5-conjugated mAb TÜ39. HLA-Cw6-GFP (green), Cy5-TÜ39 (blue), and an overlay of both is shown. (*Right*) Yellow indicates colocalization of green and red staining, white or light blue indicates colocalization of green and blue staining, and purple indicates colocalization of red and blue staining. Magnifications: (*A–C* and *E*) ×6,000; (*D*) ×4,000.

ments stained with $\beta_2$m also were stained with superantigen. To determine whether superantigen marked intracellular compartments that contain class II MHC protein, mAb TÜ39 against class II MHC protein was used. 221/Cw6 cells first labeled with

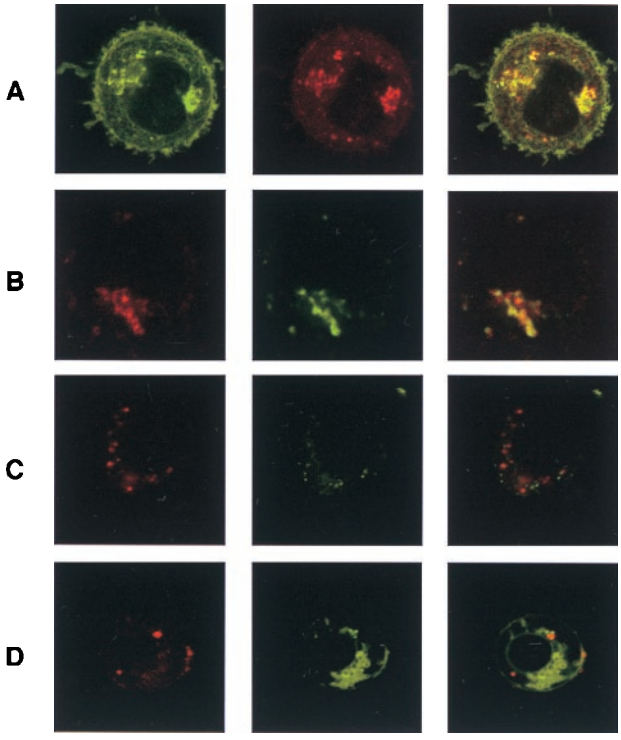


**Fig. 3.** Class I MHC protein internalizes through endosomes with transferrin. (*A*) 221/Cw6-GFP cells incubated with TR-transferrin for 2 hr. HLA-Cw6-GFP (green), TR-transferrin (red), and an overlay of both is shown. (*B*) 221/Cw6 cells incubated with TR-$\beta_2$m (red) and FITC-transferrin (green) for 2 hr. (*C*) 221/Cw6 cells incubated with TR-$\beta_2$m (red) and Bodipy-FL LDL (green) for 24 hr. (*D*) 221/Cw6 cells incubated with TR-$\beta_2$m (red) for 2 hr and $DiOC_6$ (green) for 10 sec. (*Right*) Yellow indicates colocalization of green and red staining. Magnification: ×6,000.

exogenously exchanged TR-$\beta_2$m were fixed, permeabilized, and stained with Cy5-labeled mAb TÜ39. Clearly, $\beta_2$m colocalized with class II MHC protein in these cells (Fig. 2*C*). Similarly, when 221/Cw6-GFP transfectants were incubated with fluorophore-conjugated SEB, the vast majority of intracellular compartments containing HLA-Cw6-GFP also contained SEB (Fig. 2*D*). HLA-Cw6-GFP also colocalized with mAb TÜ39 (Fig. 2*E*). The brightness of staining in these intracellular compartments varied, with some containing more class I MHC and less class II MHC protein or vice versa. However, the location of the stained compartments clearly overlapped.

**MHC Protein Internalizes Through Endosomal Compartments.** Transferrin, which binds transferrin receptor in clathrin-coated pits (37), was used as a selective marker for endosomal trafficking. TR-transferrin was incubated with 221/Cw6-GFP for 2 hr and imaged by confocal microscopy. A vast majority of compartments with GFP fluorescence also contained TR-transferrin (Fig. 3*A*). Internalized TR-$\beta_2$m also was found to localize in the same compartments as transferrin (Fig. 3*B*). Thus, nearly all endocytosed HLA-C is internalized in transferrin receptor-containing vesicles. In contrast, 221/Cw6 cells stained with fluorescent $\beta_2$m and LDL, a marker for late endosomes and lysosomes (38), did not show any colocalization even after 24 hr (Fig. 3*C*).

$DiOC_6$ (green) was used to mark the ER, cellular membrane, and nuclear membrane (39). After incubation with TR-$\beta_2$m, 221/Cw6 cells were briefly stained with $DiOC_6$, clearly illuminating the distinct web-like structure of the ER (Fig. 3*D*). Though often in close proximity, none or very little of the

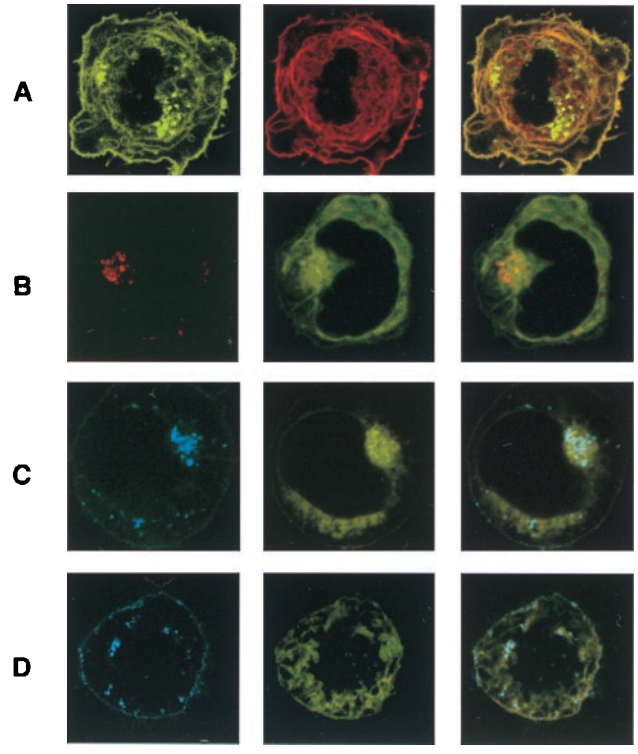


**Fig. 4.** MHC protein internalizes to a para-Golgi region. (*A*) 221/Cw6-GFP cells incubated with Bodipy-TR ceramide (red) for 30 min. HLA-Cw6-GFP (green), Bodipy-TR ceramide (red), and an overlay of both is displayed. (*B*) 221/Cw6 cells incubated with TR-$\beta_2$m (red) for 90 min and then with Bodipy-FL $C_5$-ceramide (green) for 30 min. (*C* and *D*) 221/Cw6 cells incubated with Cy5-SEB (blue) for 90 min and subsequently with Bodipy-FL $C_5$-ceramide (green) for 30 min. (*Right*) Yellow indicates colocalization of green and red staining, and white or light blue indicates colocalization of blue and green staining. Magnification: ×6,000.

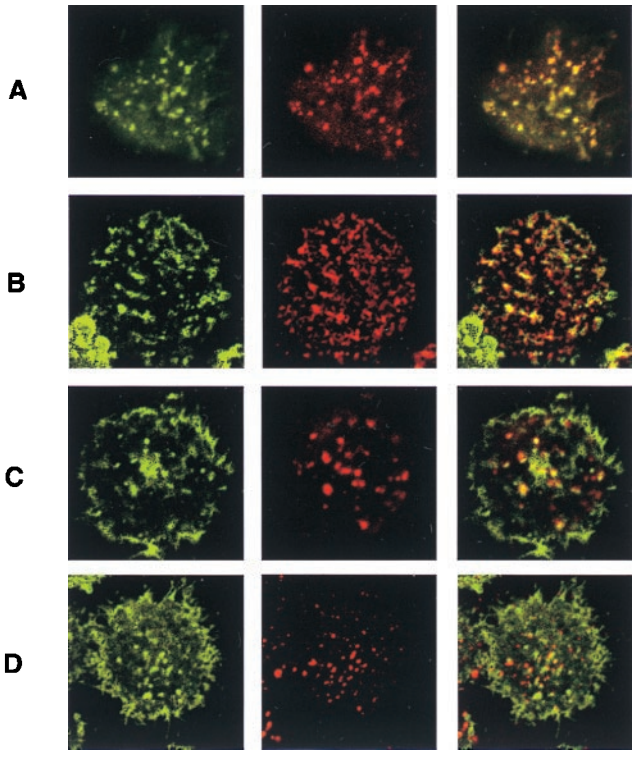


**Fig. 5.** At the cell surface, MHC protein accumulates in distinct microdomains. 221/Cw6-GFP cells were stained with (*A*) phycoerythrin-labeled TÜ39 mAb (red), (*B*) TÜ39 mAb followed by Alexa 568 goat anti-mouse IgG mAb (red), (*C*) TR-transferrin (red), or (*D*) biotinylated cholera toxin with phycoerythrin-labeled streptavidin (red). No staining was seen with phycoerythrin-labeled streptavidin alone. The green fluorescence of HLA-Cw6-FP, each red fluorescence, and an overlay of both is shown. (*Right*) Yellow indicates colocalization of green and red staining. Magnification: ×6,000.

TR-$\beta_2$m colocalized with the $DiOC_6$-stained ER even after 24 hr of incubation. $\beta_2$m was found only in round-shaped intracellular compartments and not in the web-like structure of the ER.

To stain the Golgi apparatus, Bodipy-FL $C_5$-ceramide (green) and Bodipy-TR ceramide (red) were used (40). In 221/Cw6-GFP cells, colocalization of Bodipy-TR ceramide, marking the dispersed structure of the Golgi, was seen with presumably newly synthesized HLA-Cw6-GFP (Fig. 4*A*). However, in addition, round-shaped class I MHC-positive compartments were found in very close proximity to the Golgi. To determine whether these round-shaped vesicles contained endocytosed HLA-C, 221/Cw6 cells were incubated with TR-$\beta_2$m for 90 min and then stained with Bodipy-FL $C_5$-ceramide for an additional 30 min. Internalized TR-$\beta_2$m (red) did not take on the shape of the Golgi (green) but rather localized in round-shaped vesicles that often were juxtaposed to or overlapped with the Golgi apparatus (Fig. 4*B*). Cy5-SEB also localized in round-shaped compartments juxtaposed to the Golgi, stained by Bodipy-FL $C_5$-ceramide in 221/Cw6 cells (Fig. 4*C*). Out of the plane of focus of the most dense region of the Golgi, intracellular class I and II MHC-containing vesicles were seen dispersed throughout the cytosol (Fig. 4*D*). However, in general, compartments containing endocytosed class I and class II MHC protein accumulated where the Golgi apparatus was particularly dense (Fig. 4 *B* and *C*). Within the resolution of the confocal microscope used (about 0.1 $\mu$m), it was not clear whether these vesicles were attached to or juxtaposed to the Golgi apparatus.

**Distinct Membrane Microdomains at the Cell Surface Contain Both Class I and Class II MHC Protein.** HLA-Cw6-GFP cells were stained with phycoerythrin (red)-conjugated anti-class II MHC protein mAb TÜ39 or with unlabeled mAb TÜ39 followed by Alexa 568 (red)-conjugated goat anti-mouse IgG secondary antibody. At the cell surface, HLA-Cw6-GFP and mAb TÜ39 localized in the same membrane microdomains (Fig. 5 *A* and *B*). These domains occupied diverse shapes, some quite circular and others more rod-shaped, each approximately 1–2 $\mu$m diameter or width. TR-transferrin also was localized into these HLA-C-GFP-containing cell surface microdomains, implying that they were clathrin-coated pits (Fig. 5*C*). Neither fluorophore-conjugated mAb against the glycosylphosphatidylinositol-anchored protein CD59 (not shown) nor biotinylated cholera toxin $\beta$-subunit with fluorophore-conjugated streptavidin (Fig. 5*D*), which both mark cholesterol-rich membrane microdomains (often called lipid rafts, ref. 41), colocalized with class I MHC protein at the cell surface. Nonetheless, many of these lipid rafts were in close proximity or adjacent to the membrane microdomains rich in class I MHC protein. Taken together, these data further suggest that MHC protein primarily is internalized with transferrin through clathrin-coated pits rather than through other mechanisms.

## Discussion

In determining the intracellular route of class I MHC protein, the minimally invasive method of exchanging fluorescent $\beta_2$m at the surface was used to eliminate possible artifacts arising from crosslinking or GFP attachment. The intracellular location of endocytosed fluorophore-conjugated $\beta_2$m specifically marked compartments containing HLA-C-GFP (Fig. 1). These intracellular vesicles also contained class II MHC protein, revealed by

mAb (Fig. 2) and could be stained with exogenously added fluorophore-conjugated superantigen that presumably marks endocytosed class II MHC protein. Nearly all vesicles containing endocytosed HLA-Cw6-GFP and $\beta_2$m also were found to colocalize with transferrin (Fig. 3). Thus, class I MHC protein endocytosed via the same route as class II MHC protein into transferrin receptor-containing early endosomes.

Because transferrin receptor rapidly recycles back to the cell surface (37), endocytosed class I and class II MHC protein may similarly recycle back to the cell surface. Moreover, because class II MHC protein can be loaded with exogenous antigen within recycling endosomes (21, 22), antigens processed in the same early endosomes also might be loaded onto endocytosed class I MHC protein. Thus, recycling class I MHC protein, at least in B cells, may facilitate the presentation of exogenous antigen processed in early endosomes. A function of $\beta_2$m, which dissociates at the pH of early endosomes (42), may be to facilitate peptide exchange and subsequent reassembly of the MHC trimer before recycling back to the cell surface.

It remains to be seen whether exogenous antigen presentation by class I MHC protein plays a significant role *in vivo.* For example, it is unknown whether MHC polymorphism and the stringent requirements for a 9-mer peptide really allow class I MHC protein to significantly complement the repertoire of exogenous antigen that can be presented by class II MHC protein. Understanding the mechanism by which class I MHC protein presents exogenous antigen could be exploited in the development of novel vaccines or in other therapeutic applications. For example, DNA or protein vaccines could be constructed to contain antigen(s) linked with appropriate signal sequences that direct the antigen to the endosomal compartment where both class I and II MHC protein are present. The colocalization of class I with class II MHC protein also may explain why so many peptides derived from class I MHC heavy chain are presented as self peptides by class II MHC protein (43). Some fraction of class I MHC protein may be degraded by endosomal proteases after dissociation of $\beta_2$m and peptide from the MHC heavy chain at endosomal pH.

Previously, studies in T cells have shown that class I MHC protein marked with mAbs internalize via clathrin-coated pits and end up in lysosomes (14, 15). In the current study of B cells, internalized class I MHC protein marked by fluorescent $\beta_2$m did not reach lysosomes or late endosomes, which were specifically marked by fluorescent LDL (Fig. 3*C*). Even after 24 hr of incubation, $\beta_2$m, although present in significant intracellular amounts, did not colocalize with LDL. Thus, class I MHC proteins may have distinct endocytic pathways in B cells and T cells.

$\beta_2$m associated with HLA-C did not endocytose to the ER (Fig. 3*D*). In the major class I MHC antigen presentation route, endogenous peptide is transported to the ER for binding nascent class I MHC protein. At least some exogenous peptide also is transported to the ER after internalization where it may be loaded into nascent class I MHC protein (8). However, as shown here in B cells, recycling class I MHC protein cannot bind exogenous or endogenous peptide in the ER. Internalized class I MHC did localize to compartments very close to or overlapping with the Golgi apparatus, though not into the stack-like shape of Golgi structure (Fig. 4).

Class I MHC protein was found to accumulate in microdomains at the cell surface that colocalized with transferrin and with class II MHC protein, supporting the idea that these endocytose together. The nonclassical class I MHC protein HFE was shown to traffic by specific association with transferrin receptor (44). It is unknown whether any similar association exists between transferrin receptor and other MHC proteins. The class I MHC-rich cell surface microdomains were juxtaposed to cholesterol-rich lipid rafts (Fig. 5), which might facilitate a role for lipid rafts in the motility of class I MHC protein. Indeed, T cell receptors are recruited to detergent-insoluble cholesterol-rich rafts after activation (45, 46). Thus such macromolecular organization of MHC protein at the cell surface may be important for intracellular signaling and intercellular interaction.

This study complements the work recently published by Gromme *et al.* (29). In that work, HLA-A2-GFP was found to colocalize with anti-HLA-DR mAb in acidic perinuclear vesicles within IFN-$\gamma$-stimulated melanoma cells. HLA-A2-GFP presumably was endocytosed into these acidic vesicles, because the vesicles remained rich in HLA-A2-GFP after treatment with brefeldin A. The current confocal microscopy study demonstrates that endocytosed class I MHC protein, directly marked by exogenously exchanged fluorophore-conjugated $\beta_2$m, enters vesicles often associated with the Golgi and that both class II MHC protein and transferrin receptor may be endocytosed by the same route. Gromme *et al.* further established that processing of measles virus F protein into peptides, and subsequent presentation by class I MHC protein to cytotoxic T lymphocytes, is independent of TAP (transporter associated with antigen processing) and sensitive to $NH_4Cl$, suggesting that processing and loading of peptide into class I MHC protein occurs in the endosome (29). Other cells also have been shown to recycle class I MHC protein (47).

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