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Author/s:
Kittlesen, DJ;Brown, LR;Braciale, VL;Sambrook, JP;Gething, MJ;Braciale, TJ

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Presentation of Newly Synthesized Glycoproteins to CD4+ T Lymphocytes. An Analysis Using Influenza Hemagglutinin Transport Mutants

By David J. Kittlesen,*‖ Lawrence R. Brown,‖ Vivian L. Braciale,*‡ Joseph P. Sambrook,** Mary-Jane Gething,¶** and Thomas J. Braciale*‡§

*From the Beirne B. Carter Center for Immunology Research, and the Departments of ‡Microbiology and §Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908; the ‖Immunology Graduate Program, Washington University School of Medicine, St. Louis, Missouri 63110; and the ¶Howard Hughes Medical Institute, and the **Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235*

Summary

Human lymphoblastoid cells transiently expressing the hemagglutinin (HA) glycoprotein of influenza virus are rapidly and efficiently recognized by CD4+ HA-specific T lymphocytes. This endogenous presentation pathway is sensitive to chloroquine and is therefore likely related to the classical class II major histocompatibility complex (MHC) exogenous pathway of antigen presentation. In this study we have examined a series of transport-defective HA mutants. We correlate the intracellular fate of the native antigen with its presentation characteristics. We have found that the native antigen must enter the secretory pathway since a cytosolic form is not presented. However, surface expression and normal trafficking through the Golgi apparatus are not required for efficient presentation. Instead, escape of native antigen from the endoplasmic reticulum appears to be both necessary and sufficient for gaining access to a compartment where antigen is processed and binds class II MHC molecules.

The last decade has seen major advances in our understanding of the pathways by which protein antigens are processed to peptide fragments and presented by class I and II MHC molecules. The major features of these two pathways have been described (for reviews see references 1–4). A current paradigm implicates access of the antigen to specific intracellular compartments as being crucial in determining whether it is presented in association with MHC class I or II molecules. Newly synthesized proteins (5), or intact protein molecules artificially introduced to the cytosolic compartment (6), are processed and efficiently presented in association with class I MHC molecules (7, 8). Proteins entering the endosomal compartment are processed and preferentially charge class II MHC molecules (5).

This division between processing pathways is not absolute, and apparent exceptions have been documented. For example, specialized cells in the thymus and spleen may be able to process and present preformed exogenous proteins in association with class I MHC molecules, albeit with a low efficiency (9–12). Additionally, newly synthesized cytosolic and secretory proteins have been reported to charge class II molecules (13–20). A critical issue in interpreting these findings is whether these results represent antigen-processing events in novel compartments, e.g., MHC class II charging in the endoplasmic reticulum (ER),[1] or instead, reflect alternative mechanisms of targeting antigen to previously described processing and presentation compartments, e.g., the late endosome/lysosome.

Recently, we described the recognition of the influenza A/Japan/305/57 hemagglutinin (HA) glycoprotein by human class II–restricted T lymphocytes (21). In this report we have examined in further detail the pathway by which this HA is presented to these CD4+ class II–restricted T cells. In contrast to earlier studies in the murine system (5), these lymphocytes efficiently recognize newly synthesized HA acutely expressed using a viral expression vector. Using a series of transport-defective HA mutants, we have found that newly synthesized A/Japan/57 HA must gain access to the ER but does not need to transit to the cell surface, or transit through the Golgi stacks to be presented to the T cells. These data suggest that there is a mechanism by which proteins present in the secretory pathway can be sampled from a transitional element between the ER and Golgi stacks, or an early Golgi compartment, and be delivered to an endosomal compartment for processing and presentation by class II MHC mole-

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; HA, hemagglutinin; wt, wild type.

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cules. A potential pathway of targeting proteins from the early secretory compartments to the site of class II MHC charging is described, and implications of this pathway for tolerance and autoimmunity are discussed.

Materials and Methods

Cell Lines. V1 is a CD4$^+$ MHC class II–restricted human cytolytic T cell clone. Its specificity has been mapped for HAs of the H2 subtype (which include A/Japan/305/57), and the peptide VKILPKDRWTQH, which corresponds to amino acids 129–140 of the HA protein, in association with HLA-DRw11 (21). This clone was isolated as described in detail elsewhere (22) and is maintained by weekly restimulation with irradiated (2,600 rad) allogeneic PBMC (isolated using Histopaque 1077; Sigma Chemical Co., St. Louis, MO) + OKT3 (anti-CD3 mAb, 6.25 ng/ml final; Ortho Diagnostic Systems, Inc., Westwood, MA) in RPMI 1640 supplemented to 10% with heat-inactivated FCS, 4 mM glutamine, and human rIL-2 (20 U/ml final; Biosource International, Camarillo, CA). TBLCL is an EBV-transformed B lymphoblastoid cell line isolated from the same individual as V1. It was transformed using standard procedures (23) and is maintained by serial passage every 3–4 d in RPMI 1640, 10% heat-inactivated FCS, 4 mM glutamine. This medium was used for all experiments unless otherwise noted.

HA Mutants. All of the HA constructs described below have been cloned into the vaccinia virus thymidine kinase gene by homologous recombination and are expressed under the control of the P7.5 early/late viral promoter. Details of the construction of recombinant vaccinia viruses and their growth and purification are described elsewhere (24–26). Because vaccinia virus does not incorporate influenza proteins into its virion, the only HA protein available is that synthesized in the target cell during infection. Although crude virus stocks could contain some HA protein from cellular debris, demonstration of the requirement for de novo HA synthesis was verified by testing sucrose gradient-purified virions (with identical results) and UV-inactivated virus preparations (negative). HA refers to wild-type (wt) HA of the H2 subtype from A/Japan/305/57; the vaccinia virus carrying this gene was a kind gift from Dr. Bernard Moss (National Institute of Allergy and Infectious Disease). Signal-minus HA (HA-S$^-$) has been engineered such that the 15-amino acid leader sequence NH_2 terminal of the signal cleavage site is replaced by amino acid residues MELG (27). Anchor HA HA-A$^-$ is a form of HA generated by deleting nucleotides coding for the 38 amino acids comprising the transmembrane region and cytoplasmic tail and replacing them with a sequence coding for the short, relatively hydrophilic sequence PPPGSRHDKIH (27). HA164 was generated by cutting the HA gene at the coding junction between the transmembrane and cytoplasmic domains and blunt-end ligating ClaI linkers (28). This alters the reading frame, creating a novel cytoplasmic tail of the residues IDGSAGSAYDYKSFYN (wt tail sequence is NGSLQCRICI). HAC67S is identical to wt, with the exception of a single mutation leading to an amino acid substitution at position 67 of the mature protein (position 82 from methionine initiation) (29). A cysteine residue involved in an intramolecular disulfide bond is replaced by serine. HAΔR1 was created by cutting the wt HA cDNA at two EcoRI sites to form an in-frame deletion mutant (30). This removes amino acids 248–409 to form a protein of 398 amino acids. HA-EEKKMP was designed to be an ER retention mutant and was constructed by engineering the COOH-terminal six amino acids of the adenovirus E3/19K glycoprotein onto the cytoplasmic tail of HA. Previous studies have demonstrated the role of these amino acids for the retention of adenovirus E3/19K and other transmembrane glycoproteins, to which this sequence has been transplanted (31, 32). Briefly, wt HA was removed from the plasmid pMTHA (26) by digesting with HindIII and NdeI. NdeI cuts within the 3′ coding region for the cytoplasmic tail. The oligonucleotides (5′ to 3′) TAGAAGAGAAAAAGATGCCTTGAGC and TCAAGGCATCTTTTTCTCTTC were synthesized using standard automated procedures, annealed, phosphorylated, and ligated to the HA fragment generated above and to pSC11.3 (26) (digested with HindIII and SacII, and dephosphorylated with calf intestinal phosphatase). These oligos were chosen so as to finish coding the wt HA sequence, then code for the amino acids EEKKMP, and finally a stop codon. The finished construction was verified by sequencing. Vaccinia recombinants were generated as with the other mutants, plaque purified, grown as high-titer stocks, and sucrose gradient was purified. Note that the T cell epitope (129–140) is retained in all of these mutants.

Infections. TBLCL were washed and then resuspended in serum-free RPMI 1640 and infected with the recombinant vaccinia viruses at 50 PFU/cell for 10 min on ice, then 60 min at 37°C. Cells were then washed and resuspended in medium with serum and incubated at 37°C. The time of incubation before and during the assay varied between assays and is noted in individual results.

Antibodies. 3CSBB9 is a polyclonal rabbit anti-HA antiserum. This Ab works efficiently for immunoprecipitation and recognizes both the wt and the mutant HA molecules. Fc125 is a mouse anti-HA mAb and is used in the form of hybridoma culture supernatant. It does not recognize the HAΔR 1 construction by immunoprecipitation, so it was not used for those studies. The secondary Ab used for flow cytometry is a FITC-conjugated goat Ab to murine IgG2b (Southern Biotechnology Associates, Birmingham, AL) that shows very low nonspecific binding in flow cytometry.

CTL Killing Assay. TBLCL were labeled at 10^6 cells/ml for 2–3 h at 37°C with ~100 mCi/ml ^{51}Cr sodium chromate (DuPont-NEN, Wilmington, DE). Before the assay, they were washed three times to remove released ^{51}Cr and resuspended at 0.5 or 1 × 10^5 cells/ml. Target cells were plated at 100 μl/well in 96-well plates, and an equal volume of clone (experimental), medium (spontaneous release), or 2% Triton X-100 (maximum release) was added. Final E/T ratios are indicated in the figure legends. Plates were spun, 2–3 min at 50 *g* and then incubated at 37°C with 10% CO_2. 100 μl/well of supernatant was harvested and counted in a gamma counter (Pharmacia/LKB, Uppsala, Sweden). Percent specific killing was calculated by the following formula: 100× (experimental − spontaneous)/(maximum release − spontaneous). All groups were performed in quadruplicate. SD was usually 2–3%, and the spontaneous release was routinely 10–15%.

Flow Cytometry. TBLCL were infected as noted above and incubated for various times (as indicated in the figure legends) before staining. Cells were then plated at 2.5–5 × 10^5/well in 96-well plates and maintained at 4°C. An equal volume of Fc125 culture supernatant diluted 1:10 with medium was then added to each well. Cells were incubated 30 min and then washed three times. Cells were resuspended in the secondary Ab diluted 1:20 with medium and incubated a further 30 min. Cells were then washed again three times and resuspended in 1% buffered formalin phosphate. Cells were analyzed on FACScan® using Consort 30 and Lysys software (Becton Dickinson & Co., Mountain View, CA).

Metabolic Labeling and Immunoprecipitation. Cells were pulse radiolabeled in MEM (minus cysteine/methionine) supplemented with 5% heat-inactivated FCS, 4 mM glutamine, 100 mCi/ml [^{35}S]methionine (EXPRE^{35}S^{35}S protein labeling mix; DuPont-NEN) for 30 min at 37°C. Cells were washed and then chased in serum-containing medium supplemented with 1 mM methionine and 0.2

mM cysteine at 37°C for the times indicated. Cells were then washed and quick frozen with liquid nitrogen. Cells were thawed and resuspended in lysis buffer containing 0.5% NP-40 and the protease inhibitor PMSF (0.2 mM final) for 30 min on ice. Nuclei were spun out and lysates precleared twice with protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Anti-HA Ab was added to the lysates, followed by protein A–Sepharose. Precipitated complexes were washed three times and eluted into 0.2 M citric acid/sodium phosphate buffer (pH 6.2) + 0.2% SDS. Samples were exposed to 1 mU Endo H (Oxford Glycosystems, Foster City, CA) or 7 mU Endo D (Sigma Chemical Co.), or mock digested overnight (2 d for Endo D experiments) at 37°C. Reducing (2-ME) sample buffer was then added, and samples were boiled before 10% (5% stacking gel) SDS-PAGE. Gels were then fixed, impregnated with fluorograph (Fluorohance; Research Products International, Rochester, NY), dried, and exposed to X-ray film at −80°C before development.

Results

CD4⁺ T Cell Recognition of Newly Synthesized HA. We have examined the presentation of the influenza A/Japan/57 HA glycoprotein by human B lymphoblastoid cells (LCL) to CD4⁺ MHC class II–restricted T lymphocytes. As previously reported (21), a clonal population of MHC class II–restricted T cells recognizes EBV-transformed lymphoblastoid cells treated with either infectious influenza virions, isolated HA protein, or a synthetic peptide spanning amino acids 126–145 (minimal peptide is 129–140) of the mature HA protein in association with HLA-DRw11. In addition, we found that the human T lymphocytes efficiently recognize newly synthesized HA protein expressed by a recombinant vaccinia vector (Fig. 1). This observation is in contrast with our earlier observations of murine CD4⁺ T cells where B cells poorly present newly synthesized HA expressed in these APC (5).

New viral protein synthesis was required for sensitization of cells expressing HA by infection with the recombinant vaccinia virus. As Fig. 2 demonstrates, the presence of the protein synthesis inhibitor emetine at the initiation of infection virtually abolishes recognition. However, target cells treated with influenza virion preparations (containing virion-associated HA protein, unlike the vaccinia virus) efficiently processed and presented HA in the presence of the protein synthesis inhibitor. These data also imply that in these cells processing and presentation of exogenous HA may not require newly synthesized MHC class II molecules. Presentation of newly synthesized HA expressed after vaccinia virus infection was also sensitive to the weak base chloroquine, as shown in Fig. 3. As expected, chloroquine had no effect on

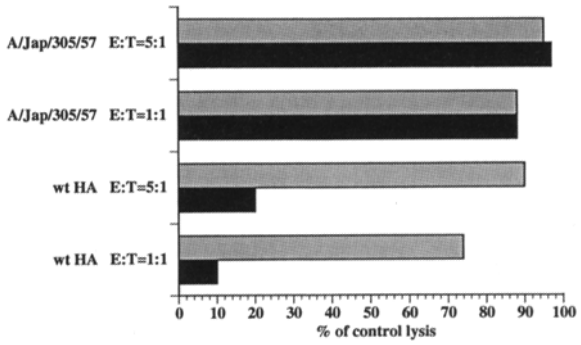

Figure 2. Target cell sensitization by vaccinia HA requires de novo protein synthesis. ^{51}Cr-labeled TBLCL were infected with the A/Japan/305/57 influenza virus or an HA-expressing recombinant vaccinia vector (*wt HA*) and subsequently incubated at 37°C for 3 h in the absence (*stippled bars*) or presence (*filled bars*) of 10^{-5} M emetine. Both emetine-treated and untreated cells were incubated with the CTL clone VI in the presence of 10^{-6} M emetine for 2 h at 37°C at the indicated E/T ratios. Values are the percent of control lysis of infected target cells in the absence of any emetine treatment during preincubation or assay. Control lysis values (defined as 100%) for A/Japan/305/57-infected and vaccinia-expressing wt HA-infected cells were 63 and 30%, respectively, at an E/T cell ratio of 5:1.

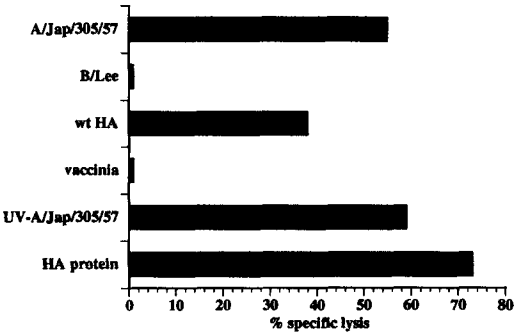

Figure 1. MHC class II–restricted T cell recognition of exogenous and endogenous HA. TBLCL were infected with influenza viruses (A/Japan/305/57 [*B/Lee*]), the recombinant vaccinia expressing the A/Japan/305/57 HA gene (*wt HA*), vaccinia alone, or treated either with noninfectious UV-irradiated A/Japan/305/57 virus (*UV-A/JAP/57*), or with detergent-solubilized HA (*HA protein*) at a final concentration of 5 μg/ml. HA-specific DRw11-restricted CTL (V1) were incubated with ^{51}Cr-labeled target cells for 2.5 h at an E/T ratio of 5:1 as described in Materials and Methods.

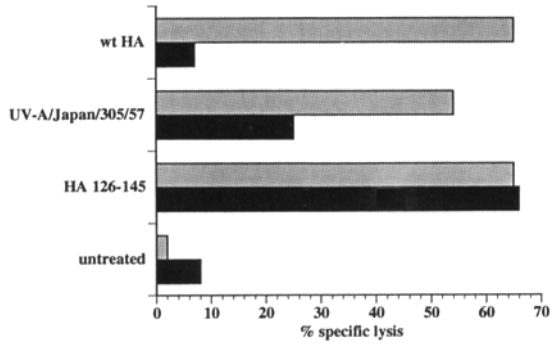

Figure 3. Chloroquine inhibits presentation of newly synthesized HA. TBLCL were preincubated for 30 min in the absence (*stippled bars*) or presence (*filled bars*) of 150 μM chloroquine and subsequently infected with vaccinia virus expressing wt HA (*wt HA*), or treated with UV-inactivated A/Japan/305/57, the HA 126–145 synthetic peptide, or left untreated. The targets were ^{51}Cr-labeled for 3 h at 37°C before a 3-h incubation with the CTL clone VI. The chloroquine was absent or present at 150 μM until incubation with the CTL was commenced, at which point its concentration was decreased to 10 μM. The E/T ratio was 20:1.

the recognition by the DRw11-restricted T cells of the preprocessed HA 126–145 synthetic peptide.

Presentation of Transport-defective HA Mutants. Processing and presentation of newly synthesized HA in these lymphoblastoid cells could reflect MHC class II charging via a cytosolic or an ER processing event and subsequent DRw11 charging in the ER. Alternatively, processing could occur in a late endosome/lysosome compartment after cell surface expression and subsequent internalization of the HA, as suggested by the chloroquine sensitivity of presentation. To further explore possible presentation pathways and to better define the intracellular site of processing, we have examined the processing and presentation of a series of transport-defective HA mutants to the DRw11-restricted T cells. Acute expression of these mutants from recombinant vaccinia virus vectors allowed us to determine the kinetics and relative efficiency of presentation, parameters that are more difficult to assess when studying a constitutively expressed antigen.

As noted above, newly synthesized HA might be presented in association with MHC class II molecules through a cytosolic processing mechanism analogous to MHC class I presentation (5, 10, 20, 33), or by fragmentation of HA derived from the secretory pathway but targeted to a conventional class II processing compartment, e.g., the lysosome, through endocytosis of cell surface HA. To discriminate between these two pathways, we examined the presentation of two HA mutants, HA-S$^-$ and HS-A$^-$. The HA-S$^-$ was engineered so as not to encode the NH_2-terminal signal peptide of the A/Japan/305/57 HA polypeptide. This HA mutant remains cytosolic and is degraded very quickly in the cytosol (27). The HA-A$^-$ mutant was similarly engineered by deleting the 3′ region of the HA cDNA encoding the hydrophobic transmembrane and hydrophilic cytoplasmic tail regions. Cells expressing this mutant secrete a glycosylated product into the medium that can easily be detected by immunoprecipitation (27; and D. J. Kittlesen, unpublished observation). As seen in Fig. 4, LCL cells infected with the vaccinia virus recombinant expressing HA-S$^-$ are not recognized by the T cells. This construction is functional and makes a translation product that is processed and efficiently presented to CD8$^+$ MHC class I–restricted T cells (M. Sweetser and T. J. B., unpublished observation). The failure of the HA-S$^-$ to charge DRw11 argues against the idea of the cytoplasm as an efficient source of peptide for presentation of this unstable cytosolic HA to CD4$^+$ T cells. In contrast, cells infected with the recombinants expressing the HA-A$^-$ gene product are recognized as efficiently as wt HA (Fig. 4), implying that targeting to a class II processing compartment does not require anchoring of the glycoprotein into the cell's lipid bilayer and is not dependent on the presence of the intracytoplasmic HA tail. These data raised the possibility that cell surface HA expression may not be necessary for MHC class II charging, and that a compartment between the ER and cell surface might be the site of HA processing and presentation in association with DRw11.

To better define this putative compartment, a series of HA mutants that are delayed or blocked in their egress from the ER and that differ in their susceptibility to intracellular proteolysis were examined for recognition by the DRw11-

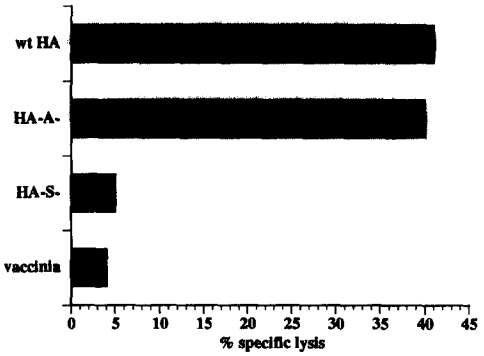

Figure 4. Recognition of a secreted, anchor-minus HA but not of a signal-minus cytolytic form. TBLCL were infected with vaccinia virus–expressing wt HA, anchor-minus HA (*HA-A*$^-$), signal-minus HA (*HA-S*$^-$), or vaccinia alone. Targets were ^{51}Cr labeled overnight at room temperature before incubation for 2 h at 37°C and then 3 h with the CTL clone VI. The E/T ratio was 10:1.

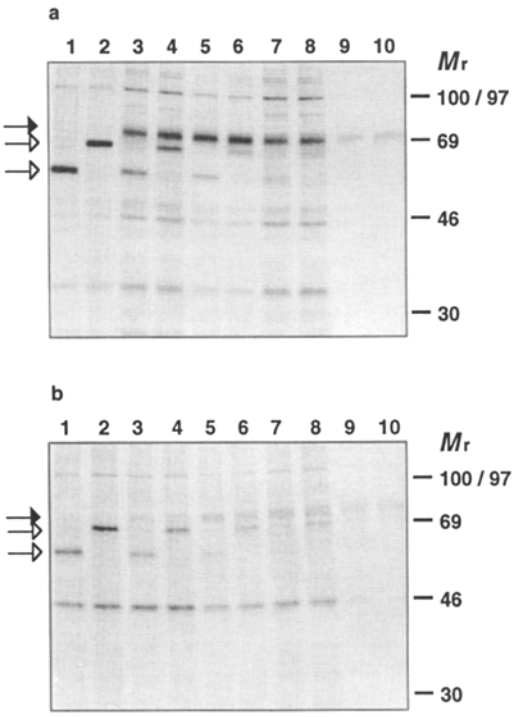

Figure 5. The kinetics of conversion of wt and HA164 to Endo H resistance. TBLCL were infected with vaccinia virus expressing wt HA (*a*) or HA164 (*b*) and metabolically labeled as described in Materials and Methods. A 30-min pulse radiolabeling was initiated 3 h after infection. Chase times are as follows: 0 min (lanes *1* and *2*), 30 min (Lanes *3* and *4*), 60 min (lanes *5* and *6*), 90 min (lanes *7* and *8*), and 120 min (lanes *9* and *10*). Odd-numbered lanes are with Endo H treatment; even-numbered lanes were mock treated. Open arrows indicate immature core glycosylated forms and the filled arrows indicate mature molecules with complex oligosaccharides. The positions of molecular weight markers are indicated. The band with a M_r of 45,000 is contaminating actin.

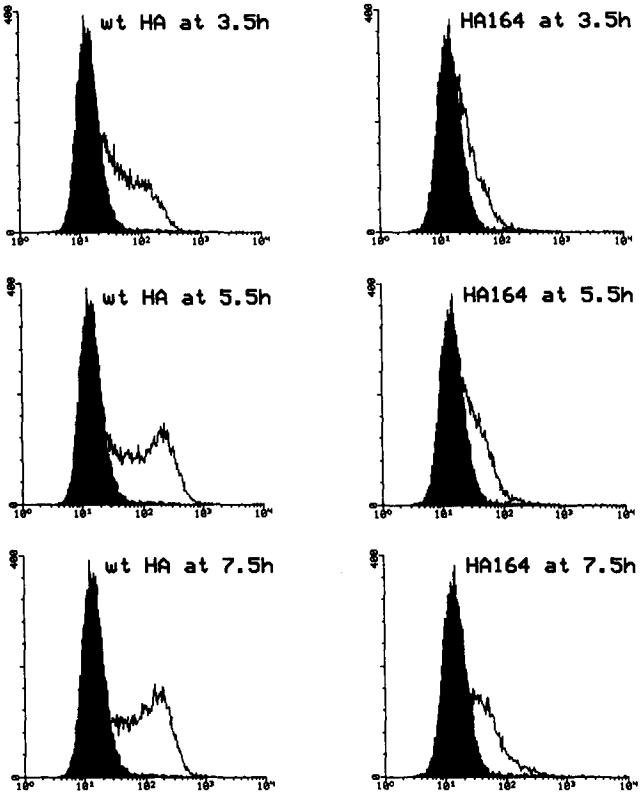

Figure 6. Flow cytometry comparison of wt HA and HA164. TBLCL were infected with the HA-expressing vaccinia viruses as indicated. Cells were subsequently stained and analyzed as described in Materials and Methods. The time points indicate hours after initiation of infection. The filled area indicates fluorescence with the secondary Ab alone; the open area indicates fluorescence with the primary and secondary antibodies.

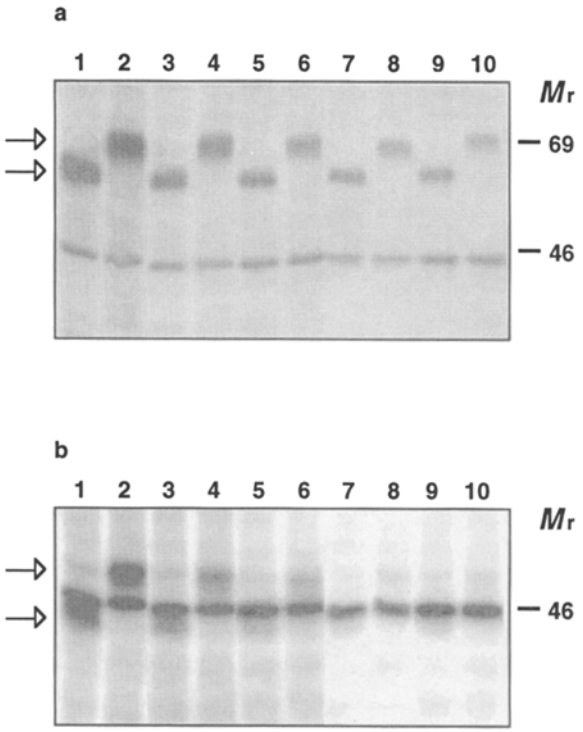

Figure 7. Endo H sensitivity and degradation of HAC67S and HAΔR1. TBLCL were infected with vaccinia virus expressing HAC67S (*a*) or HAΔR1 (*b*) and then metabolically labeled as described in Materials and Methods. The 30-min pulse was initiated 3 h after infection. Chase times are as follows: 0 min (lanes *1* and *2*), 2 h (lanes *3* and *4*), 4 h (lanes *5* and *6*), 6 h (lanes *7* and *8*), and 8 h (lanes *9* and *10*). Odd-numbered lanes are with Endo H treatment; even-numbered lanes were mock treated. The positions of molecular weight markers are indicated. The band with a M_r of 45,000 is contaminating actin.

Table 1. *Presentation Kinetics of wt and Mutant HA Molecules*

Exp.	Time	E/T	wt HA	HAC67S	HA164	HA-ΔR1	Vaccinia	
	h							
1	3.5–6.5	10:1	40	17	41	3	ND	
	5.5–8.5	10:1	45	38	44	7	ND	
	7.5–10.5	10:1	49	49	51	10	0	
			wt HA	HAC67S	HA-EEKKMP	HA-ΔR1	Untreated	HA 126-145
2	3.5–7	15:1	67	15	71	7	0	74
		7:1	60	14	64	4	1	66
		2:1	47	10	51	5	1	50
	6.5–10	15:1	63	42	65	12	0	61
		7:1	55	38	11	58	0	57
		2:1	42	25	41	7	0	43
	9.5–13	15:1	70	58	66	18	0	60
		7:1	61	53	59	15	0	56
		2:1	45	40	43	11	0	39

APC infected with recombinant vaccinia viruses expressing various HA forms, treated with HA 126–145 peptide, infected with vaccinia alone, or left untreated (as indicated), were incubated with specific CTL for the time periods indicated (postinfection). Numbers indicate percent specific lysis. Experiments were performed as in Fig. 7.

Table 2. *Chloroquine Sensitivity of HAC67S*

Time	E:T	− Chloroquine	+ Chloroquine
h			
4–7.5	15:1	22	3
	7:1	16	3
	2:1	9	2
9.5–13	15:1	59	18
	7:1	46	12
	2:1	25	8

APC infected with recombinant vaccinia viruses expressing HAC67S were incubated with specific CTL for time periods indicated (postinfection). Numbers indicate percent specific lysis. Experiments were performed as in Fig. 3.

restricted T cells. Included among these mutants is HA164, which has an altered cytoplasmic tail composed of 16 novel residues. This mutant is defective in its transit to the cell surface and may accumulate in a post-Golgi compartment (28). Fig. 5 shows a pulse-chase analysis of the acquisition of resistance to Endo H by wt HA and the HA164 mutant in LCL cells. Both wt HA (Fig. 5 *a*) and the HA164 mutant (Fig. 5 *b*) rapidly acquire resistance to Endo H, although HA164 is slightly delayed (by 30–60 min) in this conversion in the human LCL. When we examined cell surface expression of HA164 late in infection (6 h) by flow cytometry (Fig. 6), we found that the level of cell surface HA in the HA164 mutant was substantially reduced compared with wt HA. The diminished cell surface expression of HA164, in spite of rapid acquisition of Endo H resistance, is consistent with our earlier data on expression of HA164 in epithelial cells, suggesting that this mutant is significantly delayed in a post-Golgi compartment during its transit to the cell surface [28]. Although, from our data, we cannot formally rule out the possibility that this mutant is rapidly internalized.

When we examined CD4+ T cell recognition of HA164, we found that target cells expressing this mutant were recognized with the same efficiency and kinetics as targets expressing wt HA (Table 1, Exp. 1). The rapid and efficient processing and presentation of HA164, despite low cell surface expression, reinforced the view that internalization of cell surface HA into an endosomal/lysosomal compartment might not be the pathway for processing and presentation of newly synthesized HA, and that processing was occurring deeper within the cell.

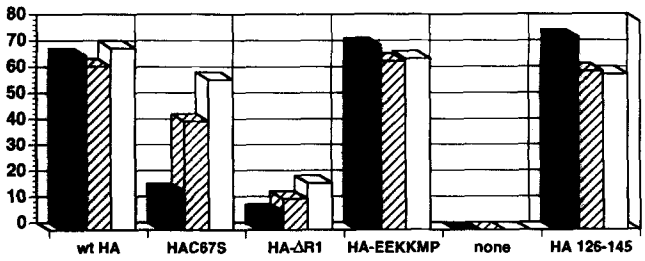

Figure 8. The kinetics of presentation of wt and mutant HAs. TBLCL were infected with recombinant vaccinia viruses expressing the various forms of HA, treated with the HA 126–145 synthetic peptide, or left untreated (as indicated). Cells were incubated for either 3.5 h (*filled bars*), 6.5 h (*striped bars*), or 9.5 h (*open bars*) before a 3-h incubation with the CTL clone V1. An E/T ratio of 15:1 was used.

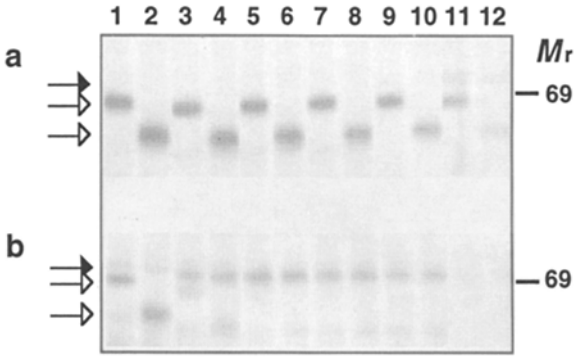

Figure 9. Pulse-chase analysis of HA-EEKKMP vs. wt HA. TBLCL were infected with vaccinia virus expressing HA-EEKKMP or wt HA and then metabolically labeled as described in Materials and Methods. The 30-min pulse was initiated 3 h after infection. Chase times are as follows: 0 min (lanes *1* and *2*), 30 min (lanes *3* and *4*), 1 h (lanes *5* and *6*), 2 h (lanes *7* and *8*), 4 h (lanes *9* and *10*), and 8 h (lanes *11* and *12*). Even-numbered lanes are with Endo H treatment; odd-numbered lanes were mock treated. Open arrows indicate immature core glycosylated forms, and the filled arrows indicate mature molecules with complex oligosaccharides. The positions of molecular weight markers are indicated.

The ER has been proposed as a site of proteolytic fragmentation of newly synthesized secretory proteins, as well as the site of charging of MHC class II molecules by the liberated peptides (17). To explore the proximal secretory pathway as a site of HA processing and charging of DRw11, we examined the presentation of three HA mutants: HAC67S,

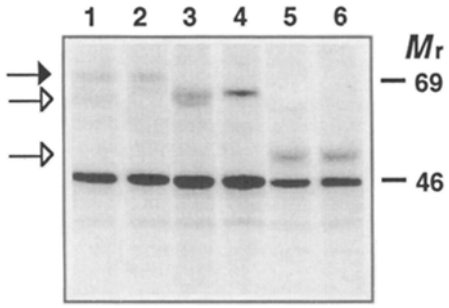

Figure 10. Endo D sensitivity of wt and mutant HAs. TBLCL were infected with vaccinia virus expressing wt HA (lanes *1* and *2*), HAC67S (lanes *3* and *4*), or HAΔR1 (lanes *5* and *6*), and metabolically labeled as described in Materials and Methods. A 30-min pulse and 30-min chase was initiated 2 h after infection. Odd-numbered lanes are with Endo D treatment; even-numbered lanes were mock treated. Open arrows indicate immature core glycosylated forms and the filled arrows indicate mature molecules with complex oligosaccharides. The positions of molecular weight markers are indicated. The band with a M_r of 45,000 is contaminating actin.

HAΔR1, and HA-EEKKMP. HAC67S has a single amino acid substitution in a site outside of the HA 129–140 epitope (serine for cysteine at position 67 in the mature protein). This mutant is unable to form a critical intramolecular disulfide bond necessary for stable HA trimer formation (29). As the pulse-chase analysis in Fig. 7 *a* shows, this molecule retains its Endo H sensitivity throughout the period of the chase, suggesting that this molecule is retained in a premedial Golgi compartment. In addition, the HAC67S molecule is extremely stable. It is still detectable in the Endo H–sensitive form after 8 h of chase and is slowly degraded in LCL cells with a half-life of 4–6 h (Fig. 7 *a* and D. J. Kittlesen, unpublished observation). As expected, this mutant does not transit to the cell surface and is not detectable on infected cells by flow cytometry (data not shown). When we examined HAC67S recognition by HA 129–140–reactive T cells, we found efficient presentation, albeit with somewhat delayed kinetics relative to wt HA (Fig. 8). As with wt HA, presentation of this mutant is also chloroquine sensitive (Table 2). These results implied that egress of the A/Japan/57 HA molecule through the secretory pathway is not required for its processing and charging of MHC class II molecules, and raised the possibility that MHC class II molecules might be charged in the ER by HA peptides derived from ER proteolysis. Our studies with the HA-EEKKMP and HAΔR1 mutants do not support this view.

In an effort to examine the ER as an antigen processing compartment for HA, we generated an ER retention mutant, referred to in this report as HA-EEKKMP. While KDEL retention sequences have been shown to be responsible for the retention of soluble ER proteins (reviewed in reference 34), this signal does not function for transmembrane proteins with their COOH termini located in the cytosol [35]. Instead, type 1 transmembrane proteins utilize a different COOH-terminal signal for ER retention. The best characterized of these is that of the adenovirus E3/19k protein (31). The presence of two lysine residues at positions −3 and −4 (or −5) from the COOH terminus has been demonstrated on numerous resident ER proteins and has been shown to be sufficient to retain other normally nonretained proteins when engineered onto their COOH termini (32). We chose therefore to express a form of wt HA containing the last six amino acids (−EEKKMP) of the adenovirus 5 E3/19k protein. As shown in Fig. 9, this molecule is in fact strongly retained relative to wt HA. However, leakage can be detected as a small pool of Endo H–resistant molecules detected at late chase times. Not surprisingly then, surface expression can be detected by flow cytometry and is ∼25% of wt at 9 h postinfection (not shown). This leak-through of proteins containing a retention sequence has been previously demonstrated, and was suggested to be a characteristic of the individual protein or of different cell types used for expression (35, 36). This molecule is presented as well as wt HA (Fig. 8 and Table 1). Although ER proteolysis and charging of class II MHC could account for the efficient recognition of the HA-EEKKMP mutant by T cells, the mutant did egress from the ER. Therefore, processing of HA in a post-ER compartment remained a possibility. We were able to test the contribution of ER processing using HAΔR1, a mutant that fortuitously is retained even more efficiently than HA-EEKKMP.

The HAΔR1 mutant was generated by a large in-frame deletion of the sequences encoding residues 248–409 of the A/Japan/57 HA (30). This mutant retains the HA 129–140 site recognized by the $CD4^+$ T cells. When expressed in LCL, the truncated polypeptide is core glycosylated but does not mature to an Endo H–resistant form in pulse-chase experiments (Fig. 7 *b*). Furthermore, the protein is rapidly degraded (with a half-life of ∼2 h) in a chloroquine-resistant manner, implying that it is susceptible to proteolysis in the ER-linked salvage/intermediate compartment (Fig. 7 *b*, and D. J. Kittlesen, unpublished observations). When tested for $CD4^+$ T cell recognition, this mutant polypeptide was only poorly processed and presented (Fig. 8 and Table 1). While recognition was poor relative to wt HA, at later time points its recognition was detectable (up to 26% of wt; Table 1), indicating that this HA deletion mutant can charge class II MHC, but does so inefficiently.

The difference in presentation between the two HA mutants that remain intracellular (HAC67S and HAΔR1) may be explained by differences in their ability to escape from the ER and progress further along the secretory pathway. The enzyme Endo D can be used to distinguish these molecules. This enzyme recognizes and cleaves oligosaccharides of the structure $(Man)_5(GlcNAc)_2$ (37). Trimming of oligosaccharides to this level is accomplished by α-mannosidase I, presumably acting in the *cis*- or *medial*-Golgi compartment (38) and is, therefore, a marker for this compartment. Fig. 10 shows the susceptibility of these molecules to this enzyme. HAC67S (and an immature form of the wt HA) are recognized and trimmed by this enzyme. Approximately one-half of the HAC67S is in an Endo D–recognizable form with doublet formation (Fig. 10, lane *3*); while none of the HAΔR1 was recognized and remains a single species (Fig. 10, lane *5*). Therefore, HAC67S, unlike the HAΔR1, has likely progressed further through the secretory pathway and been modified in the Golgi compartment by α-mannosidase 1.

Discussion

In this report we have examined the pathway by which the newly synthesized A/Japan/57 influenza virus HA glycoprotein is processed and presented in association with MHC class II molecules in human APC. We have found that processing and presentation of the HA requires translocation of the HA glycoprotein into the ER, but transit of the HA glycoprotein through the secretory pathway to the cell surface is not required for recognition of the processed protein by $CD4^+$ T lymphocytes.

Two intracellular compartments, the cytosol and the late endosome/lysosome, have been implicated as major sites of antigen processing for presentation to T lymphocytes. Although the cytosol is a major site of peptide formation for charging of MHC class I molecules, its role as a source of

processed antigens for MHC class II charging is not clear. We (7) and others (39) have previously provided evidence that proteins localized to the cytoplasm are inefficiently processed and presented in association with MHC class II molecules, while other investigators have reported presentation of newly synthesized cytosolic proteins to CD4$^+$ T cells (14, 16, 20). In the present report, the failure of the cytosolic signal-minus A/JAPAN/57 HA (Fig. 4) to be presented to HLA-DRw11-restricted T lymphocytes supports the view that, for this HA at least, localization of the HA polypeptide in the cytoplasm does not lead to MHC class II charging.

Although wt influenza HA does not efficiently cycle from the cell surface into the cell (40), we initially considered cycling of cell surface HA into an endosomal/lysosomal processing compartment as the likely mechanism for the processing and presentation of the newly synthesized HA. Our experimental observations, however, did not substantiate this view. Notably, we found that the anchor-minus secreted form of HA, and the HA164 and HA-EEKKMP mutants, which appear to be delayed in transport to the cell surface, are recognized by the HA 129–140–reactive T cells with efficiencies and kinetics comparable to wt HA. None of these forms of HA would be expected to cycle into an endosomal processing compartment from the cell surface with the efficiency of wt HA. More importantly, the HAC67S mutant, which does not transit to the cell surface and does not acquire resistance to Endo H , is also processed and presented in association with MHC class II molecules. This latter finding is incompatible with HA transit to the cell surface as a necessary requirement for HA processing and presentation.

Based on studies with ER-retained proteins, Weiss and Bogen (39) have proposed that the ER is a site of antigen processing and charging of the MHC class II molecules. Our data on the recognition of HAC67S by CD4$^+$ T cells are consistent with this concept, but our findings with the HAΔR1 mutant are not. Both HAC67S and HAΔR1 retain the 129–140 HA site. Both HA products remain in an Endo-H–sensitive state in lymphoblastoid target cells. The HAC67S mutant is relatively stable and is degraded with a half-life of ∼4–6 h. The HAΔR1 mutant is unstable and has a half-life of ∼2 h. This pattern of Endo H sensitivity and rapid degradation of the HAΔR1 mutant (which is insensitive to treatment of the cells with 50 μM chloroquine; D. J. Kittlesen, unpublished observation) are consistent with HAΔR1 proteolysis in the ER (41). If proteolytic fragmentation in the ER were an important source of peptides for MHC class II charging, then the unstable HAΔR1 mutant should be readily processed and presented. It is not, even at late times after expression of the mutant protein in the target cells (Fig. 8). Rather, MHC class II charging and T cell recognition correlate more directly with HA stability in the ER, with the stable wt HA being most efficiently processed.

One explanation for the difference in the presentation of HAC67S and HAΔR1 is that the stable HAC67S can transit from the ER to a compartment where it can be processed and presented in association with MHC class II molecules, while the unstable HAΔR1 is primarily retained in the ER and degraded there. Consistent with this view, we have found that HAC67S, but not the HAΔR1, acquires Endo D sensitivity by 90 min postsynthesis in target cells (Fig. 10). Since Endo D sensitivity is believed to be acquired during glycoprotein transit into an early Golgi compartment (37, 38), HAC67S could transit further than HAΔR1, where it can gain access to the MHC class II processing pathway. One attractive hypothesis consistent with our data is that proteins entering this ER to Golgi intermediate, or early Golgi, compartment are directly targeted to a late endosome/lysosome for fragmentation and subsequent charging of MHC class II molecules. Proteins have been engineered containing the KDEL ER retention sequence for soluble lumenal proteins and have been shown to be presented efficiently to MHC class II–restricted T cells (39). The mechanism of retention in the ER for these proteins is thought to be through retrieval from a post-ER compartment (34). This could explain how proteins with ER retention signals gain access to the MHC class II processing compartment, i.e., they reach an intermediate compartment or the *cis*-Golgi compartment before capture and retrieval back to the ER. Alternatively, low levels of escape through these retention sequences are likely sufficient for antigen presentation, as seen with our HA-EEKKMP mutant.

The findings reported here suggest that there is a mechanism for directing proteins from an early compartment in the secretory pathway to the late endosome/lysosome, where fragmentation of the protein and subsequent MHC class II charging can occur. This may be a specific pathway for shunting proteins normally retained in early secretory compartments, e.g., ER enzymes, chaperones, etc., to the lysosome. This shunt mechanism does not require protein transit through the medial or *trans*-Golgi compartment. Such a pathway might be crucial during development of the T lymphocyte repertoire to provide peptide fragments of these intracellular proteins for T lymphocyte–negative selection in the thymus. This pathway would therefore provide a mechanism for establishing CD4$^+$ T cell tolerance to these self-proteins. Alternatively, these early secretory compartment proteins, as well as viral proteins like HA, could gain access to the lysosome via an ER-associated autophagocytic process ongoing in APC. Although to date we have been unable to specifically inhibit presentation of wt or mutant HA using inhibitors of autophagy (42) (D. J. Kittlesen, unpublished observations), autophagy could also account for the processing and presentation of the HA gene products described in this and other reports.

Peptides observed from proteolysis in the endosome, ER, and cytosol may all contribute to the array of antigenic fragments displayed by MHC class II molecules on the surface of APC. Our findings reinforce the view that the endosome/lysosome is a primary site of antigen processing and presentation to CD4$^+$ T cells even for proteins retained in early compartments of the secretory pathway. The relative importance of cytosol, ER, and endosome as sources of processed peptides for self- and non-self discrimination, and foreign antigen presentation in APC in vivo, remains to be

determined. Recent advances in the isolation and characterization of processed peptide fragments associated with MHC class II molecules (43) should provide a framework for defining the contribution of these processing compartments to the formation of the peptide pool associated with MHC class II molecules.

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Address correspondence to Thomas J. Braciale, Beirne Carter Center for Immunology Research, University of Virginia Health Sciences Center, MR4 Box 4012; Charlottesville, VA 22908.

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