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Amino Acid Sequences in the α1 Domain and Not Glycosylation Are Important in HLA-A2/β2-Microglobulin Association and Cell Surface Expression

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The role of the single carbohydrate moiety present on the HLA-A2 molecule was studied by introducing several amino acid substitutions (by site-directed mutagenesis of the HLA-A2 gene) in the consensus glycosylation sequence Asn-X-Ser. Two different amino acid substitutions of the asparagine residue at position 86 (glutamine and aspartic acid) resulted in the synthesis of ca. 39,000-molecular-weight nonglycosylated heavy chains that were detected in the cytoplasm but not on the surface of mouse L-cell transfectants. However, a low level of surface expression was detected following transfection of human (rhabdomyosarcoma) cells or mouse L cells containing human β2-microglobulin. The defect in surface expression was not due to the absence of the glycan moiety, since the substitution of a glycine for a serine at amino acid 88 did not have the same drastic effect in the presence of human β2-microglobulin. These and other data suggest that the asparagine residue may play a critical role in the conformation of the HLA heavy chain and its interaction with β2-microglobulin. Immunofluorescence microscopy following permeabilization of the transfectants demonstrated that the unglycosylated HLA heavy chains are sequestered in an unidentified cellular compartment that is different from the Golgi structure.

The human major histocompatibility complex class I antigens HLA-A, HLA-B, and HLA-C (42) are cell surface proteins composed of two polypeptide chains, a ca. 43,000-dalton polymorphic heavy chain (α) that is N-glycosylated at position 86, and a nonpolymorphic, nonglycosylated ca. 12,000-dalton polypeptide (β2-microglobulin [β2m]) that is noncovalently associated with the heavy chain. The genes coding for these molecules have been mapped to chromosomes 6 (13) and 15 (16), respectively.

Studies on the biosynthesis and intracellular transport of class I antigens indicate that the α chains are synthesized on membrane-bound ribosomes in the rough endoplasmic reticulum, where they receive the core *N*-acetylglucosamine and mannose sugars and associate with β2m shortly (5 to 10 min) after their synthesis (28). HLA heavy chains appear to be conformationally competent to associate with β2m during this short period in vivo (28, 41). However, in vitro-isolated denatured heavy chains will not reassociate with β2m to form the mature molecule (29). Heavy-chain–β2m complexes are transported to the Golgi complex, where the carbohydrate chain is modified to the terminally sialylated complex form (27) before traveling to the cell surface.

Association with the invariant β2m has been shown to be important in the conformation, stability (29), intracellular transport (47), terminal glycosylation (46), and surface expression (36) of the HLA molecule after its synthesis in the rough endoplasmic reticulum. This is best exemplified in the Burkitt lymphoma line Daudi, in which the lack of surface expression of HLA antigens is due to a failure in the synthesis of the β2m subunit as a result of a point mutation in the initiation codon of its mRNA (44). Somatic cell hybrids demonstrate that the Daudi heavy chains can be rescued by human or mouse β2m supplied by the fusion partner (22). Thus, mouse β2m may act as an effective substitute for its human counterpart in cell hybrids (23) as well as in DNA-mediated gene transfer transfectants (2), and human or mouse β2m can be exchanged in tissue culture by bovine β2m from the medium normally used to grow the cells (5, 25).

Although the biosynthesis of major histocompatibility complex molecules has been studied in great detail over the past few years, the exact events and the requirements for the association of both subunits (40) in the surface expression and stability of the complex are still of interest, since very little is known concerning the molecular mechanisms of their intracellular transport (26). The importance of the unique asparagine-N-linked glycosylation site in HLA molecules has been previously investigated by an enzymatic approach with glycosidases or by treatment in vivo with tunicamycin (an inhibitor of N-linked glycosylation). Although the results of these experiments suggested that the carbohydrate moiety does not play a central role in the membrane insertion, assembly, rate of expression (36, 43), or antigenic properties (37) of the HLA-A2 or HLA-B7 molecule, its biological role remains unclear. Since the use of these procedures has several disadvantages including potential secondary effects (cytotoxicity, inhibition of protein synthesis, general effect on all cellular proteins, etc.), we decided to use site-directed mutagenesis by using mismatched oligonucleotides to make specific changes in the signal on the HLA-A2 molecule recognized by glycosyl transferases and create a new molecule devoid of carbohydrate. This approach permits the study of the nonglycosylated HLA-A2 molecule without affecting the general protein synthesis of the cell.

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MATERIALS AND METHODS

Site-directed mutagenesis with mismatched oligonucleotides. Synthetic oligonucleotides were synthesized on an automated synthesizer, purified by high-performance liquid chromatography and electrophoresis on a 20% polyacrylamide gel containing 7 M urea, and sequenced by a modification of the Maxam-Gilbert procedure (31) before use. Site-directed mutagenesis was performed on double-stranded DNA plasmids by the following protocol (see Fig. 1A). The supercoiled plasmid DNA purified from a cesium chloride gradient was digested in two different reactions with one (to linearize) or two (to open the region to be mutated) restriction enzymes. The resulting molecules were mixed on equimolar basis, denatured by boiling (to create a single-strand template to which the oligonucleotide would hybridize), and renatured with the oligonucleotide at different ratios (1:20 and 1:200) at 10°C below its calculated dissociation temperature [melting temperature, T_m = 4(G+C) + 2(A+T) (52)] for 2 h. After the reaction mixture was cooled on ice, the gap was filled and the plasmid was covalently closed by addition of deoxynucleotides (0.5 mM), *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 (57). Different dilutions of the ligation mixture were then used to transform *E. coli* previously made competent (HB101 or JM83) (19). Ampicillin-resistant colonies were transferred to nylon membranes (Biotrans; ICN Pharmaceuticals, Inc.), and those harboring the mutation were detected by colony hybridization (16) with the 5'-end-labeled ([γ-^{32}P] ATP, ca. 6,000 Ci/mmol; Amersham Corp.) oligonucleotide at 10°C below the calculated T_m for 14 to 16 h by using 2 × 10^6 cpm/filter. Hybridization was carried out in 6× NET (1× NET is 0.15 M NaCl, 15 mM Tris hydrochloride [pH 8.3], 1 mM EDTA)–10% Denhardt solution–0.1% sodium dodecyl sulfate followed by washes at increasing temperatures with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) and 0.05% sodium PP_i. The filters were exposed for 1 to 4 h at −70°C to XAR-5 film (Eastman Kodak Co.) with intensifying screens. The DNA from positive colonies was isolated from 10-ml cultures (20), and the correct localization of the primer to the α1 domain was identified by Southern blot transfer (49) with appropriate restriction enzyme digests. Since primary transformants harbor both strands of DNA (normal and mutated), a second bacterial transformation (with the same DNA obtained from the minipreparations) was required to further purify the plasmid DNA. The frequency of generation of the desired mutation ranged from 2 to 5%. All the enzymes used were purchased from New England BioLabs and used as recommended.

DNA sequence analysis. Sequence determination was carried out by the dideoxy chain termination method (6, 45) after subcloning (9) the corresponding fragments into the appropriate M13 vectors (mp18 or mp19) (34). Sequence experiments were performed with [α-^{35}S]dATP (specific activity, 500 Ci/mmol; Amersham).

Cell culture and DNA-mediated gene transfer. The mouse fibroblasts LMtk− (H-2K^k), LJ26 (LMtk+ cells transformed with human DNA and expressing human β2m) (24), Ltk/c13, and the human rhabdomyosarcoma cell line (RD: HLA-A1, Bw51, B14) were maintained in alpha minimal essential medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (Hazleton Research Products), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. LMtk− and LJ26 cells were kindly provided by Len Herzenberg. Transfections were performed by using calcium phosphate precipitation (54) of monolayer cultures as described previously (2). Cotransfection (ratio of HLA to marker, 20 to 30:1) was carried out with the recombinant plasmid containing either the HLA-A2 plasmid (pUCA2) (P. A. Biro, B. W. Duceman, R. Srivastava, J. Pan, A. U. Sood, and S. M. Weissman, unpublished results) or the mutated plasmids HLA-A2M$_{86Gln}$, HLA-A2M$_{86Asp}$, or HLA-A2M$_{88Gly}$ together with the plasmid containing either the herpes simplex virus thymidine kinase (11) gene (pTKX1) or the Tn*5* neomycin resistance gene (pSV2neo) (50). Additional high-molecular-weight recipient cell DNA was used as carrier. Appropriate selection medium containing either HAT (0.1 mM hypoxanthine, 0.01 mM aminopterin, 0.03 mM thymidine) or Geneticin (G418-sulfate, 0.5 to 1 μg/ml; GIBCO Laboratories) was added 48 to 60 h after transfer, and fresh selection media were replaced every 2 to 3 days. Individual colonies or entire flasks of colonies were harvested 10 to 15 days after selection.

MAbs. Monoclonal antibodies (MAbs) used were MAb W6/32 (anti-HLA-A, HLA-B, HLA-C monomorphic determinant) (3), MAb PA2.1 (anti-HLA-A2 plus -HLA-A28*) (38), MAb BB7.2 (anti-HLA-A2 plus -HLA-A28*) (39), MAb MA2.1 (anti-HLA-A2 plus -HLA-B17) (32), MAb 4B3 (anti-HLA-A2 plus -HLA-A28) (55), and MAb A131 (HLA-A locus) (51). The reactivity of the rabbit anti-heavy-chain serum with nonassociated heavy chains has been previously described (28), and the serum was a gift of M. Krangel and H. Ploegh. Mouse anti-Golgi MAb was kindly provided by Lan Bo Chen (personal communication).

Indirect immunofluorescence and fluorescence-activated cell sorter analysis. Indirect immunofluorescence was carried out with saturating amounts of antibody at 4°C for 45 min. After several washes with media (alpha minimal essential medium with 2% FCS and 0.1% NaN_3), cells were incubated with fluorescein isothiocyanate-conjugated goat F(ab')$_2$ anti-mouse immunoglobulin G (Cappel Laboratories) at 4°C for another 45 min and then washed with the media again. Evaluation of immunofluorescence and cell sorting were carried out with an EPICS V laser system and MDADS multichannel analyzer (Coulter Electronics, Inc.). When gamma interferon (Biogen, Inc., [human] or Genentech, Inc. [mouse]) treatment was used, 10^6 cells were incubated for 48 h in media containing 100 to 200 U of the corresponding gamma interferon per ml, and indirect immunofluorescence and fluorescence-activated cell sorter analyses were performed as previously described (2).

Cell labeling, immunoprecipitation, and gradient sodium dodecyl sulfate gels. Cells (2 × 10^6 to 5 × 10^6) were labeled by incubation in methionine-free alpha minimal essential medium (Select-Amine kit; GIBCO) containing 10% fetal bovine serum in the presence of 250 μCi of [^{35}S]methionine (500 Ci/mmol; New England Nuclear Corp.) for 14 to 16 h at 37°C. When used, 3 μg/ml of tunicamycin was added to the media 4 to 6 h earlier and remained throughout labeling. Labeled cells were washed and lysed in 1.0 ml of lysis buffer (1% Nonidet P-40, 10 mM Tris hydrochloride [pH 7.5], 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride) on ice for 10 to 30 min. The lysates were centrifuged for 10 min in an Eppendorf centrifuge, and the supernatants were stored at −70°C if not used immediately. Immunoprecipitation with the specific MAb or the anti-heavy-chain rabbit antiserum was carried out as described in reference 27, and the samples were analyzed by electrophoresis on a 7 to 15% linear-gradient sodium dodecyl sulfate-polyacrylamide gel electro-

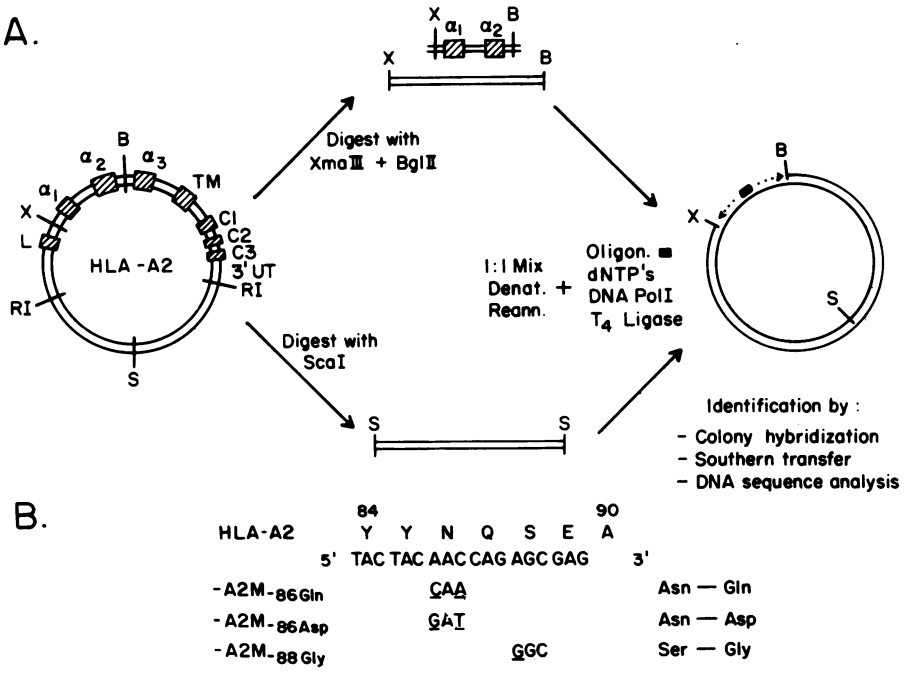

FIG. 1. Schematic diagram of the mutagenesis protocol used. (A) The recombinant plasmid was digested with one (*Sca*I) or two (*Xma*III and *Bgl*II) restriction enzymes. The two reaction mixtures were mixed with the mismatched oligonucleotide, denatured by boiling, and reannealed. The gap created was then filled by polymerase I in the presence of deoxynucleotides and the molecule ligated by T4 DNA ligase. (B) The sequences of the oligonucleotides used and their corresponding mismatches with the original HLA-A2 sequence.

phoresis gel. Radioactive bands were detected after exposure on Kodak XAR-5 X-ray film.

Internal staining and immunofluorescence microscopy. Cells (10^6 cells per well) were grown on glass cover slips, fixed with 3.5% formaldehyde in phosphate-buffered saline at room temperature for 20 min, and permeabilized with acetone or methanol at −20°C for 2 min. Alternatively, methanol alone or 1% formaldehyde in methanol at −20°C for 5 min was also used (8). The cells were then rinsed sequentially with double-distilled water–phosphate-buffered saline and incubated with the specific MAb (W6/32, 4B3), anti-heavy-chain rabbit antiserum, or mouse anti-Golgi MAb. When double-immunofluorescence labeling was performed, the mouse MAb and the rabbit antiserum were mixed. The second antibody (fluorescein-conjugated goat anti-rabbit, rhodamine-conjugated goat anti-rabbit, or rhodamine-conjugated goat anti-mouse; Cooper Biomedical, Inc.) was also used for 30 min at 37°C in a humidified chamber. Each incubation was followed by a 5-min wash in phosphate-buffered saline. Cover slips were mounted on glass slides with glycerol-gelatin containing 0.1 M *n*-propyl gallate (Sigma Chemical Co.). Fluorescence microscopy was carried out with a Zeiss Photomicroscope III.

RESULTS

Construction and expression of the nonglycosylated molecule A2M$_{86Gln}$ in mouse fibroblasts. A mismatched oligonucleotide (5′-GGCTACTACCAACAGAGCGAG-3′) was used to construct an HLA-A2 molecule altered by the substitution of 86Gln for 86Asn, as described in Materials and Methods and Fig. 1B. Plasmid DNA isolated from positive colonies (Fig. 2A) was digested with several diagnostic restriction enzymes, and the localization of the mutation to the α1 domain was confirmed following Southern blot transfer and hybridization with the labeled oligonucleotide to the filter by using the same differential-temperature washes as those that detected positive colonies (data not shown). DNA sequence analysis of the α1 and α2 exons following excision with *Sma*I from the purified mutant DNA (HLA-A2M$_{86Gln}$) confirmed that the appropriate base changes had been introduced at position 86, corresponding to the sequence of the oligonucleotide (Fig. 2B). No other base changes were observed in these exons, and the *Xma*III and *Bgl*II restriction enzyme sites used were regenerated. Because the remainder of the HLA gene was not open to mutagenesis by this protocol, it is unlikely that any other alteration occurred elsewhere in the molecule. This was further confirmed by exon swap experiments with the normal HLA-A2 gene (see below).

Once the presence of the mutation was confirmed, the plasmid was cotransfected with appropriate selectable marker genes (pTKX1 or pSV2neo) into mouse Ltk/c13 cells by the calcium phosphate precipitation procedure (54). The selection medium (HAT or G418) was added to the cells after 48 to 60 h. The expression of the HLA molecules in the stable HAT or G418 resistant colonies (mass population or clones) was analyzed by indirect immunofluorescence and flow cytometry (fluorescence-activated cell sorter) with the HLA-A2 specific monomorphic and polymorphic MAbs listed in Materials and Methods. None of these MAbs was able to detect the presence of the HLA-A2M$_{86Gln}$ gene product on the surface, while the normal HLA-A2 molecule was easily detected. Figure 3 illustrates the result with two MAbs, PA2.1 and W6/32.

Because this lack of expression could result from the unlikely possibility that other alterations were introduced elsewhere in the HLA gene by an error created during the mutagenesis procedure, an exon swap experiment was designed to interchange the sequenced region of the HLA-

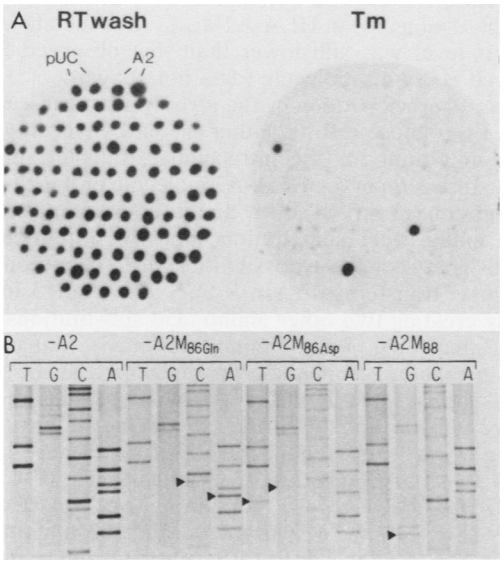

FIG. 2. Identification and characterization of the mutants. (A) Differential colony hybridization analysis. DNA from colonies transferred and grown on nylon membranes was hybridized with the ^{32}P-end-labeled oligonucleotide. The presence of the plasmid carrying the mutation is detected under differential washing conditions. Tm refers to the melting temperature of the oligonucleotide. (B) DNA sequence analysis of the glycosylation mutants. The sequence of the glycosylation mutants was determined by the dideoxy method after the α1 domain was subcloned into the mp19 vector. The arrows show the corresponding base differences between the mutants and the original HLA-A2 molecule (see also Fig. 1B). No other base changes were observed in the entire exon.

$A2M_{86Gln}$ with the original HLA-A2 gene. Both DNAs were digested with the enzymes originally used (*Xma*III and *Bgl*II [Fig. 1A]), the fragments were isolated from a low-melting agarose gel and religated to generate two new hybrid molecules: A2/MUT (normal gene but containing α1 + α2 from the mutated plasmid) and MUT/A2 (the mutated plasmid with the α1+α2 segment from the normal gene). Upon DNA-mediated gene transfer in the mouse L cells, the A2/MUT antigen was not detected on the surface, while MUT/A2 was now expressed at normal levels (data not shown). This, together with the complete sequence of α1 and α2 from the mutant showing only the appropriate changes at position 86, excludes the possibility that any other alteration in the molecule caused the lack of surface expression observed.

The presence of the appropriate ca. 39,000-molecular-weight nonglycosylated heavy chain in the cytoplasm of the mouse cells transfected with these hybrid molecules was demonstrated by metabolic labeling with [^{35}S]methionine and subsequent immunoprecipitation with a rabbit anti-heavy-chain antiserum (Fig. 4). Although tunicamycin treatment produced the appropriate shift (ca. 43,000 to ca. 39,000) in the normal HLA-A2 molecule (Fig. 4; lanes 4 and 5) and the hybrid MUT/A2 (lanes 10 and 11), the heavy chains of the mutant at position 86 (lanes 8 and 9) and the hybrid A2/MUT remained unaltered (lanes 6 and 7).

Cell surface expression of the nonglycosylated molecule associated with human β2m. Since there are several differences between mouse (14) and human (10) β2m and since the intracellular transport and cell surface expression of class I heavy chains can be affected by the association with the species-specific β2m subunit, the mutated molecule was introduced into the human rhabdomyosarcoma cell line (RD) and also into mouse L cells previously transfected with the human β2m gene (LJ26). A low level of expression (50 to 100 times less than for the normal HLA-A2 molecule) was

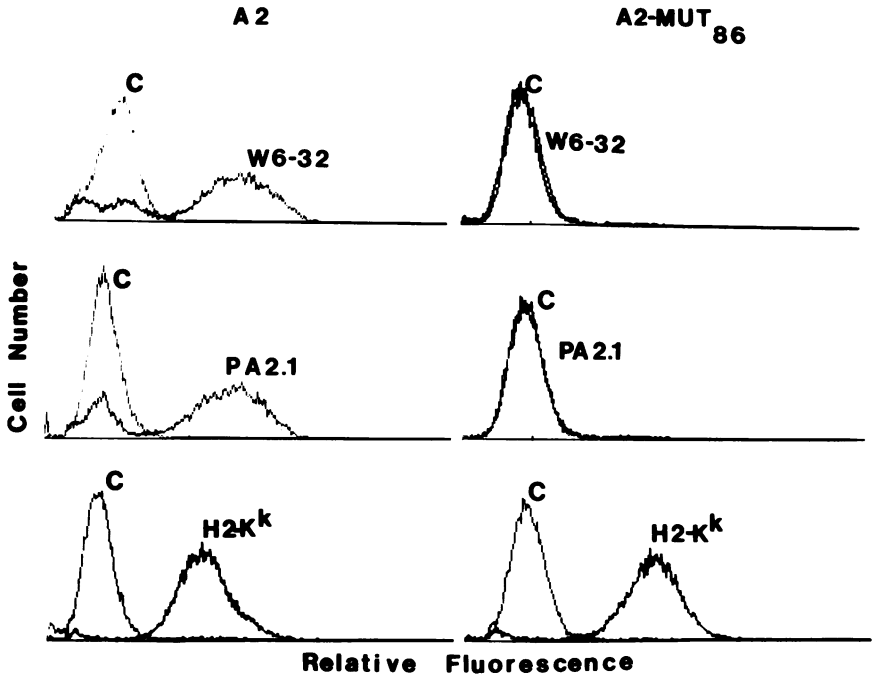

FIG. 3. Immunofluorescence and fluorescence-activated cell sorter analysis of HLA-$A2M_{86Gln}$ expression in mouse Ltk/c13 cells. The histograms represents a mass population analysis of transfected Ltk/c13 cells stained with two different HLA-A2 reactive MAbs W6/32 and PA2.1. Mouse H-$2K^k$ was used as a control of mouse endogenous class I antigen expression. Control (C) fluorescein-conjugated goat anti-mouse immunoglobulin G alone. Fluorescent signals converted to logarithmic values are displayed on a multichannel analyzer.

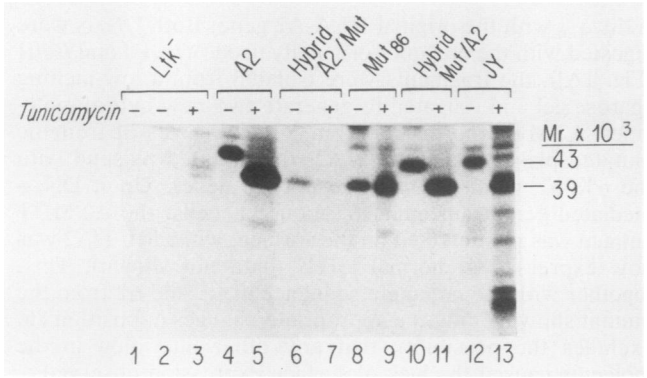

FIG. 4. Immunoprecipitation analysis of HLA heavy chains from transfected L cells. The transfectants were labeled with [^{35}S]methionine in the presence (+) or absence (−) of tunicamycin. A rabbit anti-class-I heavy-chain antiserum was used to precipitate the heavy chains from the lysates, and the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5 to 15% linear-gradient gel. Lanes: 1 to 3, immunoprecipitation of the lysate from the Ltk− cell line used with normal rabbit serum (lane 1) or anti-H (lanes 2 and 3); 4 and 5, original HLA-A2 transfectant before (−, ca. 43,000) and after (+, ca. 39,000) treatment with tunicamycin; 6 and 7, α1 + α2 domains from the mutant introduced in the normal HLA-A2 gene (hybrid A2/MUT); 8 and 9, HLA-A2 M_{86Gln}; 10 and 11, α1 + α2 domains from the normal HLA-A2 gene introduced into HLA-A2M_{86Gln} (hybrid MUT/A2); 12 and 13 (controls), immunoprecipitation of the class I polypeptides from the lymphoblastoid cell line JY from which the HLA-A2 gene was obtained.

observed in both cell lines (Fig. 5, upper and middle panels). Moreover, to investigate the relation between quantity of the light chain and the level of expression of the mutated HLA-A2M_{86Gln} molecule, the mouse cell transfectants were treated with mouse gamma interferon. The increased levels of α and β2m chain transcripts did result in greatly enhanced surface expression of the normal HLA-A2 molecule in HLA-A2 transfectants as well as some surface expression of the mutated molecule in HLA-A2M_{86Gln} transfectants. However, this level was still lower than that observed for the uninduced HLA-A2 molecule (data not shown).

Further characterization of the glycosylation consensus sequence. From these results, either the carbohydrate moiety itself is important for the intracellular transport and cell surface expression of the HLA-A2 molecule or the alteration of expression observed is a direct consequence of the specific amino acid substitution that we introduced. To distinguish between these possibilities, the recognition signal for glycosyl transferases (Asn-X-Ser) was studied in more detail by creating two other amino acid substitutions in the HLA-A2 molecule, i.e., a nonconservative change (with respect to charge) at position 86 replacing the asparagine residue with aspartic acid (HLA-A2M_{86Asp}) and the conservative substitution of serine by glycine at position 88 (HLA-A2M_{88Gly}). The oligonucleotides (a 21-mer, 5′-GGC TACTACGATCAGAGCGAG-3′, and a 19-mer, 5′-CTACA ACCAGGGCGAGGCC-3′) containing one or two mismatches (Fig. 1B) were synthesized and the mutant DNA was identified, sequenced (Fig. 2B), purified, and transfected by DNA-mediated gene transfer into mouse and human cell lines as previously described for HLA-A2M_{86Gln}. Alteration of the mobility of the heavy chains was also observed after immunoprecipitation of the lysates obtained from these transfectants, consistent with the lack of carbohydrate moiety. Tunicamycin treatment of the cells did not affect the mobility of the molecules (data not shown).

Immunofluorescence and fluorescence-activated cell sorter analysis of these transfectants shows that the new substitution (Asp) at position 86 had an identical effect as had been seen previously for the substitution of Gln at position 86 (no surface expression on L cells and a low level on human or mouse cells carrying human β2m). In contrast, the specific change at position 88 had little effect on the surface expres-

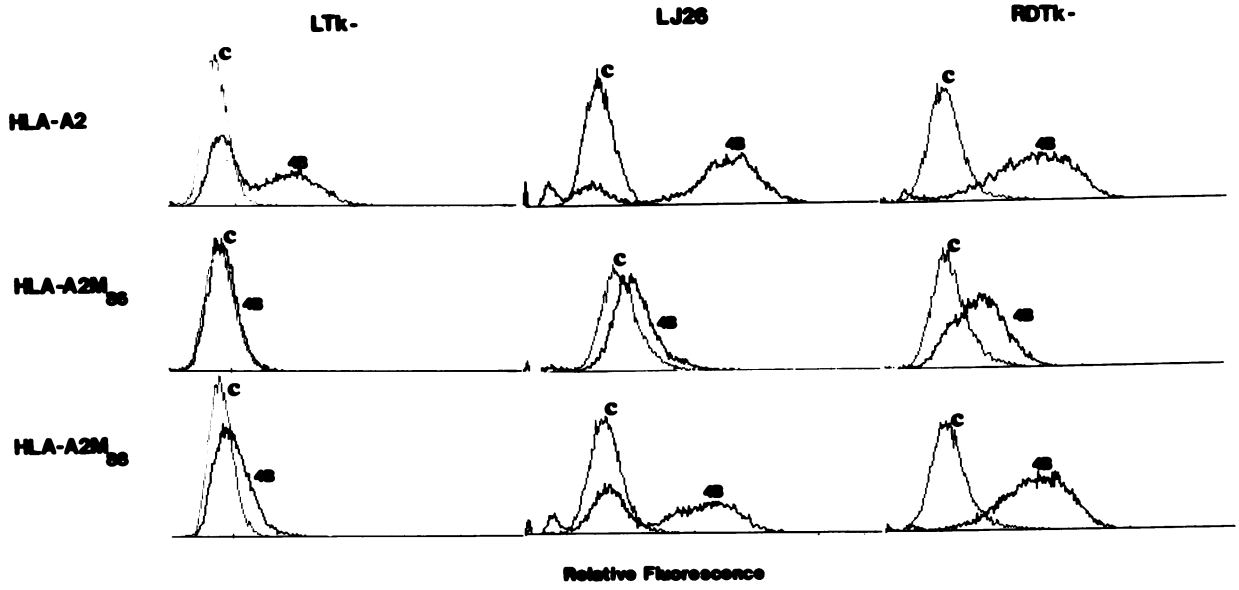

FIG. 5. Immunoflourescence and flow cytometry comparison analysis of HLA expression in different recipient cell lines. Each histogram represents the expression of the original HLA-A2 and the glycosylation mutants HLA-A2M_{86} and HLA-A2M_{88} in mouse L cells (Ltk−), LJ26, and the human RD cell line. The corresponding mass population of resistant clones was analyzed with a panel of HLA-specific mono- and polymorphic MAbs. Reactivity with MAb 4B is shown. The control (C) was the fluorescence obtained with the fluorescein-conjugated goat anti-mouse immunoglobulin alone.

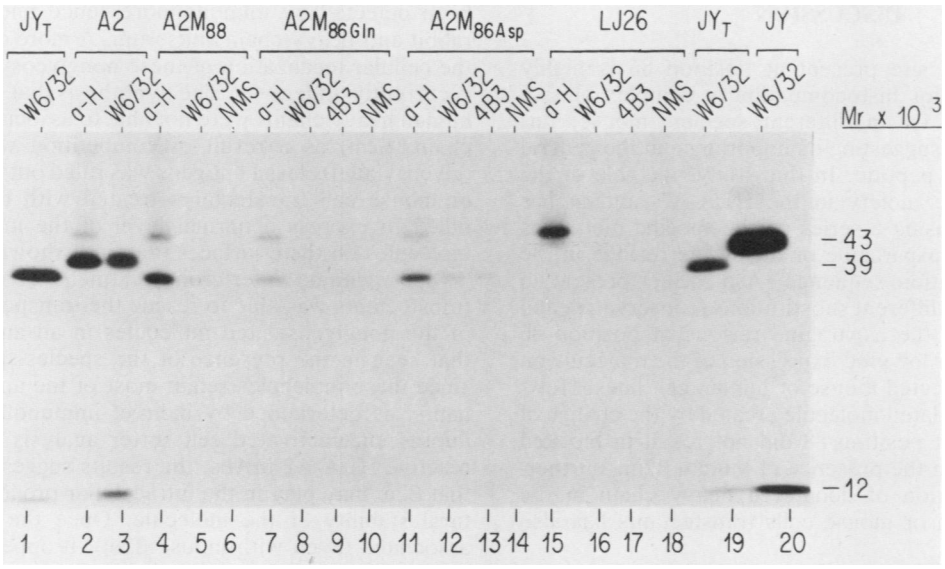

FIG. 6. Immunoprecipitation of HLA heavy chains from transfected LJ26 cells. The autoradiogram illustrates the difference between the immunoprecipitated material from the lysates of the transfectants with the rabbit serum or the specific MAbs. The cells were labeled in the presence of [^{35}S]methionine for 4 h. The transfectants were previously sorted to eliminate negative cells and to obtain a similar level of expression for the glycosylation mutants. Lanes 2 and 3 show the immunoprecipitation of the lysate from the HLA-A2 normal transfectant with the anti-heavy-chain antiserum (a-H) or W6/32. Similarly, the lysate from the transfectants HLA-A2M$_{88}$ (lanes 4 to 6), HLA-A2M$_{86Gln}$ (lanes 7 to 9), or HLA-A2M$_{86Asp}$ (lanes 11 to 13) can also be compared by using a-H, W6/32, or 4B3. Lanes 14 to 16 show the immunoprecipitation from the LJ26 cell line used. Normal mouse serum (NMS; lanes 6, 10, 14, and 18) was used on each lysate as a control. Class I polypeptides from the lymphoblastoid cell JY with (JY$_T$, lanes 1 and 19) or without (JY, lane 20) treatment with tunicamycin were used as a control.

sion of the molecule when human β2m was present. Thus, in mouse (LJ26) and human (RD) cells, the levels of expression were comparable to those for the normal HLA-A2 gene. A lowered level of surface expression was nevertheless detected in mouse Ltk− cells, although this effect was not as marked as in the HLA-A2 molecule having the substitution at position 86 (Fig. 5). The alteration in the association between the heavy and light chain of the nonglycosylated molecules is illustrated in Fig. 6 after metabolic labeling and immunoprecipitation with W6/32 and anti-(a-H) of the lysates obtained from the LJ26 transfectants. In contrast to the equal amount of immunoprecipitated material obtained in the normal HLA-A2 transfectant, the nonglycosylated mutants, HLA-A2M$_{88Gly}$ (lanes 5 and 6), HLA-A2M$_{86Gln}$ (lanes 8 to 10), and HLA-A2M$_{86Asp}$ (lanes 12 to 14), all showed a much smaller quantity of associated (as detected by the MAbs W6/32 or 4B3) than of nonassociated (using the anti-heavy-chain rabbit antiserum, a-H) HLA heavy and light chains. Thus, amino acid changes in the consensus glycosylation sequence (especially at position 86) have a drastic effect on the association of the heavy and light chains and the subsequent cell surface expression of the complex. The alteration is more dramatic when the human HLA heavy chain must associate with mouse β2m and in this case is observed also for the mutation at position 88. The relatively small amount of material seen on immunoprecipitation of A2M$_{88Gly}$ with W6/32 (Fig. 6) compared with the amount seen by fluorescence-activated cell sorter analysis (Fig. 5) could reflect this alteration in association between the human heavy chain and mouse β2m (since the experiment was done with mouse cells). Alternatively, the MAbs used may not react as well with this mutated molecule in internal membranes associated with mouse β2m as opposed to the surface in which the mouse β2m exchanges readily with bovine β2m (11).

Intracellular localization of the nonglycosylated heavy chains. The cellular localization of the nonglycosylated HLA-A2 molecules in the transfectants was further investigated by indirect immunofluorescence microscopy analysis after permeabilization of the membrane. In contrast to the uniformly distributed immunofluorescence pattern present in the cytoplasm of the cells transfected with the original HLA-A2 molecule, the glycosylation mutants at position 86 show a characteristic internal staining pattern (in both human and mouse cells) with the anti-heavy-chain antiserum (Fig. 7A). These fluorescent granules were seen after four different fixation procedures (8) (see Materials and Methods) and therefore are unlikely to be due to fixation artifacts. Similar concentration of MAb staining was not observed with W6/32 or another MAb (4B3) identifying mature β2m-associated heavy chains (data not shown). This result confirms that the heavy chains of the mutants at position 86 were unable to associate with β2m and that their transport to the surface was blocked.

To address whether these chains were localized in the Golgi compartment, dual immunofluorescence staining (using simultaneously the anti-heavy-chain rabbit antiserum and a MAb recognizing the Golgi structure) was performed. The HLA heavy chains were not located within the Golgi complex, since fluorescein and rhodamine staining were present in different locations (compare the fluorescence localization in Fig. 7A and B). A similar result was observed for the HLA-B7 molecule mutated at the same position (J. Barbosa et al., unpublished results). Furthermore, the pattern of staining is not typical of the punctate stain usually observed for lysosomal staining (L. B. Chen, personal communication). These experiments pose the questions of the role of β2m in transport of the heavy chain and where and why the α chain becomes arrested in the absence of association with β2m.

DISCUSSION

The glycosylation site present at position 86 is highly conserved in all major histocompatibility complex class I antigens studied so far in different species: mouse, rat, rabbit, and humans, suggesting its importance in the general structure of the polypeptide. In this study, the role of the unique carbohydrate moiety in the HLA-A2 antigen has been addressed by using a series of site-specific mutations involving either the asparagine or the serine residue in the consensus glycosylation sequence (Asn-X-Ser) present in the α1 domain. Two different substitutions (conservative and nonconservative) of the asparagine residue at position 86 produced a lack of or lowered expression of the molecule on the surface of transfected mouse or human cell lines. However, the nonglycosylated molecule created by the change of the serine residue at position 88 did not result in blocked surface expression in the presence of human β2m. Furthermore, an accumulation of denatured heavy chain in the cytoplasm of human or mouse cells transfectants has also been detected by immunofluorescence microscopy with a rabbit anti-heavy-chain antiserum. A more detailed study of the cellular localization of these nonglycosylated molecules is currently under way. The hypothesis that nonglycosylated human heavy chains were not able to associate with the light chain (β2m) as a result of competition with endogenous (glycosylated) class I antigens was ruled out when the human or mouse cell transfectants treated with tunicamycin still failed to express a normal level of the mutated HLA-A2 molecules on their surfaces (data not shown).

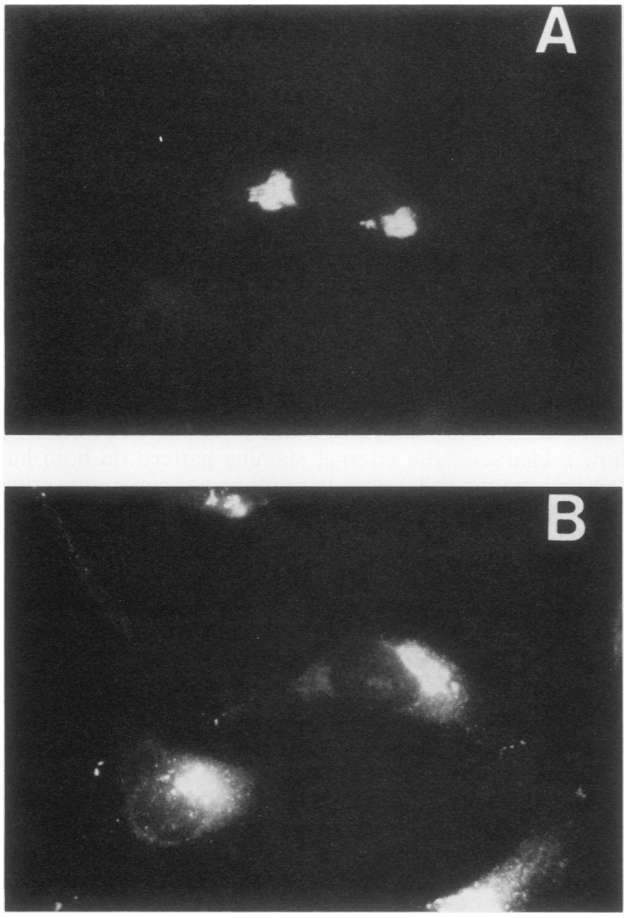

FIG. 7. Double-label indirect immunofluorescence microscopy analysis showing internal staining for HLA-A2M_{86Gln}. RD cells transfected with the mutant DNA were permeabilized and fixed, and the denatured heavy chain was labeled with rabbit anti-heavy-chain and fluorescein-conjugated goat anti-rabbit immunoglobulin G (A). The Golgi structure was labeled with a mouse MAb and rhodamine-conjugated goat anti-mouse immunoglobulin G (B). Panels A and B correspond to the same field photographed with two different filters to demonstrate the distribution of fluoresceinated anti-heavy-chain antiserum or the anti-Golgi MAb detected with rhodamine.

Since gamma interferon treatment of the mouse cell transfectants was able to rescue the transport to the surface of the nonglycosylated molecules in an amount similar to that seen in the presence of the species-specific β2m and since these molecules retain most of the antigenic determinants, as determined by indirect immunofluorescence and fluorescence-activated cell sorter analysis with a panel of reactive HLA-A2 MAbs, the results suggest the active role that β2m may play in the intracellular processing and structural stability of the molecule. Once the heavy chain is associated (even with mouse β2m), it appears able to continue to the surface. The low level of expression may be due to the inability to form this complex. Thus, the α1 domain, and possibly amino acid 86 in particular, provides some capacity for the association between newly synthesized heavy and light chains. Alteration of the asparagine residue may reduce this interaction, and if β2m does not associate by the time the entire heavy chain is synthesized, a conformational change may occur, after which the association is no longer possible. This would explain the improved expression capability resulting from both gamma interferon treatment (greater quantity of HLA heavy and light chains) and the presence of the species-specific β2m. Support for this notion has some precedent (29).

It is not surprising that lack of association between both subunits results in a block of the transit of the heavy chain through the cytoplasm, as occurred in Daudi cells owing to a complete absence of the β2m. The results presented in this study suggest, furthermore, that specific amino acid changes in a highly conserved region of the molecule may also affect the association of the two subunits, seen more dramatically in the association of the heavy chain with mouse β2m. Although it has been generally accepted that the major site of β2m interaction is the α3 domain (56), several earlier results had suggested a possible role for the α1 and α2 domains (29, 53). In addition, small changes in other regions of the molecule (especially the α1 domain, J. Santos-Aguado et al., unpublished results) may have a major effect in the association between both subunits. The different requirements of the association of class I molecules with the species-specific β2m is also exemplified by the fact that, although HLA-A2 and HLA-B7 associate with mouse β2m in their transit to the surface, their rates of dissociation (21) and extent of exchange with bovine or human β2m from the culture medium containing bovine or human serum varies; i.e., most of the HLA-B7 molecules on the surface become associated with either bovine or human β2m, while only 40 to 60% of HLA-A2 exchanges (4).

The presence of carbohydrate moieties on cell surface glycoproteins has been linked to several cellular functions such as maintenance of correct polypeptide conformation, protection from proteolytic degradation, signals for intracellular targeting, and components of receptors systems (35). Experiments involving the use of different inhibitors nevertheless indicate a molecule-specific role for these glycan moieties, since some nonglycosylated proteins fail to reach

their correct cellular destination (glycoprotein G of vesicular stomatitis virus [15]) or are degraded more rapidly while others are not (7). Recently, it was suggested that carbohydrate moieties may play an important role as recognition signals in the intracellular transport of cell surface glycoproteins (1, 18, 30, 33). However, present results suggest that in the HLA-A2 molecule, in addition to any other role of the carbohydrate moiety itself, certain residues in the glycosylation sequence are important in the conservation of the general structure of the polypeptide. It is curious that the mutant mouse H-2L^d molecules containing lysine at position 86 and destroying the consensus glycosylation sequence were not drastically affected in expression (48). Alteration in surface expression was only observed when all three glycosylation sites present in the H-2L^d molecule were mutated (33). It is possible that a positively charged amino acid introduced at position 86 in the HLA-A2 antigen will not result in dramatic expression differences. Alternatively, the structural requirements at position 86 may not be the same for mouse or human class I antigens. The nonglycosylated mutants produced in this study may provide excellent tools for a more detailed study of the biosynthesis, intracellular transport, and cell surface expression of major histocompatibility complex class I antigens.

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