SITE-DIRECTED MUTAGENESIS OF CLASS I HLA GENES
Role of Glycosylation in Surface Expression and Functional Recognition

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The carbohydrate moieties present on membrane-bound glycoproteins have
been implicated in a variety of cellular functions including protection from
intracellular degradation, stabilization of protein conformation and assembly of
oligomeric structures, sorting of proteins within the cell from those destined to
be exported (secreted or membrane bound), binding sites for intercellular
interactions, and determinants of immunological specificity (see references 1 and
2 for review). The contribution of glycan moieties to any of these functions can
vary widely for different glycoproteins, with no general consensus evident.

Studies investigating the functional role of carbohydrates on cell surface
proteins have been based on methods that eliminate or alter glycan moieties.
These methods include the generation of glycosylation-deficient mutant cells (3),
treatment with inhibitors of glycosylation such as tunicamycin (4), or sugar
analogs (5), and treatment with chemicals or glycosidases (5, 6). The disadvantage
of these nonspecific approaches, especially in the study of intercellular interac-
tions, is that all cell-surface glycoproteins are affected. This global deglycosylation
can result in a large increase in the net hydrophobicity and disorganization of
the membrane environment. Furthermore, the majority of these methods are
usually incomplete and have inhibitory or cytotoxic effects on the cells. Therefore
it is not surprising that conflicting reports have been published describing the
importance of glycosylation on the expression and function of various membrane
proteins (1, 2).

One family of cell surface glycoproteins expressed on all nucleated cells is the
major histocompatibility complex class I antigens (7). MHC class I molecules play
an important role in immunologic recognition and can function as target mole-
cules for allospecific cytotoxic T lymphocytes (CTL). Class I HLA molecules are
heterodimers consisting of a highly polymorphic, glycosylated transmembrane
heavy chain of ~44 kD, noncovalently associated with β2-microglobulin (β2m),1
an invariant, nonglycosylated polypeptide of 11.5 kD. Determinants recognized

1Abbreviations used in this paper: β2m, β2 microglobulin; HIFCS, heat-inactivated fetal calf serum;
IIF, indirect immunofluorescence.

J. Exp. Med. © The Rockefeller University Press 0022-1007/87/11/1329/22 $2.00 1329
Volume 166 November 1987 1329–1350
Glycosylation plays a minimal role in HLA recognition

**FIGURE 1.** Schematic diagram of N-linked glycosylation sites on human HLA and mouse H-2 class I antigens.

by T cells appear to map to the first two external globular domains of the HLA heavy chain, those that show the greatest amino acid variation (8–13). Carbohydrate moieties are also present in these segments, N-linked to asparagine residues at position 86 on all human HLA antigens, and at positions 86 and 176 on all murine H-2 antigens (see Fig. 1). An additional glycan is present at residue 256 on murine H-2K^d, D^b, and L^d antigens (11).

Glycosidases and inhibitors of glycosylation have been used to investigate the biosynthesis of class I HLA and β2m molecules (14, 15). In the presence of tunicamycin, chain association occurs at about the same rate as in its absence, and the deglycosylated class I complex reportedly reaches the surface at the same rate as normal glycosylated molecules, without a noticeable difference in proteolytic degradation. Furthermore, most serologic determinants remain intact (14, 16). However, the role of the class I oligosaccharide in the recognition of the molecule by allogeneic or virus-specific HLA-restricted CTL remains unclear. Although no data is available in humans, modification of the carbohydrate on intact stimulator or target cells in mice has been observed to inhibit (17–19), stimulate (20, 21), or have no effect (19, 22, 23) on T cell responses. Recently, Goldstein and Mescher (24) using liposomes carrying murine H-2K^k antigens
devoid of carbohydrates, suggested that the glycans on MHC class I molecules are not required to trigger the maturation of precursor to effector CTL. Here we have taken another approach in which we tailor altered HLA proteins at the DNA level by eliminating or creating the recognition signal for N-linked glycosylation, (Asn-X-Ser/Thr), using oligonucleotide-directed mutagenesis, and analyze the expression, conformation, and functional recognition of these mutant HLA molecules after gene transfer into appropriate recipient cells.

Materials and Methods

Oligonucleotide-directed Mutagenesis. Synthetic oligonucleotides were synthesized by the solid-phase phosphotriester method (25) using an automated synthesizer (Applied Biosystems, Foster City, CA), purified through 20% polyacrylamide gel electrophoresis, and 5'-phosphorylated using T4 polynucleotide kinase. The oligonucleotides used in this study are listed in Table I. Site-specific mutagenesis was performed on double-stranded DNA plasmids using the protocol outlined in Fig. 2. Supercoiled plasmid DNA purified from a cesium chloride gradient was digested separately in two reactions using one (to linearize the plasmid in the vector sequence) and two (to gap the region in the HLA sequence to be mutated) restriction enzymes, respectively. The resultant molecules were mixed in equimolar ratio, denatured by boiling, and renatured with the oligonucleotide at molar ratios of 1:20 or 1:200 pmol at 10°C below its calculated disassociation temperature [melting temperature, \( T_m = 4^\circ(G + C) + 2^\circ(A + T) \)] for 2 h. After cooling the reaction on ice, the single-stranded gap was filled by primer extension and the plasmid was covalently closed by adding 0.5 mM deoxynucleotides, Escherichia coli DNA polymerase I Klenow fragment, and T4 DNA ligase at 15°C for at least 20 h, as recommended by Zoller and Smith (26). The ligation mixture was then used to transform competent E. coli HB101. Ampicillin-resistant colonies were transferred to Biotrans nylon membranes (ICN Biomedicals, Inc., Irving, CA) and the colonies harboring the mutant plasmid were detected by colony hybridization (27) using the 5’ end-labeled oligonucleotide (\( \gamma^\text{32P} \))-ATP; 6,000 Ci/mmol; New England Nuclear, Boston, MA). Hybridization was carried out at 10°C below the calculated \( T_m \) for 12–16 h using 2 × 10^6 cpm/filter in 6× SSC (1× SSC is 15 mM NaCl, 15 mM sodium citrate, pH 7.0); 10% Denhardt’s solution, and 0.1% SDS followed by increasing temperature washes using 6× SSC and 0.05% sodium pyrophosphate. The filters were exposed at −70°C to Kodak XAR film with Cronex intensifying screens for 1–3 h.

DNA from positive colonies, isolated from 5 ml cultures (28), was digested with appropriate restriction enzymes and analyzed by Southern blot hybridization (29) with the 5’ \( ^\text{32P} \) end-labeled oligonucleotide to assure correct localization of the primer to the appropriate HLA domain. Finally, since primary colonies harbor both strands of DNA (normal and mutated), retransformation and rescreening by colony hybridization were

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**Table I**

*Carbohydrate Moiety*

| HLA gene | First protein domain | Second protein domain | Amino acid residues |
|----------|----------------------|-----------------------|--------------------|
| HLA-B7   | +                    | -                     | Asn Lys Lys        |
| B7M86   | -                    | -                     | Gln Lys Lys        |
| B7M176* | +                    | +                     | Asn Asn Thr        |
| B7M86*/176* | -          | +                     | Gln Asn Thr        |

**HLA gene** 5’GGGCTACTACAACCAGGCGGAG 3’  
**Oligo 86**  
**Asn**  
**B7** 5’GGGCTACTACAACCAGGCGGAG 3’  
**Gln**  
**Asn**  
**Thr**  
**Gln**  
**Asn**  
**Thr**
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Figure 2. Schematic diagram for oligonucleotide-directed mutagenesis in double-stranded plasmid molecules. The recombinant plasmid containing the HLA-B7 gene is digested with one (Hind III) or two (Kpn I and Sma I) restriction enzymes (for mutagenesis in the α-2 domain). The two reactions are mixed with the mismatched oligonucleotide, denatured by boiling, and renatured. Only 25% of the resulting molecules are appropriately gapped. The gap created is then filled by polymerase I (Klenow) in the presence of deoxynucleotides and the molecule ligated by T4 ligase. The ligated mixture is transformed into E. coli and drug resistant colonies are screened by colony hybridization using 32P end-labeled oligonucleotide. Appropriate colonies are further purified and characterized.

performed to obtain purified mutant plasmid DNA. The frequency of generation of the desired mutation ranged from 1 to 10%, and colonies were usually homogeneous after the second transformation. All enzymes used were purchased from New England Biolabs (Beverly, MA) and used as recommended.

Final confirmation of the desired mutation was obtained by DNA sequence analysis using a modification of the dideoxy chain-termination method (30), after subcloning the fragments corresponding to the appropriate HLA exon into M13 Mp18 or Mp19 vectors. Sequencing primers and kit were purchased from New England Biolabs. Sequence experiments were performed using α-35S-dATP (500 Ci/mmol; Amersham Corp., Arlington Heights, IL).

Cell Culture and DNA-mediated Gene Transfer. All monolayer cell lines were maintained in α-MEM (Gibco, Grand Island, NY), supplemented with 10% heat-inactivated FCS (HIFCS)(M.A. Bioproducts, Bethesda MD), penicillin/streptomycin, and 2 mM glutamine. LJ26 (mouse L cells expressing human β2m) (31), was a gift from L. Herzenberg (Stanford University, Palo Alto, CA). The human rhabdomyosarcoma cell line, RD, was HLA-typed by M. Pollack (Baylor College of Medicine, Waco, TX) as HLA-A1;Bw51,B14 (32). Transfected cell lines were maintained in the appropriate selectable medium as described (32). Cotransfection (HLA plasmid/selectable marker plasmid mass ratio of 20:1) was carried out using the recombinant plasmid containing the HLA-B7 gene (p158R1) or the mutated plasmids B7M86−, B7M176+, or B7M86−/176+, together with the plasmid containing the Tn5 neomycin resistance gene (pSV2neo) (33). Additional high-molecular-mass recipient cell DNA was used as carrier. Selection medium containing 0.5–1 mg/ml G418/geneticin sulfate (Gibco) was added 48–60 h after transfer, and fresh selection
media was replaced every 2–3 d. Entire flasks of colonies or individual colonies picked using cloning cylinders were harvested 10–14 d after selection.

**Monoclonal Antibodies.** mAb-producing hybridoma cells were grown in BALB/c mouse ascites. The specificities and reference to the original characterizations of the HLA mAb have been described (34–37). mAb recognizing monomorphic HLA-A,B,C determinants were W6/32 and 9.12. The panel of allotypic mAb used to characterize mutant HLA-B7 gene expression included BB7.1 (HLA-B7), ME1 (HLA-B7, -B27, -B22), MB40.2 (HLA-B7, -B40), MB40.3 (HLA-B7, -B40), B27M1 (HLA-B7, -B27). B locus-specific mAb included 4E and 1.23. Other mAb that bind to HLA-B7 but have a wider reactivity included BB7.6 and BB7.7. Rabbit anti-heavy chain serum, anti-H, was a gift of Dr. Hidde Ploegh, Netherlands Cancer Institute (Amsterdam, The Netherlands).

**Cell Surface Immunofluorescence and Flow Cytometry.** Cells were detached from monolayer culture by using 0.03% EDTA in PBS without calcium or magnesium, and were washed with media containing 2% filtered HIFCS and 0.02% sodium azide. Standard indirect immunofluorescence was performed as described (32) on 10^6 cells/assay with saturating amounts of antibody in 50 μl medium at 4°C for 45 min. The cells were washed twice with media and incubated at 4°C for another 45 min with fluorescein isothiocyanate–conjugated goat F(ab')2 anti-mouse IgG antibody (Cappel Laboratories, Cochranville, PA) diluted 1:20. After three washes, the cells were either fixed in 1% paraformaldehyde for analysis or analyzed directly. Evaluation of immunofluorescence and sterile cell sorting was performed on an Epics V system interfaced with a MDADS multichannel analyzer (Coulter Electronics, Hialeah, FL). Relative linear fluorescence values were calculated from peak logarithmic channel numbers as described by Muirhead et al. (38), using polystyrene microspheres of known fluorescence intensities as standards (Coulter Electronics).

**Metabolic Cell Labeling, Immunoprecipitation, and Gradient SDS Gel Electrophoresis.** Cells (2–5 × 10^6) were labelled by incubation in methionine-free α-MEM containing 10% HIFCS in the presence of 250 μCi of [35S]methionine (500 Ci/mmol, New England Nuclear) for 12–16 h at 37°C. When used, tunicamycin (3 μg/ml) was added to the media 4–6 h previously, and remained throughout the time of labeling. Labeled cells were removed from monolayer culture using 0.03% EDTA in PBS and washed twice before lysing in 1.0 ml of lysis buffer (1% NP-40, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, and 0.1 mM PMSF) on ice for 30 min. The lysates were centrifuged for 10 min in an Eppendorf centrifuge to remove nuclei, and the supernatants were used immediately or stored at −70°C. Preclearing and immunoprecipitation with specific mAb was carried out as described (15), and the immune complexes were precipitated with protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ), followed by extensive washes. The samples were boiled in elution buffer containing SDS and 2-ME and analyzed by electrophoresis on a 7–15% linear gradient SDS-polyacrylamide gel followed by fluorography and drying for exposure at −7°C to Kodak XAR-5 X-ray film.

**Generation of Human Alloreactive CTL Clones and Cytotoxicity Assays.** Isolation and culture of CTL clones used in this study have been described elsewhere (39). CTL clones were isolated from a single volunteer (HLA-A11, Aw32; B27, Bw51; Cw2; DR7). Isolated mononuclear cells at 2 × 10^6 cells/ml were stimulated in culture with 10^6 cells/ml irradiated JY lymphoblastoid cells (HLA-A1, B7; DR4,6), or murine L cell transfectants expressing HLA-B7 antigens, in 2 ml wells (Linbro, McLean, VA) at 37°C. Bulk cultures were maintained by stimulation with irradiated JY cells every 1–2 wk, and stringently cloned after 6 wk in culture by limiting dilution in 96-well round-bottom microtiter plates (Linbro) using irradiated JY cells as a feeder layer. RPMI 1640 medium (Gibco) was supplemented with 10% HIFCS, 5 × 10⁻⁵ M 2-ME, 10 mM Hepes, penicillin/streptomycin, and 10% IL-2 conditioned supernatants from PHA and MLC-activated PBL. Clone specificity was determined by a panel of HLA-typed human lymphoid lines and mAb blocking.

51Cr-release assays were performed as described (40). Target cells were labeled overnight as monolayers using 0.1 mCi 51Cr (Na₂¹⁶CrO₄; New England Nuclear) in 1.8 ml complete media at 37°C. Labelled cells were detached with EDTA and were washed
three times. CTL-mediated lysis assays were performed in triplicate in V-bottom microtiter wells (Linbro). Effectors were added in threefold dilutions to $10^3$ target cells/well. Microtiter plates were centrifuged and incubated at 37°C for 4 h before being recentrifuged, and the supernatants were assayed for $^{51}$Cr release. Specific cytotoxicity was calculated as percent cytotoxicity = $100 \times \frac{(\text{experimental release}) - (\text{spontaneous release})}{(\text{total release}) - (\text{spontaneous release})}$. When blocking with mAb were used, they were added at the start of the assay. Standard deviation of the triplicate wells rarely exceeded 2–4% of the specific lysis.

Results

Creation of Glycosylation Mutants in the HLA-B7 Molecule by Oligonucleotide-directed Mutagenesis. Two oligonucleotide sequences were synthesized (see Table I) and used in a modification of standard oligonucleotide-directed mutagenesis techniques (see Materials and Methods and Fig. 2) to create the novel HLA-B7 mutant molecules described in Table I. A 21-mer oligonucleotide containing two mismatches compared with the HLA-B7 genomic sequence was designed to create the specific amino acid substitution of glutamine for asparagine at position 86. This conservative amino acid substitution destroys the recognition sequence for N-linked glycosylation in the first external domain. A second 20-mer oligonucleotide also containing two mismatches compared with the HLA-B7 genomic sequence was designed to create the specific amino acid substitutions of asparagine for lysine at position 178, thus replacing two highly charged amino acids and creating a recognition sequence for N-linked glycosylation at amino acid position 176 in the second external domain.

After mutagenesis, ampicillin-resistant colonies harboring the mutated plasmid were detected by colony hybridization using the appropriate 5' $^{32}$P-labeled oligonucleotide under differential washing conditions (Fig. 3). Plasmid DNA isolated from positive colonies was digested with several diagnostic restriction enzymes. The site mutated by the oligonucleotide was confirmed by Southern blot transfer and hybridization of the filters using the appropriate end-labelled oligonucleotide followed by differential temperature washes (data not shown). The vast majority of mutants analyzed hybridized to the restriction fragment encompassing the appropriate annealing site. DNA fragments, corresponding to the exons open to the mutagenesis procedure, were isolated from purified mutant plasmid DNA and subcloned into M13 for DNA sequence analysis. Complete sequencing of these regions confirmed that the appropriate base changes were introduced in the sites 86 and 176, corresponding to the sequence of the oligonucleotide used (Fig. 4). No further base changes were present in the remaining exon sequences.

A further HLA-B7 mutant was constructed lacking the glycosylation site at 86 but containing one at 176 by subsequently mutating the HLA-B7M86$^-$ mutant with the oligonucleotide 176. Colony and Southern blot hybridizations, and DNA sequence analysis (Fig. 4) confirmed both mutations. An equivalent mutant B7 molecule was also constructed by exchanging the mutated $\alpha$-2 domain of HLA-B7M176$^+$ with the normal $\alpha$-2 domain of HLA-B7M86$^-$ using Sma I–Kpn I restriction sites (data not shown).

Surface Expression and mAb Epitope Mapping of Glycosylation Mutants B7M86$^-$ and B7M176$^+$ in Mouse and Human Cells. Intracellular processing and surface
FIGURE 3. Differential colony hybridization screening. DNA from colonies transferred and grown on nylon membranes was hybridized with the appropriate $^{32}$P end-labeled mutagenic oligonucleotide. Colonies containing mutant plasmid DNA were detected by increasing temperature washes, followed by autoradiography. Examples of both B7M86$^-$ and B7M176$^+$ are shown. RT refers to room temperature wash, and p328, pB7, and pA2 refer to control colonies containing the vector only, HLA-B7 and HLA-A2 genes, respectively.

expression of the native and mutant HLA-B7 molecules were studied using DNA-mediated gene transfer. The HLA genes were cotransferred with pSV2neo into the human rhabdomyosarcoma cell line (RD), as well as mouse L cells (LTK), and mouse L cells expressing human $\beta_2$m (LJ26). Stable G418-resistant colonies taken as a mass population were expanded and analyzed for HLA-B7 surface expression by indirect immunofluorescence (IIF) and flow cytometry using a panel of HLA-B7-reactive monomorphic and polymorphic mAb (Table II). Fig. 5 shows the binding of mAb ME1 to the surface of normal HLA-B7, B7M86$^-$, and B7M176$^+$ transfected cells in G418-resistant mass populations derived from the three recipient lines.

Native HLA-B7 molecules were easily detected on the surface of each human and murine cell line. In contrast, the substitution of glutamine for asparagine at residue 86 had a dramatic effect on the surface expression of the mutated HLA-B7 molecule. No surface expression of B7M86$^-$ was detected on mouse L cells by any of the mAb used. When the B7M86$^-$ mutant gene was introduced into human RD or mouse L cells expressing human $\beta_2$m, a markedly decreased level of expression, 50-fold less than that of the normal HLA-B7 gene product, could be detected by all mAb. Therefore, the reduction or absence of expression was
FIGURE 4. DNA sequence analysis of the glycosylation mutants. Dideoxy chain termination sequencing was performed after subcloning the DNA fragment corresponding to the appropriate HLA exon into M13 MP18 or MP19 vectors. Autoradiography of 8% polyacrylamide gels comparing the sequence of the mutants and wild type DNA around the region of the mutation is shown. Arrows indicate the corresponding base differences between the mutants and original HLA-B7 molecule. No other base changes were present throughout the entire exon.

not due to the alteration of any one epitope. A similar low level of surface B7M86^- antigens could be induced on mouse L cells after treatment with mouse IFN-γ (data not shown). Three independent clones of mutant B7M86^- DNA were purified, and each displayed identical results after gene transfer (data not shown). This, together with the complete sequencing of the region open to mutagenesis, eliminates the possibility that an additional alteration was inadvertently introduced elsewhere in the HLA gene during the mutagenesis procedure.

After metabolic labelling with [35S]methionine and subsequent immunoprecipitation, the normal HLA-B7 44 kD band was easily detected in HLA-B7 transfecteds with mAb ME1 I (Fig. 6). The presence of the appropriate 40 kD nonglycosylated heavy chain in the cytoplasm of B7M86^- transfectants was detected but at a lower intensity. Further studies have shown that larger quantities of B7M86^- can be detected in transfectants using a rabbit anti-heavy chain serum, anti-H, which detects denatured heavy chains not in association with β2m.
Table II

mAb Screening

| Recipient cell | DNA | BB7,1 B7, BW42 | ME1 B7, B27, BW22 | MB40.2 B7, B40 (BW60, BW41) | MB40.3 B7, B40 (BW60, BW61) | B27M1 B27, B7 | 4E, B, C locus | 1.25 B, C locus | BB7.6 BW6-related | BB7.7 Mono-morphic* | W632 Mono-morphic | 9.12 Mono-morphic | BBM1 Human βm |
|----------------|-----|----------------|-------------------|-----------------------------|-----------------------------|----------------|----------------|----------------|------------------|------------------|------------------|------------------|------------------|
| LTK            | HLA-B7 | +              | ++                | ++                          | ++                          | ++             | −              | ++             | +                | +                | −                | +                | −                |
|                | B7M86* | ±              | ±                 | ±                           | ±                           | ±              | ±              | ±              | ±                | ±                | ±                | ±                | ±                |
|                | B7M176* | +              | +                 | −                           | −                           | −              | −              | −              | −                | +                | +                | −                | −                |
| LJ26           | HLA-B7 | +++            | +++               | +++                         | +++                         | +++            | +              | +++            | +                | +                | +++              | +++              | +++              |
|                | B7M86* | +              | +                 | +                           | +                           | +              | +              | +              | +                | +                | +                | +                | +                |
|                | B7M176* | +++            | +++               | +++                         | +++                         | +++            | +              | +++            | +                | +                | +++              | +++              | +++              |
| RD             | HLA-B7 | +++            | +++               | +++                         | +++                         | +++            | +              | +++            | +                | +                | +++              | +++              | +++              |
|                | B7M86* | +              | +                 | +                           | +                           | +              | +              | +++            | +                | +                | +++              | +++              | +++              |
|                | B7M176* | +++            | +++               | +++                         | +++                         | +++            | −              | +++            | −                | +                | +++              | +++              | +++              |

Relative peak channel difference between specific and control staining from a three-log, full-scale, 256-channel analyzer: −, no difference; +, 1–10 channels; ++, 10–50 channels; ++++, 50–100 channels; ++++, >100 channels.

* Combinatorial determinant with human βm only.

† After IFN-γ treatment.

‡ Reacts also with mouse H-2 antigens bound to human βm.
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Relative Fluorescence

Figure 5. Surface expression of HLA-B7, B7M86−, and B7M176+ on mouse LTK, mouse LJ26, and human RD cells. Each histogram represents flow-cytometric analysis of a mixed population of >100 G418-resistant colonies stained by IIF using the B7-reactive mAb ME1 or normal mouse serum control (C). Fluorescent signals converted to logarithmic values are displayed on a 256 multichannel analyzer.

(49 and Barbosa et al., unpublished results). Tunicamycin treatment produced the appropriate shift in the size of the normal HLA-B7 molecule to 40 kD while the mutant B7M86− remained unaltered.

In contrast to mutant B7M86−, mouse and human recipient cells transfected with B7M176+ displayed equivalent levels of surface expression as normal HLA-B7 (Fig. 5 and Table II). This was true for 10 of 12 mAb analyzed. The binding of two mAb (MB40.2 and MB40.3), each specific for epitopes common to the HLA-B7 and -B40 molecules, was completely eliminated by the creation of the glycosylation site at site 176 (Fig. 7). Identical results were seen in transfectants of all three mouse and human recipient lines analyzed. These two mAb are thought to recognize spatially separate epitopes on the HLA-B7/1140 molecules, as suggested by competitive binding experiments (41–43) and differential reaction with the cell line Sweig, which expresses a variant HLA-B40 antigen.

LJ26 and RD transfectants were treated for 48 h with 100 U/ml of mouse or human IFN-γ, respectively. Normal HLA-B7, mutant B7M86−, and mutant B7M176+ transfectants all showed a similar 8–10-fold induction of surface expression. Lack of binding of MB40.2 or MB40.3 to B7M176+ transfectants persisted.

Metabolic labelling with [35S]methionine and subsequent immunoprecipitation with both rabbit anti-H sera (data not shown) and mAbs W6/32 or ME1 demonstrated equivalent quantities of the appropriate 48 kD dual glycan-containing heavy chain in the cytoplasm of B7M176+ transfectants as that seen for the normal 44 kD single glycan-containing chain in HLA-B7 transfectants (Fig. 6). Tunicamycin treatment produced the appropriate shift in B7M176+
FIGURE 6. [35S]Methionine labeling and immunoprecipitation analysis confirms the presence of appropriate molecule mass changes in mutant HLA-B7 molecules. Normal mouse serum control (C) and mAb ME1 (M) precipitates of tunicamycin treated (+) and nontreated (−) cells were analyzed by a 7.5–15% gradient SDS-polyacrylamide gel. Normal HLA-B7 was precipitated from both the human lymphoblastoid cell JY and HLA-B7-transfected RD cells, while B7M86− and B7M176+ antigens were precipitated from appropriately transfected RD populations.

molecules to the 49 kD band (Fig. 6; incomplete inhibition reveals the 44 kD single glycan-containing molecule also).

Tunicamycin Treatment Does Not Rescue the Expression of B7M86-Antigens to the Cell Surface nor Allow MB40.2 or MB40.3 mAb to Bind B7M176+ Mutant Anti-
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A

Relative Fluorescence

B

Cell Number

C

Figure 8. Tunicamycin treatment did not rescue expression of B7M86\(^{-}\) antigens to the surface or unmask MB40.2 or MB40.3 binding to B7M176\(^{+}\) mutant antigens. LJ26 clonal transfectants expressing B7, B7M86\(^{-}\), and B7M176\(^{+}\) respectively were treated overnight with 3 \(\mu\)g/ml tunicamycin and harvested alone with nontreated duplicates for IIF and flow cytometry analysis using mAb ME1, MB40.2, and MB40.3 (identical results as MB40.2). Replicate flasks harvested for immunoprecipitation indicated that tunicamycin treatment was \(\sim 90\%\) complete under the conditions used. Identical results were obtained with the LTK (e.g., B7M86\(^{-}\) transfectants remained completely negative) and RD transfectant cell lines.

gens. The reduced surface expression observed for mutant B7M86\(^{-}\) unglycosylated antigens contradicts previous data obtained using the glycosylation inhibitor, tunicamycin (14). Therefore, tunicamycin experiments were initiated to address whether tunicamycin could have a second unknown effect allowing transport of deglycosylated HLA antigens to the cell surface, or whether the nonglycosylated B7M86\(^{-}\) heavy chains might be unable to compete with endogenous glycosylated HLA or H-2 chains for association with either mouse of human \(\beta_{2m}\). In addition, we wished to determine whether the elimination of binding of mAbs MB40.2 and MB40.3 to B7M176\(^{+}\) mutant antigens was due to the presence of the carbohydrate glycan or due to the alteration of two positively charged amino acids at the 176 and 178 positions.

Clonal populations of LJ26 transfectants expressing B7, B7M86\(^{-}\), and B7M176\(^{+}\) antigens, respectively, were treated overnight with 3 \(\mu\)g/ml tunicamycin and were harvested along with nontreated duplicates for IIF and flow cytometry and analysis using mAbs ME1, MB40.2, and MB40.3. Duplicate flasks of tunicamycin-treated and -untreated cells were also labelled overnight with \([^{35}\text{S}]\)methionine and harvested for immunoprecipitation with mAb ME1 and anti-H serum to monitor the extent of deglycosylation by tunicamycin. Tunicamycin treatment was \(\sim 90\%\) complete under the conditions used (data not shown). Results shown in Fig. 8A confirm that deglycosylated normal HLA-B7 antigens are fully capable of reaching the cell surface. Small decreases in expression levels
are likely due to a general inhibitory effect of tunicamycin on protein synthesis at the concentrations needed for nearly complete inhibition. Tunicamycin treatment, however, was not able to rescue expression of B7M86- antigens to the cell surface (Fig. 8B). In addition, mAbs MB40.2 and MB40.3 did not bind to B7M176+ mutant antigens after tunicamycin treatment that would have eliminated the glycan moieties on the majority of B7M176+ antigens (Fig. 8C). Identical results were obtained with LTK and RD transfectant cell lines (data not shown). Thus, the reduction or lack of surface expression of mutant B7M86- antigens is not due to either cell type or to competition with endogenous glycosylated HLA or H-2 antigens, and is more likely due to conformational alterations created by the amino acid change at position 86. Second, the loss of binding to B7M176+ mutant antigens by mAb MB40.2 and MB40.3 is not due to the presence of the carbohydrate specifically, but most likely due to conformational alterations either at that site or at another site affected by the replacement of two lysines at positions 176 and 178.

**Creation of a Glycan Moiety in the Second Domain Partially Rescues Surface Expression of B7M86- Unglycosylated Mutant Molecules.** The B7M86- mutant molecule was further mutated by the oligonucleotide 176 to address whether the presence of a glycan moiety at another location could provide a function necessary to rescue surface expression. As seen in Fig. 9, mutant B7M86-176+ showed two- to three-fold increased levels of surface expression over those seen by mutant B7M86-. These levels were still quite low as compared to normal HLA-B7. Similar results were observed in LTK and RD transfectants, as well as for a similar mutant created by an exon swap between mutants B7M86- and B7M176+ (data not shown). mAb MB40.2 and MB40.3 also did not bind B7M86-176+ mutant antigens. All other mAb analyzed bound to B7M86-176+ mutant antigens with levels similar to W632. We suggest that the small two- to three-fold increase in expression of B7M86-176+ as compared with B7M86-
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FIGURE 10. Specific lysis of human RD cells expressing transfected HLA-B7 glycosylation mutants by allo-specific CTL. Cloned human RD transfectants expressing HLA-B7 (■), B7M176+ (○), B7M86- (□), or mock-transfected (♦) were used as targets in standard 4-h 51Cr-release assay with human allo-specific CTL clones generated as described in Materials and Methods. RDB7 and RDB7M176+ expressed nearly identical surface antigen levels while RDB7M86- expressed ~50-fold lower levels. A and C are representative HLA-B7 allo-specific clones, while D is a representative HLA-A2 allo-specific clone. B represents a bulk CTL population stimulated with the human lymphoblastoid cell line JY and shows both HLA-A2 and -B7 specificity.

may be due to small structural changes that counteract those created by mutant B7M86- and not specifically due to the presence or absence of a glycan moiety.

Allo-specific CTL Recognition of B7M86- and B7M176+ Glycosylation Mutants. Human CTL clones allo-specific for HLA-B7 antigens have been generated to analyze the recognition and lysis of mouse and human target cells expressing transfected HLA-B7 gene products (32, 39, 40). Only a few of the clones isolated were capable of lysing mouse cell as well as human cell transfectants, while the vast majority were capable of only lysing human or monkey cell transfectants expressing HLA-B7 antigens.

To assess whether the recognition of HLA-B7 was affected by the absence of glycosylation or the presence of an additional glycan in the second protein domain, cloned human RD transfectants expressing B7, B7M176+, and B7M86- surface antigens were used as targets for various HLA-B7 specific CTL. Representative results are shown in Fig. 10. RD7B7 and RDB7M176+ expressed nearly identical surface antigen levels, while RDB7M86- expressed ~50-fold lower levels of antigen. CTL clones 303 and PT8 lysed RDB7 and RDB7M176+ cells nearly equivalently. RDB7M86- target cells were lysed also, although less effectively. Similar results were obtained repeatedly and are representative of all CTL analyzed. Moreover, a bulk population of CTL stimulated with the human
FIGURE 11. Specific lysis of mouse LTK cells expressing transfected HLA-B7 glycosylation mutants. Cloned mouse LTK transfectants expressing high (□) and low (■) levels of HLA-B7, and high (●) and low (○) levels of B7M176+ were used as targets in standard 4-h $^{51}$Cr-release assay using representative B7 allospecific CTL clones previously shown capable of lysing murine transfectants expressing HLA-B7 (CTL clone 147) or CTL clones incapable of lysing mouse cell transfectants expressing HLA-B7 (CTL clone 304). High B7 and B7M176+ expressors were equivalent and expressed approximately three times higher levels than equivalent B7 and B7M176+ low-level expressors. Mock transfectant (×) was not lysed by either clone.

Lympoblastoid cell line JY lysed all three transfectants equivalently. An HLA-A2-specific CTL clone was unable to recognize and lyse any of the transfectants. Because the level of expression of B7M86+ antigens is 50-fold lower than HLA-B7 levels, 10-fold higher levels of B7M86+ antigens were induced by human IFN-γ. These cells could be lysed with similar efficiencies as those seen with untreated RDB7 transfectants (data not shown). Thus, neither mutant dramatically affected recognition by the CTL clones analyzed, and analysis of B7M86+ transfectants served to demonstrate the low threshold of surface HLA required for recognition and lysis on human cell transfectants.

CTL lysis of LTK and LJ26 transfectants expressing equivalent surface levels of HLA-B7 or B7M176+ antigens is shown in Figs. 11 and 12, respectively. CTL clone 147 is a high-affinity CTL clone previously shown capable of lysing murine transfectants expressing HLA-B7 (39). Clones 303 and 304 represent the major-
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ity of CTL that are unable to lyse mouse cell transfectants expressing HLA-B7. Similar results were obtained for CTL clone 147 as was observed on human RD transfectants. LTK or L26 transfectants expressing B7M176+ antigens were lysed as well as HLA-B7 transfectants. LJB7M86− (Fig. 12) expressed ~50-fold lower levels of B7M86− antigens as determined by IIF and flow cytometry analysis. LJB7M86− was lysed, but with a lower efficiency. LTKB7M86− showed no detectable surface expression of B7M86− (but low levels were inducible by IFN), and was not lysed above background. CTL clones like 303 and 304 remained unable to lyse mouse LTK or L26 transfectants expressing any of the HLA-B7 antigens. Thus, the presence of an additional glycan at amino acid position 176, as seen in all murine H-2 antigens, did not rescue murine transfectants from the species restriction previously observed for the majority of human allospecific CTL clones isolated.

Discussion

To study the structure/function relationship of MHC class I antigens, standard oligonucleotide-directed mutagenesis procedures (26, 44) have been modified to develop a relatively simple and efficient protocol, making precise changes directly in double-stranded plasmids in which only a small single-stranded region of the DNA is accessible to the mutagenic oligonucleotide (Fig. 2). This protocol should reduce random errors introduced by polymerase I activity and the amount of DNA sequencing necessary to characterize the mutant. Combined with recent improvements for DNA sequencing in double-stranded vectors (45), M13 steps may be eliminated entirely.

Here, HLA-B7 proteins have been altered at the DNA level by either eliminating or creating a recognition signal for N-linked glycosylation, (Asn-X-Ser/Thr), to address the role of the glycan moiety in protein conformation, surface expression, and recognition of the molecule by mAb and allospecific CTL. Using two oligonucleotides, a set of four HLA-B7 molecules were analyzed that had N-linked glycans present or absent at those sites that are highly conserved in HLA and H-2 class I antigens (Fig. 1). Gene transfer of the mutant HLA-B7 molecules into human and mouse cell lines demonstrated that the presence or absence of a carbohydrate moiety at these amino acid positions had little or no effect on the recognition of the HLA-B7 molecule by available mAb or allospecific CTL.

This work is in strong agreement with recent studies by Shiroshiet al. (46), Miyazaki et al. (47), and Goldstein and Mescher (24) using similar or different approaches for murine H-2 antigens. Together, these studies confirm the lack of effect of deglycosylation on serologic recognition after tunicamycin treatment (14), and may help clarify studies using inhibitors of glycosidases to address carbohydrate’s role in T cell recognition and function (17–23). The preservation of antigenicity after the removal of glycan moieties in both HLA and H-2 antigens raises the question as to the biological significance of these highly conserved structures.

Although an in vitro mutagenesis approach eliminates the technical problems inherent in using glycosylation inhibitors or glycosidases (global effect on all glycoproteins, toxicity, incompleteness, etc.), and allows the role of particular
glycosylation sites to be individually addressed, the substitution of different amino acids in the primary structure of antigens may inadvertently alter the secondary structure of the molecule. This may be evident in the H-2Ld mutant lacking a glycan at amino acid 86 (46). Four mAb showed a quantitative reduction in binding, probably not due to the removal of the carbohydrate moiety itself, but rather to the substitution of a positively charged lysine for asparagine at position 86. In the HLA-B7 molecule, a conservative change of glutamine for asparagine at the same residue 86 site had a drastic effect on surface expression, while a drastic change replacing two highly charged lysine residues at positions 176 and 178 had no effect on the level of surface expression. It is possible that the asparagine residue at position 86 may be critical for protein conformation and chain association. Recent crystallographic data on HLA-A2 (48) suggest that α-1 and α-2 domains have a stronger interaction with β2m than previously predicted (49).

Quantitative and qualitative differences in β2m expression did influence whether small amounts of mutant B7M86− protein could reach the cell surface. Further, the majority of intracellular B7M86− heavy chains were found in a denatured unassociated state (data not shown). Because β2m association is known to be critical for intracellular migration and surface expression of class I molecules, it is likely that the low surface expression seen for this mutant is due to problematic association with β2m. This could be caused by structural alteration due to either the lack of carbohydrate or the substituted amino acid. However, further mutational work altering the serine to glycine at position 88 to create a nonglycosylated molecule in HLA-A2 has suggested that alteration of the conserved asparagine at residue 86, rather than the lack of a carbohydrate moiety at this position, is important (50). This interpretation is supported by the lack of effect of tunicamycin on the expression of class I molecules (14). Despite the alteration in transport, the B7M86− molecules that reach the cell surface at low levels in human and mouse cells transfected with human β2m or after IFN-γ induction are recognized appropriately by mAb and allospecific CTL.

After creating a nonglycosylated H-2Ld molecule, Miyazaki et al. (47) observed a similar drastic reduction in surface expression while leaving intact all functional recognition by mAb, allospecific CTL, and vesicular stomatitis virus–specific, H-2Ld-restricted CTL. They showed that this low expression was not due to increased degradation or accelerated shedding. Impaired intracellular transport was suggested. However, rather than an effect on protein conformation and β2m association, they suggested that the carbohydrate moiety itself may aid specific transport mechanisms in the cell. We have not directly shown that the nonglycosylated HLA-B7M86− heavy chains are not degraded more rapidly than normal HLA-B7 molecules. However, intracellular labelling after permeabilization has demonstrated that denatured HLA-B7M86− mutant heavy chains accumulate and are sequestered in an unknown compartment in the cell other than the Golgi (50, 51).

Recent reports by Guan et al. (52) and Machamer et al. (53) using a similar mutagenesis approach support the concept of a required function of glycosylation for transport of certain molecules to the cell surface (either membrane-bound or secreted). Their results suggest that a single N-linked oligosaccharide at any one of a number of positions is sufficient for transport of vesicular stomatitis virus G
protein or a recombinant growth hormone molecule to the cell surface. We were able to rescue some surface expression of the HLA-B7M86+ mutant by creating a single N-linked glycan at residue 176 (see HLA-B7M86+/176'). Data presented elsewhere (50, 51) suggest that, for these HLA molecules, this increase in expression may be due to improved β2m association caused by a conformational change rather than due to a transport mechanism dependent on the presence of a carbohydrate moiety.

Surprisingly, the alteration of two highly charged lysines at positions 176 and 178 to create a glycosylation site did not affect the transport or expression of HLA-B7M176+ mutant molecules. Alteration of site 176 in H-2Ld, conservatively inserting a glutamine for asparagine, also did not affect surface expression (47). The presence of the carbohydrate moiety itself at position 176 on HLA-B7M176+ did not affect the binding of any mAb analyzed, nor did it affect the recognition and lysis by allospecific CTL. Furthermore, the presence of a glycan at residue 176 did not promote the lysis of murine cell transfectants by those allospecific CTL clones previously shown capable of lysing only human cells transfected with HLA-B7 antigens (32, 39, 40). Further characterization of these CTL clones suggests that their lower affinity requires greater involvement of accessory cell interaction molecules, for which murine cells may lack appropriate ligands (39). Where successful lysis did exist, as in human cells, neither the lack of glycan nor the presence of a second glycan significantly affected the blocking of lysis typically observed by antibodies to these molecules (S. J. Mentzer, unpublished results).

The loss of binding of mAb MB40.2 and MB40.3 to the B7M176+ molecules was a direct consequence of the amino acid changes at this site either because the lysine residues at positions 176 and 178 represent the epitope recognized by these mAb, or because another site is conformationally altered by these changes. Interestingly, substantial evidence suggests that mAb MB40.2 and MB40.3 recognize different epitopes on the HLA-B7 molecule (41–43). Of the known sequenced HLA proteins to date, only HLA-B7 and HLA-B40, the two antigens recognized by both of these mAb, are identical in the region of amino acids 175–180. Further oligonucleotide-directed mutagenesis in this region may be informative.

Summary

We have investigated the role of the carbohydrate moiety on the HLA-B7 molecule in mAb and CTL recognition using oligonucleotide-directed mutagenesis and gene transfer techniques. A conservative substitution of asparagine to glutamine at amino acid 86 in HLA-B7 was created to abolish the unique glycosylation site present on all HLA molecules. A second mutant B7 molecule was made by substituting asparagine–aspartic acid–threonine for the resident lysine–aspartic acid/lysine tripeptide at amino acids 176–178, thus creating an N-linked glycan at amino acid 176, which is additionally present on all known murine H-2 class I antigens. Upon gene transfer into mouse and human cell recipients, the HLA-B7M176+ mutant and normal HLA-B7 expressed identical levels of surface protein. However, the binding of two mAbs (MB40.2 and MB40.3) thought to recognize different epitopes of the HLA-B7 molecule was completely eliminated. In contrast, the HLA-B7M86+ mutant displayed no
surface expression (mouse L cells) or minimal surface expression (human RD cells or mouse L cells coexpressing human β2 microglobulin [β2m]) after indirect immunofluorescence (IIF) and flow cytometric analysis with a panel of 12 HLA-B7 mAb reactive with monomorphic and polymorphic determinants. Immunoprecipitation analysis demonstrated that intracellular denatured mutant protein was present. Tunicamycin treatment did not rescue the expression of HLA-B7M86− antigens to the cell surface; while interferon did induce higher levels of surface expression. Tunicamycin treatment also did not allow binding of the mAbs MB40.2 or MB40.3 to HLA-B7M176+ mutant antigens, suggesting that the carbohydrate moiety itself was not directly involved in the recognition or conformation of these mAb epitopes. Further mutation of the B7M86+ molecule to create a glycan moiety at amino acid position 176 (B7M86+/176+) did not rescue normal levels of surface expression. Finally, neither mutation was seen to affect recognition by a panel of 12 allospecific CTL clones. The low expression of HLA-B7M86− on the surface of human cell transfectants was sufficient to achieve lysis, albeit at a reduced efficiency, and lysis could be increased by interferon induction of higher levels of expression. Thus, the carbohydrate moiety on HLA antigens plays a minimal or nonexistent role in recognition by available mAb and allospecific CTL clones.

We acknowledge the excellent technical assistance of Ms. M. A. V. Grimmer and Ms. U. Fuhrmann. We thank Drs. M. Brenner, M. E. Kamarck, and M. Krangel for helpful criticism of the manuscript.

Received for publication 22 June 1987.

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