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## EXPRESSION AND FUNCTION OF A NONGLYCOSYLATED MAJOR HISTOCOMPATIBILITY CLASS I ANTIGEN

BY JUN-ICHI MIYAZAKI\*, ETTORE APPELLA<sup>‡</sup>, HONG ZHAO<sup>§</sup>,  
JAMES FORMAN<sup>§</sup>, AND KEIKO OZATO\*

*From the \*Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; the <sup>‡</sup>Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and the <sup>§</sup>Department of Microbiology, University of Texas, Southwestern Medical School at Dallas, Dallas, Texas 75235*

The majority of membrane proteins in mammalian cells are glycosylated. Because of their structural complexity and exclusive localization on the outer cell surface, carbohydrate moieties of membrane glycoproteins have been implicated to take part in cell to cell interaction and in molecular recognition processes (1, 2). However, the precise biological significance of carbohydrates for most membrane glycoproteins and secretory molecules has remained unsolved. The main difficulty in clarifying the role of carbohydrate components for these molecules has been the lack of experimental methods in which carbohydrates can be removed from the molecule (or altered) without affecting others in the cell. A number of studies used glycosylation inhibitors, such as tunicamycin (3), sugar analogs (4, 5), and glycosidases (6) to remove (or alter) carbohydrate moieties. These studies, however, did not provide definitive conclusions on the roles of carbohydrates, and in some cases results are conflicting, because these reagents affect all the carbohydrates in the system in an indiscriminatory manner. For the same reason the mechanistic basis of the observed effects has been difficult to ascertain. The use of mutant cells deficient in glycosylation pathways (7, 8), though advantageous for certain studies, has suffered similarly from the broad nature of the intervention.

We studied the roles of carbohydrate moieties in one of the most ubiquitous membrane glycoproteins, MHC class I antigen. The extracellular portion of the antigen is divided into three domains, and the two amino terminal domains in the mouse carry, without exception, sugar components. The positions of the glycosylation sites in the two domains are highly conserved throughout mammalian species (9–11). Some mouse class I antigens, such as the H-2L<sup>d</sup> antigen, have an additional glycosylation site in the third domain (12, 13). As in the case of other membrane glycoproteins, studies on the role of carbohydrates of the MHC class I antigens have not been conclusive (references 6, 14–17; see Discussion).

In this work, an alternative and more specific approach has been used to investigate the role of sugar moieties. We constructed mouse H-2L<sup>d</sup> genes in which codons for the N-linked glycosylation sites have been replaced by those of

TABLE I  
Synthetic Oligonucleotides Used to Mutate Glycosylation Sites of H-2L<sup>d</sup> Gene and L Cells  
Transformed by Mutant H-2L<sup>d</sup> Genes

	Domain oligonucleotides (3' → 5')*	Mutant L <sup>d</sup> genes	Transformants
N	GATGATGTT↓CGTCTCGCG (AAC)	8-3 (Asn-86 → Lys)	C8-3†
C1	TTCTTGCCCGTT↓CGCTGCGAC (AAC)	CH4-19 (Asn-86 → Lys) (Asn-176 → Gln)	CM2-2
C2	CCCTTCCTCGT↓CGTCATGTGTACGGCA (AAT)	CH13 (Asn-86 → Lys) (Asn-176 → Gln) (Asn-256 → Gln)	CM3-1, CM3-6

\* Oligonucleotides are complementary to the coding sequence cloned in M13 single-stranded DNA. Substituted nucleotides are indicated by arrows. Wild type codons are shown in parentheses.

† See Shiroishi et al. (16).

other amino acids, enabling us to study the function of the nonglycosylated H-2L<sup>d</sup> antigens expressed in the transformed L cells with high specificity. Recently Machamer et al. (18) and Guan et al. (19) reported a similar approach for studying viral glycoprotein and hybrid growth hormone, respectively.

### Materials and Methods

**Site-directed Mutagenesis.** The mutant L<sup>d</sup> gene 8-3, in which the codon for Asn-86 is replaced by Lys (16), cloned in M13 mp9 single-stranded DNA phage was used as the template for site-directed mutagenesis to change the codons for the remaining N-glycosylation sites (Asn-176 and Asn-256). The oligonucleotides used in this study are listed in Table I. They were synthesized by the solid-phase phosphotriester method (20) using an automated synthesizer (Vega Biotechnologies, Inc., Tucson, AZ) and purified by HPLC. The mutagenesis to convert the codon for Asn-176 to that of Gln was carried out as described before (16). One isolate, CH4-19, had the desired mutation as confirmed by DNA sequencing (21), and single-stranded DNA of this mutant was used as a template to replace the remaining glycosylation site in the third domain. To improve the efficiency of the mutagenesis, we constructed a gapped DNA in which most of the template (the entire M13 phage sequence and a part of the L<sup>d</sup> gene insert) had been made double-stranded by hybridizing the single-stranded CH4-19 DNA with 11-kb double-stranded CH4-19 DNA lacking a 630-bp Bgl II fragment. This gapped DNA left a 630-bp-long single-stranded region to which the mutagenic oligonucleotide was then hybridized. The subsequent procedures were the same as above. The resultant mutant CH13 was confirmed to have the desired mutations at all three glycosylation sites by DNA sequencing. Double-stranded DNAs of these mutants were introduced into the mouse L cells by the CaPO<sub>4</sub> coprecipitation method, and transformed cells were selected on the basis of thymidine kinase (*tk*)<sup>1</sup> gene cotransfection as described before (16).

**Metabolic and Surface Labeling of Cells.** Cells (~10<sup>7</sup>) were dissociated and incubated in methionine-free DME containing 10% FCS for 30 min. Then cells were incubated in the above medium plus 500 μCi/ml [<sup>35</sup>S]methionine (sp act 1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 3 h at a concentration of 10<sup>7</sup> cells/ml. For kinetic experiments, cells were labeled with [<sup>35</sup>S]methionine for 80 min at 37°C, followed by incubation in normal medium, and a portion of cells was taken at indicated times. The labeled cells

<sup>1</sup> Abbreviations used in this paper: SACI, *Staphylococcus aureus* Cowan I; tk, thymidine kinase; VSV, vesicular stomatitis virus.

were washed and lysed in 0.2 ml of lysis buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, and 0.1 mM PMSF) on ice for 15 min. Nuclei were removed by centrifugation for 10 min in an Eppendorf centrifuge. The supernatants were stored at  $-70^{\circ}\text{C}$  and used for immunoprecipitation.

Cells were surface-labeled with  $^{125}\text{I}$  by the lactoperoxidase method (22) with a minor modification. Cells were scraped from petri dishes, washed, and incubated in 3 ml of HAT medium at a concentration of  $3 \times 10^6$  cells/ml for 2 h. After washing twice, cells were surface-labeled with 0.5 mCi/ml of  $\text{Na}[^{125}\text{I}]$  (Amersham Corp., 14.5 mCi/mg) in the presence of  $\beta\text{-D}(+)\text{glucose}$ , lactoperoxidase, and glucose oxidase. Labeled cells were then washed five times, incubated in normal medium for 0, 2.5, or 5.5 h at  $37^{\circ}\text{C}$ , and lysed as described above.

**Immunoprecipitation.** Labeled lysates were precleared with protein A-bearing *Staphylococcus aureus* Cowan I (SACI) pretreated with an mAb of unrelated specificity and with SACI pretreated with unlabeled L cell lysate. Then aliquots of the lysates were incubated with an excess of the indicated mAbs (used as undiluted ascites fluid) at  $4^{\circ}\text{C}$  overnight. The immune complexes were incubated with SACI, and then extensively washed. Bound proteins were solubilized by boiling in elution buffer containing SDS and 2-ME, and analyzed by electrophoresis on 10 or 11% SDS polyacrylamide slab gel (23) followed by fluorography. Commercially available molecular weight markers were electrophoresed in parallel with the samples.

**Flow Cytofluorography Analysis.** Cells ( $5 \times 10^5$ ) were dissociated with trypsin, washed, and incubated with 50  $\mu\text{l}$  of antibodies (culture supernatants of hybridoma cells) at  $4^{\circ}\text{C}$  for 45 min. After washing with HBSS containing 0.1%  $\text{NaN}_3$  and 3% FCS, cells were incubated with 50  $\mu\text{l}$  of FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse F(ab')<sub>2</sub> and anti-mouse Fc (Cappel Laboratories, Cochranville, PA) diluted 1:40 at  $4^{\circ}\text{C}$  for another 45 min, and then washed twice with HBSS. Antibody binding was monitored by a cytofluorograph system (Ortho Diagnostic Systems, Inc., Westwood, MA) with an argon laser at wavelength 488 nm.

**Antibody Binding Assay.** Antibody binding assays were carried out as described previously (24). Cells ( $4 \times 10^5$ ) were placed in wells of 96-well microtiter plates and incubated overnight. Fifty  $\mu\text{l}$  of mAbs (culture supernatants of hybridoma cells) were added to each well, and binding was assayed by subsequent incubation with  $^{125}\text{I}$ -labeled sheep anti-mouse whole Ig (Amersham Corp.).

**Generation of Alloreactive CTL.** (B6  $\times$  DBA/2)F<sub>1</sub>, BALB/c, and BALB.K mice were bred at the University of Texas Health Science Center at Dallas. Anti-vesicular stomatitis virus (VSV) responses were generated as described previously (25). Briefly, spleen cells were obtained from mice immunized with  $10^9$  PFU of VSV 1 wk previously. These responder cells were cultured with syngeneic stimulator cells that were infected with a temperature-sensitive mutant of VSV at 25 PFU/cell for 2 h and then irradiated at 2,600 rad for 30 min. The responder and stimulator cells were mixed together at a 1:1 ratio and incubated at  $40^{\circ}\text{C}$  in complete media. 5 d later these cells were tested for cytotoxicity against  $^{51}\text{Cr}$ -labeled VSV-infected or uninfected (control) target cells.

Alloreactive CTL were obtained from coculturing BALB/c spleen cells with irradiated BALB.K stimulator cells and vice versa. Anti-H-2L<sup>d</sup> CTL clones were generated by limiting dilution as described previously (26). Clones L9.4 and L11.15 were derived from (C3H  $\times$  BALB/c-dm2)F<sub>1</sub> anti-DBA/2 effectors, clones L13D.4A, L13D.8, and L13D.17 from CBA/J anti-A.AL effectors.

## Results

### *In Vitro* Mutagenesis to Replace the Consensus Asn of the N-Glycosylation Sites in the H-2L<sup>d</sup> Gene

The H-2L<sup>d</sup> gene has three consensus Asn(-X-Ser/Thr) that represent the sites of N-linked glycosylation (27), each located in one of the three separate extracellular domains, i.e., at amino acid position 86, 176, and 256 (13). We previously prepared a mutant gene in which the N-glycosylation site of the first external

domain (amino acid 86) was converted to Lys (16). This mutant gene, 8-3, was cloned in M13 mp9 and used as the first template to sequentially remove the remaining glycosylation sites at the second and third domains. Synthetic primers used to replace these three glycosylation sites are shown in Table I. Mutagenesis experiments to replace the codon for amino acid 176 were carried out as described (16), and a mutant clone *CH4-19* containing the correct mutation was isolated. Single-stranded DNA of this mutant was then used as the template to eliminate the last glycosylation site in the third domain. For this mutagenesis we used a gapped duplex DNA construct to increase the efficiency of mutagenesis (reference 28; see Materials and Methods). A positive clone *CH13* was isolated, and subsequent DNA sequence analysis confirmed that this clone had three mutations at the expected sites and no other alterations. Double-stranded DNAs of the above mutants were introduced into mouse L cells, and transformants were isolated (Table I). Introduction of the *CH13* mutant gene lacking all the glycosylation sites resulted in the generation of transformed cells all of which expressed only very low levels of the L<sup>d</sup> antigen on their surface (see below). The cells expressing relatively high levels of the H-2L<sup>d</sup> antigen were used for the subsequent experiments.

#### *Molecular Mass Analysis of the Mutant H-2L<sup>d</sup> Gene Products*

The absence of the carbohydrate moieties in the products of the mutant genes was verified by immunoprecipitation of [<sup>35</sup>S]methionine-labeled cell lysates with mAbs specific for the H-2L<sup>d</sup> antigen. As seen in Fig. 1, SDS-PAGE analysis shows a stepwise reduction of the apparent molecular weight in the mutant H-2L<sup>d</sup> antigens that lack one (expressed in C8-3), two (CM2-2), or all three glycosylation sites (CM3-1). Removal of one glycosylation site resulted in a molecular mass reduction of ~2 kD roughly consistent with a previous estimate made for HLA antigen (6). Thus the mutant L<sup>d</sup> antigen devoid of all carbohydrates was ~6 kD smaller than the wild type H-2L<sup>d</sup> antigen. In the above experiments all the transformants were labeled and immunoprecipitated under identical conditions employing an excess amount of mAbs. The density of each L<sup>d</sup> band should, therefore, represent approximately the relative amount of antigen in the cells. Note that the amount of the nonglycosylated L<sup>d</sup> antigen found in CM3-1 was comparable to that of the wild type antigen in W-12 (Fig. 1, lanes B and E), even though the level of the cell surface antigen was much lower than the wild type counterpart (see below).

#### *Reduced Expression of the Nonglycosylated Mutant H-2L<sup>d</sup> Antigen on the Cell Surface*

The level of the mutant antigens expressed on the surface of transformed cells was examined by cytofluorography analysis using an excess of mAbs specific for each domain of the H-2L<sup>d</sup> antigen. Two examples of the analysis presented in Fig. 2 show strikingly low antibody binding to CM3-1 and CM3-6, both of which express the nonglycosylated H-2L<sup>d</sup> antigen. The binding of the antibodies to these cells was ~10–15% of the wild type cells, as estimated by the mean fluorescence values. The low binding was not explained by higher sensitivity of the nonglycosylated antigen to trypsin, which was used to harvest the cells before cytofluorography, because mechanically harvested cells showed the same staining

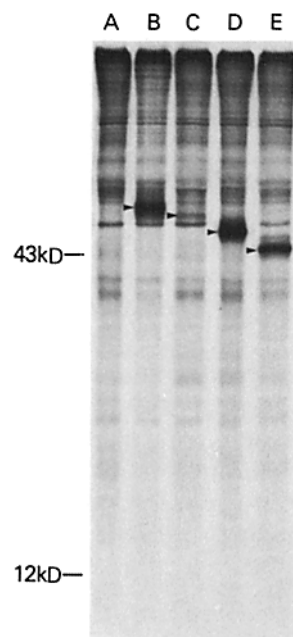


FIGURE 1. Molecular mass analysis of the mutant H-2L<sup>d</sup> antigens. Untransformed DAP-3 cells (A), wild type L<sup>d</sup> transformant W-12 (B), mutant L<sup>d</sup> transformants C8-3 (C), CM2-2 (D), and CM3-1 (E) were labeled with [<sup>35</sup>S]methionine, and immunoprecipitated with H-2L<sup>d</sup> specific mAbs 64.3.7 and 28.14.8 (16). Precipitates were analyzed by 11% SDS-PAGE. Arrows indicate bands corresponding to the L<sup>d</sup> heavy chains.

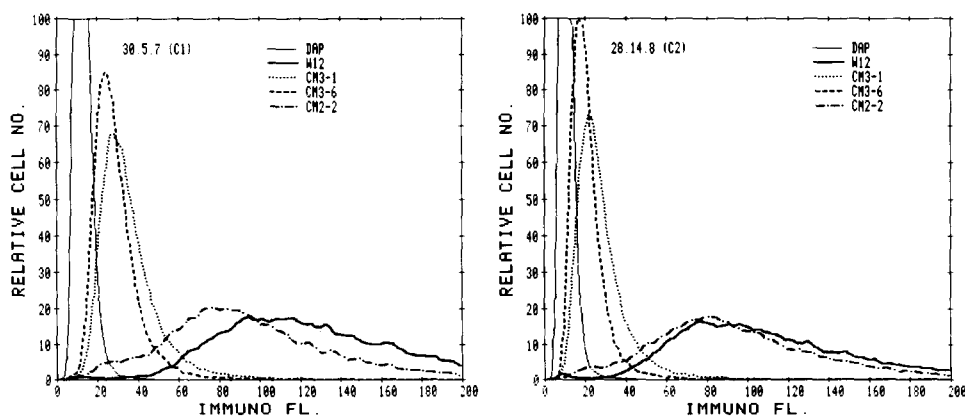


FIGURE 2. Cytofluorographic analysis of surface expression of the mutant H-2L<sup>d</sup> antigens. Untransformed DAP-3 cells, wild type L<sup>d</sup> transformant W-12, mutant L<sup>d</sup> transformants CM2-2, CM3-1, and CM3-6 were dissociated and incubated with H-2L<sup>d</sup> C1 domain specific mAb 30.5.7 (left) or C2 domain specific mAb 28.14.8 (right), followed by incubation with fluorescein-conjugated goat anti-mouse Ig. Antibody binding was monitored by flow cytofluorography.

pattern (data not shown). The reduced antibody binding was not due to a decreased affinity of the antibodies for the mutant H-2L<sup>d</sup> antigen, but rather due to reduced surface expression of the mutant antigen, since the same antibodies immunoprecipitated a comparable amount of the antigen from lysates of

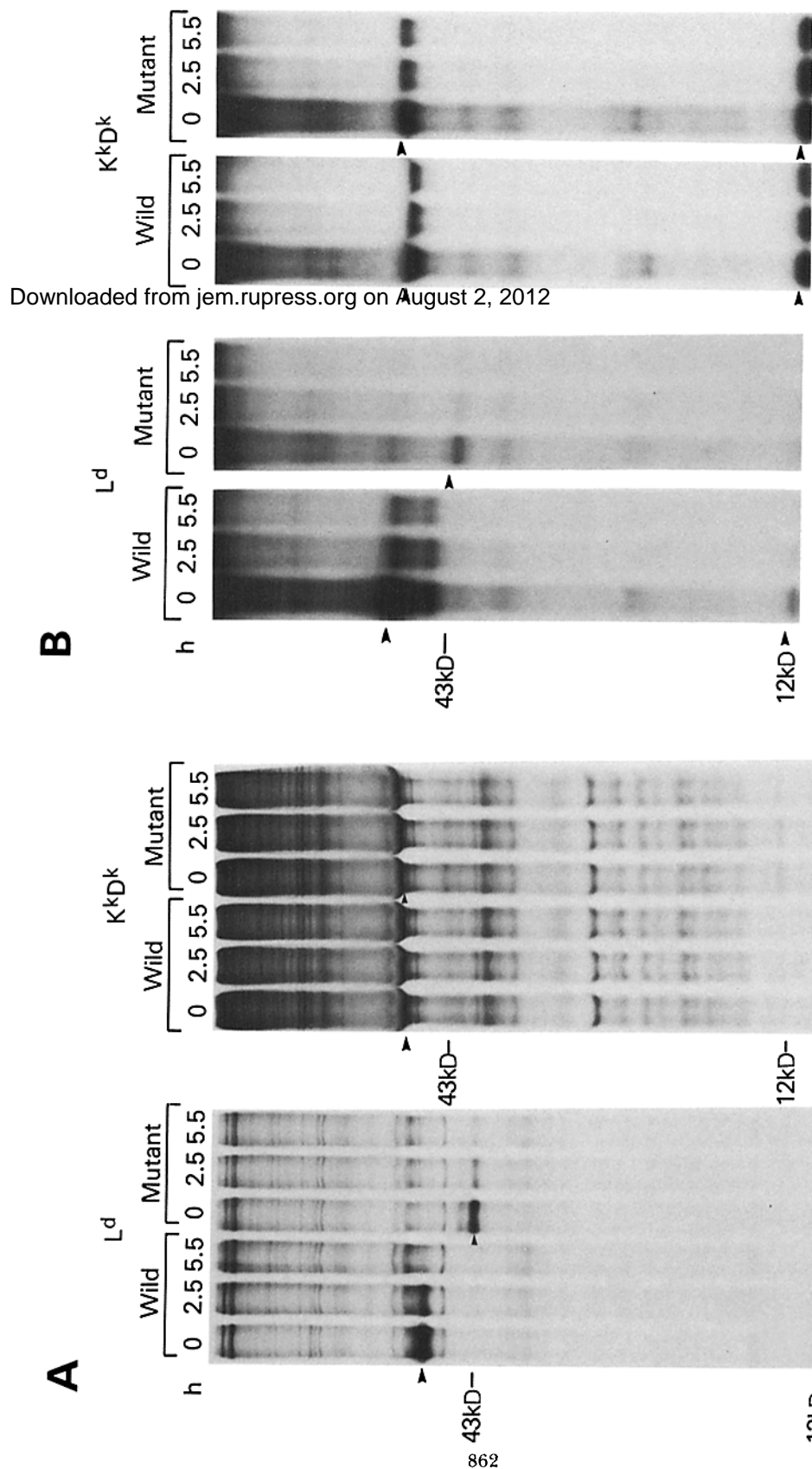
the mutant as from the wild type cells (Fig. 1). 14 other anti-H-2L<sup>d</sup> mAbs all exhibited extremely low binding to the mutant cells. The level of the cell surface H-2L<sup>d</sup> antigen in CM2-2 lacking two glycosylation sites was comparable to that in the wild type W-12, whereas C8-3 lacking one glycosylation site showed a much lower level of the antigen expression. However, the discrepancy in the ratio of surface vs. internally synthesized antigens seen in CM3-1 was not observed in CM2-2 or C8-3 cells.

#### *Turnover Rate of the Mutant and Wild Type H-2L<sup>d</sup> Antigens*

The decreased surface expression of the mutant L<sup>d</sup> antigen in CM3-1 and CM3-6 may be explained by the following two possibilities: (a) the nonglycosylated antigens are less stable and turned over faster than the wild type antigen; (b) alternatively, the mutant L<sup>d</sup> antigen is transported to the plasma membrane less efficiently than the wild type counterpart. To distinguish these possibilities, we studied the turnover of the newly synthesized and the surface-expressed H-2L<sup>d</sup> antigens. The wild type (W-12) and the nonglycosylated mutant (CM3-6) were labeled with [<sup>35</sup>S]methionine (Fig. 3A) or surface-iodinated (Fig. 3B) and then cultured for 2.5 or 5.5 h.

Immunoprecipitation and SDS-PAGE analysis were carried out for the H-2L<sup>d</sup> antigen and for the endogenous H-2K<sup>k</sup> and D<sup>k</sup> antigens. To quantitatively assess the amount of the H-2 antigens immunoprecipitated after various incubation periods, densitometric measurement was carried out, and results are summarized in Fig. 4. In this figure the density of the L<sup>d</sup> or K<sup>k</sup> and D<sup>k</sup> antigens of the wild type W-12 cells immediately after labeling (0 h) was taken as 100%. The H-2K<sup>k</sup> and D<sup>k</sup> antigens, either metabolically labeled or surface-iodinated, were very stable among all the transformants tested (Fig. 3, A and B, right); the intensity of the H-2K<sup>k</sup> and D<sup>k</sup> bands obtained after 5.5 h chase was almost comparable to that of time 0. In contrast, the H-2L<sup>d</sup> antigens were turned over much faster; in both the mutant and wild type cells, the half-life of the H-2L<sup>d</sup> antigen was ~2 h. The surface H-2L<sup>d</sup> antigens also turned over quickly, exhibiting similar kinetic patterns as those of internally labeled antigens again for both the mutant and wild type cells (Fig. 4). Another wild type transformant (24) also showed the same kinetic pattern for the L<sup>d</sup> antigen with W-12 (data not shown). The amount of the nonglycosylated mutant antigen immunoprecipitable from the cell surface at time 0, however, was <10% of the wild type antigen (Fig. 4B), even though the same mutant cells contained a much larger amount of <sup>35</sup>S-labeled antigen (Fig. 4A). The strikingly small amount of the mutant L<sup>d</sup> antigen precipitable from <sup>125</sup>I-labeled CM3-6 cell surface was not due to a lowered affinity of antibody binding to the mutant antigen, because the same antibodies were used for both experiments. It is significant that the nonglycosylated mutant and the wild type H-2L<sup>d</sup> antigens were virtually identical in turnover kinetics in terms of either surface-expressed or newly synthesized molecule. These results indicate that the low level of surface expression of the mutant antigen is not due to accelerated degradation or increased shedding, but rather to a deficiency in intracellular transport.

The turnover difference seen between the K<sup>k</sup>D<sup>k</sup> antigens and the L<sup>d</sup> antigen may not be surprising, because Emerson et al. (29) reported that turnover rates



immunoprecipitated using H-2L<sup>d</sup>-specific mAbs 64.3.7 and 28.14.8, or H-2K<sup>kDk</sup>-specific mAbs 3.83 and 11.4.1. Precipitates were analyzed by 10 or 11% SDS-PAGE.

FIGURE 3. Kinetic analysis of metabolically or surface labeled wild type and mutant H-2L<sup>d</sup> antigens. Wild type L<sup>d</sup> transformant W-12 and mutant L<sup>d</sup> transformant CM3-6 were metabolically labeled with [<sup>35</sup>S]methionine (A) or surface-iodinated with Na[<sup>125</sup>I] (B), and chased for varying times. Cells were



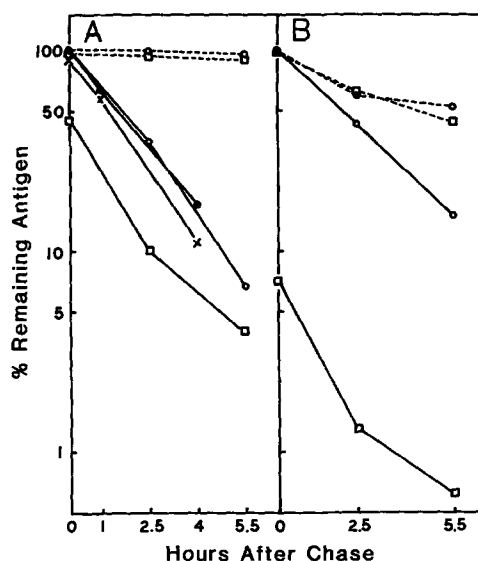


FIGURE 4. Densitometric analysis of kinetic experiments for wild type and mutant H-2L<sup>d</sup> antigens. Kinetic experiments by metabolic labeling (A) or surface iodination (B) shown in Fig. 3, and others were analyzed by densitometry using the films after a various length of exposures to obtain linear measurements. The densities of H-2L<sup>d</sup> and K<sup>d</sup>D<sup>k</sup> antigens of the wild type transformant W-12 at 0 h were taken at 100%. (----) H-2K<sup>d</sup>D<sup>k</sup>; (—) H-2L<sup>d</sup>. (O, ●) W-12; (X) CM3-1; (□) CM3-6. (A) Results of two independent experiments.

of class I antigens are highly variable among different gene products, and are affected by the combination of alleles of the class I genes.

#### *Immunological Functions of the Nonglycosylated Mutant Antigen*

**Serological Determinants.** To investigate a contribution of carbohydrate moieties to the fine serological determinants detected by B cell products we have tested the binding of 15 mAbs specific for various domains of the H-2L<sup>d</sup> antigen by indirect radioimmunoassays, and the results are shown in Table II. A variability noted in binding among different antibodies reflects differences in the isotypes and affinity of individual antibodies tested. The nonglycosylated mutant CM3-6 was universally low in binding for all the antibodies tested. CM2-2 was comparable to the wild type for most antibodies. Binding of antibody 28.11.5 was lower in both CM2-2 and CM3-6 relative to that in the wild type antigen. This antibody, however, was previously noted to bind poorly to C8-3 cells lacking one glycosylation site of the antigen (16). Antibodies 23.10.1, 1634, and 174.1 also showed reduced binding to CM2-2 and CM3-6 cells. Thus no additional serological alterations were found by removing the glycosylation sites from the third external domain. Binding of the remaining 11 antibodies was unchanged by the removal of all the glycosylation sites. These results illustrate overall preservation of serological determinants in the nonglycosylated mutant antigen.

**Reactivity with Allospecific CTL.** MHC class I antigens are recognized by alloreactive CTL through T cell receptors. It is of interest to test whether carbohydrates constitute or control antigenic determinants to be recognized by T cells. Even if carbohydrate moieties did not serve as antigenic determinants

TABLE II  
Binding of Anti-H-2L<sup>d</sup> mAbs to Transformed Cells

Anti-body	Specificity (domain)	Isotype	Cell line*			
			DAP-3	W-12	CM2-2	CM3-6
<i>cpm/well</i>						
64.3.7	L <sup>d</sup> (N)	IgG	20	480	580	240
66.3.5	L <sup>d</sup> (N/C1)	IgG	23	1,660	2,310	1,080
30.5.7	L <sup>d</sup> (C1)	IgG	21	2,490	2,780	1,070
23.10.1	L <sup>d</sup> (C1)	IgM	16	230	76	40
1634	L <sup>d</sup> (C1)	IgM	28	200	100	45
174.1	L <sup>d</sup> (C1)	IgM	38	200	74	44
28.14.8	L <sup>d</sup> (C2)	IgG	27	2,660	2,250	930
28.11.5	L <sup>d</sup>	IgM	17	260	54	30
27.11.13	L <sup>d</sup>	IgG	76	1,150	980	420
34.4.20	L <sup>d</sup>	IgG	20	1,230	1,900	640
T.0.101	L <sup>d</sup>	IgG	12	1,010	950	270
T.0.102	L <sup>d</sup>	IgG	22	790	1,770	550
T.0.103	L <sup>d</sup>	IgG	54	1,790	1,840	690
T.0.105	L <sup>d</sup>	IgG	37	1,700	2,250	890
T.0.106	L <sup>d</sup>	IgG	38	440	670	170
16.1.2	K <sup>k</sup> and D <sup>k</sup>	IgG	4,500	2,690	5,430	3,930
14.4.4	IA <sup>k</sup>	IgG	27	23	35	25
W/0 Ab	—	—	16	12	36	36

\* See Materials and Methods for details. Each value represents mean of triplicate.

for T cell receptors, they may play a significant role in providing efficient interaction with the receptor. Further, the cytolytic function of T cells requires a variety of accessory molecules (30) with which the carbohydrates of class I antigen may have significant associations. To investigate these possibilities, we tested whether CTL generated against the wild type L<sup>d</sup> antigen can lyse the transformants expressing the nonglycosylated antigen. Figs. 5B and 6B show the lytic activity of primary anti-H-2L<sup>d</sup> CTL mediated by BALB.K anti-BALB/c (H-2<sup>k</sup> anti-H-2<sup>d</sup>) effector cells. These bulk-cultured CTL lysed L cells transfected with H-2L<sup>d</sup> (W-12), H-2L<sup>d</sup> mutant genes (C8-3, CM2-2, CM3-1 and CM3-6), but not L cells transfected with the *tk* gene (Ltk) only. In most experiments the lysis directed against the mutant antigens was less than seen against the wild type H-2L<sup>d</sup> and probably reflects the diminished expression of the mutant H-2L<sup>d</sup> molecules on the cell membrane (Fig. 2). To further investigate the effect of carbohydrate on alloantigen recognition, we tested whether five anti-H-2L<sup>d</sup> CTL clones could recognize these mutant molecules. The data in Table III show that all five of these clones lyse L cells transfected with the mutant molecules to a similar extent as H-2L<sup>d</sup> transfectants. These results indicate that carbohydrates are not essential for alloantigen specific recognition. Further, while carbohydrates could possibly affect some epitopes recognized by CTL, it is likely that only a minor component of the response is directed against such determinants, since we detected little or no change in recognition by either bulk cultured or cloned alloreactive CTL on the mutant molecules.

**Function in VSV-specific, H-2L<sup>d</sup>-restricted CTL.** Viral antigens expressed on the membrane of infected cells are recognized by cytotoxic T cells in association

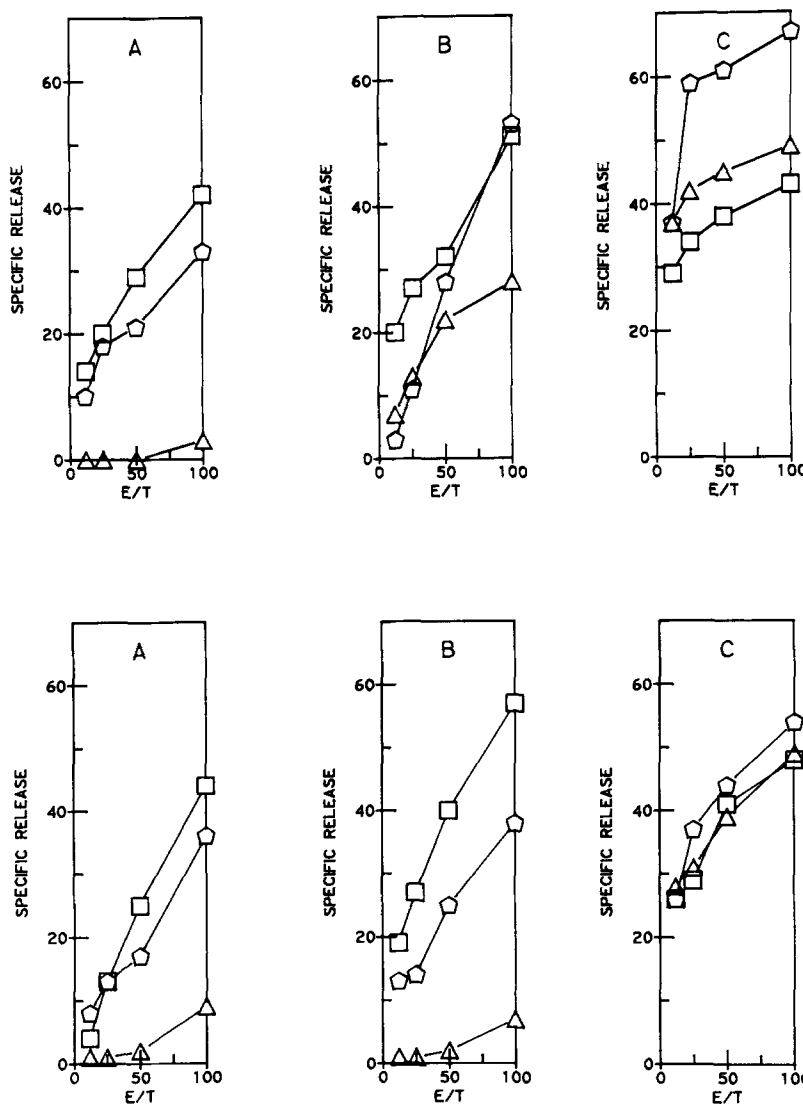


FIGURE 5. Specific cytolysis of transformed cells expressing mutant H-2L<sup>d</sup> antigens by alloreactive and H-2L<sup>d</sup>-restricted anti-VSV CTL. (B6 × DBA/2)F<sub>1</sub> anti-VSV (A), BALB.K anti-BALB/c (anti-H-2<sup>d</sup>) (B), and BALB/c anti-BALB.K (anti-H-2<sup>b</sup>) (C) CTL were tested against VSV-infected Ltk (Δ), W-12 (□), C8-3 (◇, top panels), or CM2-2 (◻, bottom panels) target cells.

with self MHC class I antigens (31), which may be the most significant function of the MHC class I antigens. Thus it was important to determine whether carbohydrates of the class I antigens are necessary for this process. It has been shown previously that in H-2<sup>d</sup> haplotype mice, VSV is recognized solely in conjunction with the H-2L<sup>d</sup> gene products, and that H-2K<sup>d</sup> and H-2D<sup>d</sup> antigens are not effective in the recognition of VSV (25). Therefore, we tested the susceptibility of the H-2L<sup>d</sup> mutant antigens to serve as restricting molecules for anti-VSV CTL. (B6 × DBA/2)F<sub>1</sub> mice were inoculated with VSV and their spleen

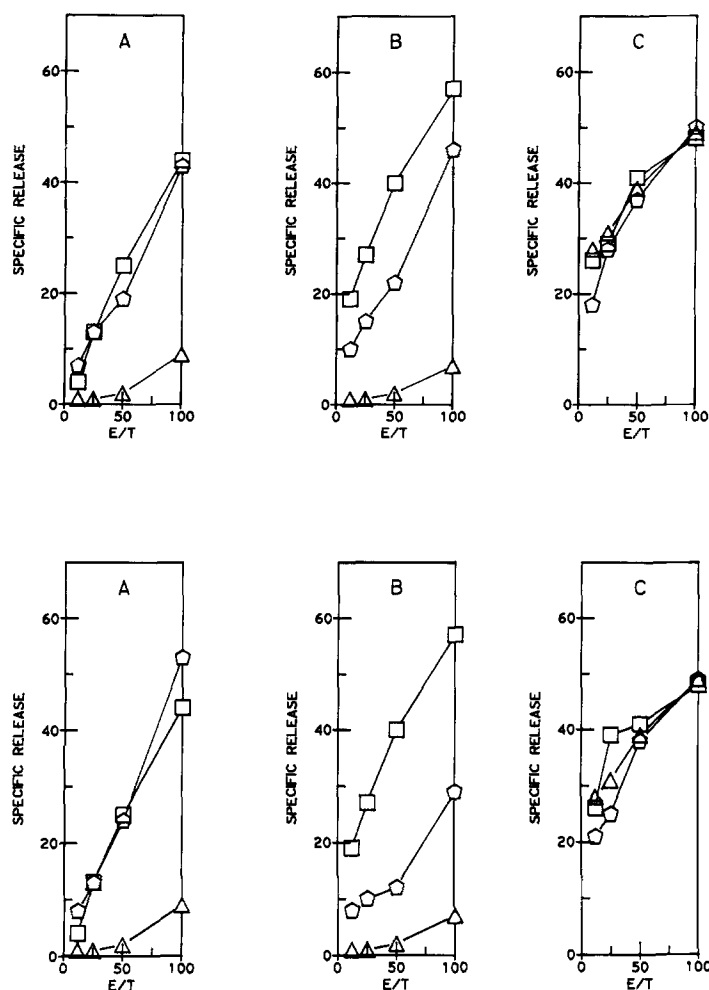


FIGURE 6. Specific cytotoxicity of transformed cells expressing mutant H-2L<sup>d</sup> antigens by alloreactive and H-2L<sup>d</sup>-restricted anti-VSV CTL. (B6 × DBA/2)F<sub>1</sub> anti-VSV (A), BALB.K anti-BALB/c (anti-H-2<sup>d</sup>) (B), and BALB/c anti-BALB.K (anti-H-2<sup>k</sup>) (C) CTL were tested against VSV-infected Ltk (Δ), W-12 (□), CM3-6 (◇, top panels), or CM3-1 (◇, bottom panels) target cells.

TABLE III  
Ability of Anti-H-2L<sup>d</sup>-specific CTL Clones to Recognize H-2L<sup>d</sup> Mutant Molecules

CTL clone	Target cells									
	Ltk		W-12		C8-3		CM2-2		CM3-6	
L9.4	1	1*	44	25	50	35	38	27	41	32
L11.15	2	1	47	35	59	46	48	36	54	36
L13D.4A	1	1	42	35	29	26	38	26	34	21
L13D.8	1	2	58	30	40	21	33	15	39	27
L13D.17	0	1	52	44	50	37	41	33	45	34

\* Specific lysis at E/T ratio of 20 and 5.

cells were tested in a secondary CTL assay. The data in Figs. 5A and 6A show that the absence of carbohydrate in the N, N and C1, or N, C1 and C2 domains had little or no effect on target cell lysis. In some experiments (not shown), lysis of the mutant H-2L<sup>d</sup> targets was less than that of the wild type L<sup>d</sup> targets. In general, anti-VSV lysis paralleled the extent of lysis mediated by BALB.K anti-BALB/c effector cells. All of the target cells were equally sensitive to anti-H-2<sup>k</sup> CTL (Figs. 5C and 6C). These results indicate that carbohydrates are not required for H-2-restricted recognition of the viral antigens of VSV by CTL effector cells. This does not, however, exclude the possible importance of carbohydrate moieties in the induction of CTL, either allo- or viral-specific.

### Discussion

This study has addressed the role of carbohydrate moieties in the expression and function of MHC class I antigens. We examined products of mutant L<sup>d</sup> genes lacking N-glycosylation sites and our observations can be summarized as follows. First, the nonglycosylated class I antigen was functionally intact in terms of reactivity with alloreactive CTL, and in terms of serological specificities detected by a number of mAbs. Further, the nonglycosylated L<sup>d</sup> antigen was capable of mediating H-2-restricted recognition by anti-VSV-specific CTL. The failure to observe a functional significance associated with carbohydrates may be somewhat surprising in view of long-standing implications for sugar moieties in cell to cell interactions proposed for general cellular differentiation (1) and for evolution of immunity (2). However, in a number of reports, retention of biological activities has been shown in proteins from which sugar moieties were removed; antiviral action of human interferon-gamma (32), infectivity of viral particles (33), and antigen binding capacity of IgG (34) are fully maintained after removal of carbohydrates by tunicamycin treatment, although in the latter case some other functions, such as complement fixation and Fc receptor binding, are affected. These findings point to the notion that carbohydrates of some membrane (and secretory) glycoproteins are not absolutely necessary for recognition processes that take place in the external milieu.

The most significant consequence of the removal of the glycosylation sites was the markedly diminished surface expression of the antigens. The reduced surface expression was due to the impaired intracellular transport of the antigen and was not attributable to increased degradation or accelerated shedding, as verified by pulse-chase experiments (Fig. 4). In accordance with these results, most, if not all, glycoproteins have been shown to exhibit reduced surface expression upon tunicamycin treatment. However, in contrast to our conclusion, in some cases reduction was explained by enhanced degradation; e.g., nonglycosylated acetylcholine receptor (35) and fibronectin (36) reportedly degrade more rapidly, although data are conflicting for the latter protein (37). Hastened degradation has been indicated also for the T25 glycoprotein (8). The basis of the discrepancy is not clear at present. It is possible that these and our results are not directly comparable as different mechanisms may operate in different systems. However, the possibility cannot be excluded that the increased degradation noted in the previous studies is caused secondarily by the general perturbation of cellular metabolism upon tunicamycin treatment.

Further evidence indicates that impaired intracellular transport is responsible for the reduced surface expression of nonglycosylated molecules (4, 37–39). Recently, Guan et al. (19) demonstrated that a membrane-anchored form of growth hormone, otherwise intracellular, becomes expressed on the surface upon acquisition of an N-linked glycosylation site. This finding also supports the general notion that carbohydrate moieties promote the transport of a molecule to the plasma membrane. It has been shown in a number of cases that the transport of nonglycosylated proteins produced by tunicamycin treatment is not completely blocked, so that a fraction of the nonglycosylated molecules reaches the plasma membrane. This phenomenon has been demonstrated for glycophorin A (39), envelope proteins of VSV (33), and HLA and H-2 histocompatibility antigens (6, 17), in addition to the H-2L<sup>d</sup> antigen in this study. In contrast, the complete blockade of membrane expression is reported for other molecules; i.e., opsin of the retinal photoreceptor (40), T25 protein in lymphoma cells (8), and murine leukemia virus (5) are not detectable on the plasma membrane without carbohydrates. Taken together, these results indicate that for many membrane glycoproteins, carbohydrates are not an absolute requirement for intracellular transport, but they act by facilitating the transport process. These data further indicate that the degree of transport blockade differs characteristically depending on the carbohydrate requirement in each glycoprotein. Similar variability has been noted for the blockade of secretory glycoproteins, for example, immunoglobulins of different isotypes (41). The defective transport caused by the absence of carbohydrates may be explained by the possible alteration of conformation and/or orientation of the molecule. In addition, carbohydrates may be required for maintaining the solubility of the protein, as has been indicated for viral glycoproteins (38). Not contradictory with the above, the following mechanism may be postulated: carbohydrate components are an important element for recognition by internal receptors that regulate translocation of nascent glycoproteins from the endoplasmic reticulum to the plasma membrane. A receptor-mediated mechanism of intracellular transport has been proposed for a number of viral and cellular membrane glycoproteins (42, 43). Based on characteristic partial blockade of the transport seen for some molecules, one may envisage that carbohydrate moieties control the affinity of the interaction with such receptors. The notion that carbohydrates play a key role in interaction with such receptors is also compatible with the mode of action of the mannose-6-phosphate receptor that translocates hydrolases from the endoplasmic reticulum to lysosomal membranes through recognition of the sugar residue (for reviews see reference 44). It is suggested that the primary function of carbohydrate for many glycoproteins is to facilitate the interaction with a variety of intracellular receptors that controls translocations between membranes, and that this function is the basis of the broadly observed evolutionary conservation of N-linked glycosylation sites, not only for MHC class I antigens, but for other membrane proteins as well.

### Summary

The major histocompatibility class I antigens, expressed in most somatic cells, have carbohydrate moieties. We constructed mutant mouse MHC class I genes in which codons for the N-linked glycosylation sites were replaced by those of

other amino acids. L cell transformants expressing the nonglycosylated class I antigens allowed us to investigate biological roles of carbohydrates with the highest specificity possible. The nonglycosylated antigen was unchanged in its overall serological specificities, and was recognized by alloreactive cytotoxic T cells. Further, the antigen was capable of mediating cytotoxic activity of vesicular stomatitis virus-specific T cells. These studies indicate that carbohydrates are not essential for immunological function of the MHC class I antigens.

Cell surface expression of the nonglycosylated antigen was markedly reduced as compared with the native antigen, which was not attributable to accelerated degradation or rapid shedding. We conclude that the primary role of carbohydrates of the class I antigens is to facilitate the intracellular transport of the nascent proteins to the plasma membrane. The possible involvement of carbohydrate-receptor interactions in this process is discussed.

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