N-Linked Glycosylation Selectively Regulates the Generic Folding of HLA-Cw1*[S]

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To resolve primary (glycosylation-assisted) from secondary (glycosylation-independent) quality control steps in the biosynthesis of HLA (human leukocyte antigen) class I glycoproteins, the unique N-linked glycosylation site of the HLA-Cw1 heavy chain was deleted by site-directed mutagenesis. The non-glycosylated Cw1S88G mutant was characterized by flow cytometry, pulse-chase, co-immunoprecipitation, and *in vitro* assembly assays with synthetic peptide ligands upon transfection in 721.221 and 721.220 cells. The former provide a full set of primary as well as secondary chaperoning interactions, whereas the latter are unable to perform secondary quality control (*e.g.* proper class I assembly with peptide antigens) as a result of a functional defect of the HLA-dedicated chaperone tapasin. In both transfectants, Cw1S88G displayed a loss/weakening in its generic chaperoning interaction with calreticulin and/or ERp57 and became redistributed toward calnexin, known to bind the most unfolded class I conformers. Despite this, and quite unexpectedly, a weak interaction with the HLA-dedicated chaperone TAP was selectively retained in 721.221. In addition, the ordered, stepwise acquisition of thermal stability/peptide binding was disrupted, resulting in a heterogeneous ensemble of Cw1S88G conformers with unorthodox and unprecedented peptide assembly features. Because a lack of glycosylation and a lack of tapasin-assisted peptide loading have distinct, complementary, and additive effects, the former is separable from (and upstream of) the latter, *e.g.* primary quality control is suggested to supervise a crucial, generic folding step preliminary to the acquisition of peptide receptivity.

Quality control in the endoplasmic reticulum (ER)[4] ensures the proper folding, oligomerization, and sorting of glycopro-teins. Primary quality control, common to all glycoproteins, is carried out by successive rounds of glucosylation and de-glu-cosylation of N-linked glycans. In this process, known as the calnexin cycle, monoglucosylated glycoproteins are the elective substrates of the lectin-like "retention-retrieval" chaperones calnexin (a transmembrane protein) and/or calreticulin (the soluble homologue of calnexin) and become folded and disul-fide-bonded through the concerted action of calnexin, calreti-culin, the thiol-dependent oxido-reductase ERp57, protein disulfide isomerase, and other members of the quality control machinery (1).

Trimmed, conformed substrates are released for intracellu-lar transport and further processing, but the maturation, assembly, folding, and intracellular transport of some glycopro-teins require specialized steps, collectively known as secondary quality control (1). It is agreed that secondary (protein-specific) quality control has two general features: (*a*) it takes place after completion of primary quality control, and (*b*) it involves the formation of dedicated complexes between glycoprotein sub-strates and ER proteins acting as either chaperones or escorts/guides for intracellular transport. To the best of our knowledge, major histocompatibility complex class I molecules are an intriguing exception in both respects, because (*a*) their primary and secondary quality control mechanisms are subtly inter-twined, and (*b*) they simultaneously bind generic and class I-dedicated chaperones (1, 2).

Class I major histocompatibility complex molecules (also called HLA-A, -B, -C in humans, H-2K, -2D, -2L in mice, and RT1 in rats) are formed upon heterotrimeric assembly of: (*a*) a highly polymorphic heavy (44 kDa) chain carrying up to three glycosylation sites, only one of which (at position 86) is highly conserved across species and present in human molecules; (*b*) a non-polymorphic, non-glycosylated light (12 kDa) chain sub-unit (β_2-microglubulin, β_2m); and (*c*) a short (8–11 mer) pep-tide antigen derived from the cytosolic degradation of cellular proteins. The heavy chain binds calnexin, associates with β_2m, and is loaded with peptide on a unique folding and quality con-trol station called "peptide loading complex," formed by the supramolecular association of generic and class I-dedicated chaperones. The former include calnexin (only in murine cells), calreticulin, ERp57, and protein disulfide isomerase, recently identified (3). The latter include the transporter associated with antigen processing (TAP), a heterodimeric membrane pump that translocates peptides from the cytosol into the ER lumen, and tapasin, a peptide editor/facilitator. Thermally stable (pep-

tide-bound), conformed class I heterotrimers are released from the peptide loading complex, leave the ER, and are exported to the cell surface for functional recognition by CD8 T lymphocytes and natural killer (NK) cells (2).

There is evidence that class I heavy chains are sequentially de-glucosylated and re-glucosylated (4), *e.g.* they undergo the calnexin cycle and become increasingly conformed upon successive interaction with calnexin and calreticulin (Ref. 5) and see Ref. 6), but the importance of *N*-linked glycosylation and the specific role of the two glycosylation-dependent chaperones remain elusive. For instance, (*a*) ER retention of misfolded class I heavy chains is mediated by calnexin when interaction with the peptide loading complex cannot take place, *e.g.* in β_2m-deficient cells and in insect cells (7–9) but is physiologically mediated by TAP and tapasin in peptide loading-sufficient cells (Refs. 10–12) and discussed in Ref. 13); (*b*) class I molecules normally mature in calnexin-deficient cells (14); (*c*) the Asn-86 class I glycan is not required for calnexin binding and, although required for calreticulin binding, its absence disrupts generic (with calreticulin and ERp57) and class I-dedicated (with TAP and tapasin) chaperoning steps altogether (15, 16).

The biochemical features of Asn-86 glycosylation-defective class I molecules do not help to clarify these issues but rather add to the complications, because several human glycosylation-defective mutants are poorly, if at all, transported to the cell surface and are non-functional (17, 18), whereas most murine Asn-86 mutants are expressed and are at least partly functional (19–21). Moreover, the only two mutants that have been studied in detail displayed two different phenotypes; the human mutant was completely refractory to peptide assembly, *in vivo* as well as *in vitro* (22), whereas the rat mutant failed to stably bind natural ligands *in vivo* but retained its ability to assemble *in vitro* with specific peptides (23), an alteration reminiscent of those seen in cells defective in calreticulin, TAP, or tapasin (24–26).

In summary, the functional redundancy of calnexin and calreticulin, the integration of generic and class I-dedicated quality control steps within the peptide loading complex, the global and/or uninformative disruptions in class I chaperoning and folding caused by an absence of the Asn-86 glycan, as well as differences among species and class I alleles, make it difficult to assess the relative contributions (and/or stepwise occurrence) of primary (glycosylation-assisted) and secondary (peptide loading-dependent) quality control events.

To address these issues, we have characterized an *N*-glycosylation-defective mutant of a class I HLA molecule (the HLA-Cw*0102 heavy chain). The Cw1 allele was selected because of its poor and very slow class I assembly (27), two features expected to be most useful in dissecting biosynthetic intermediates and steps. We show herein that the Cw1 mutant differs, in its chaperoning and folding intermediates, from all the previously described conformers of mutant as well as wild-type class I heavy chains. This reveals a crucial mergence between primary and secondary quality control events in a model glycoprotein.

EXPERIMENTAL PROCEDURES

Cell Lines—The 721.221 (221 hereafter) cell line lacks the expression of HLA-A, -B, -C but retains the expression of HLA-E and -F molecules (28, 29). The 721.220 (220) cell line (26) lacks functional tapasin and retains partial expression of the HLA-Cw1 allele.

Site-directed Mutagenesis—Site-directed mutagenesis was performed as described (30) using the GE Healthcare unique site elimination toggle selection kit. Briefly, pUC18-Cw1 (31) DNA containing the HLA-Cw*0102 gene was annealed with the 5′-GCTACTACAACCAG**G**GCGAGGCCGGTGA-3′ oligonucleotide (the single-nucleotide mismatch is boldface), extended by T4 DNA polymerase, ligated by T4 ligase, and amplified in the NM522 mutS *Escherichia coli* strain. Mutagenesis introduced a single conservative substitution at codon 88 (from GAG to GGG) of the Cw1 heavy chain, turning the *N*-glycosylation consensus NQS into NQG (HLA-Cw1S88G hereafter). The DNA sequence of the Cw1S88G construct was identical (except for the mutation) to the published Cw1 sequence (GenBank^{TM} accession number M16272). The wild-type Cw1 and the mutated Cw1S88G inserts were EcoRI/HindIII subcloned into the pcDNA3.1 expression vector (Invitrogen) and transfected by electroporation in 221 (both constructs) and 220 (the mutant only) cells.

Immunochemical Methods—All the antibodies, synthetic peptides, and methods are referenced (27). Where indicated, immunoprecipitates (in the form of Sepharose beads) were digested for 3 h in 30 μl of either phosphate 0.1 M, pH 7.2, buffer containing 10 mM EDTA, 0.2% SDS, 1% 2-mercaptoethanol, and 1 unit of PNGase F or phosphate 0.1 M, pH 5.5, buffer containing SDS 0.02% and 0.025 units of endo-β-*N*-acetylglucosaminidase H (both enzymes from Roche Applied Science). Immunoprecipitates were washed three times in buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.25% Nonidet P40, eluted, and electrophoresed. Underexposed autoradiographs of filter blots (chemiluminescence) and dried gels ([^{35}S]methionine) were analyzed by densitometry using the public domain NIH Image program, following image calibration to an optical density step reference marker (serial 2-fold dilutions of class I immunoprecipitates).

RESULTS

Biochemical Analysis of the HLA-Cw1S88G Glycosylation-defective Mutant—The 221 cell line, lacking classical class I molecules, was stably transfected with DNAs encoding wild-type Cw1 and glycosylation-defective Cw1S88G. Parental 221 cells and the two transfectants were compared by biochemical methods and flow cytometry (Fig. 1, *A* and *B–D*, respectively). Nonidet P-40 lysates from metabolically labeled cells were immunoprecipitated in parallel with antibodies known (6) to bind unfolded, β_2m-free Cw1 heavy chains (L31) and folded, β_2m-associated Cw1 heavy chains (W6/32 and F4/326). In the SDS-PAGE slab shown in Fig. 1*A*, Cw1 and Cw1S88G polypeptides of the expected 44- and 40-kDa sizes, respectively, were detected by all the antibodies in transfectants (*lanes 4–7* and *12–15*) but not in parental 221 cells (*lanes 2* and *3*), whereas a third component migrating between these two bands was
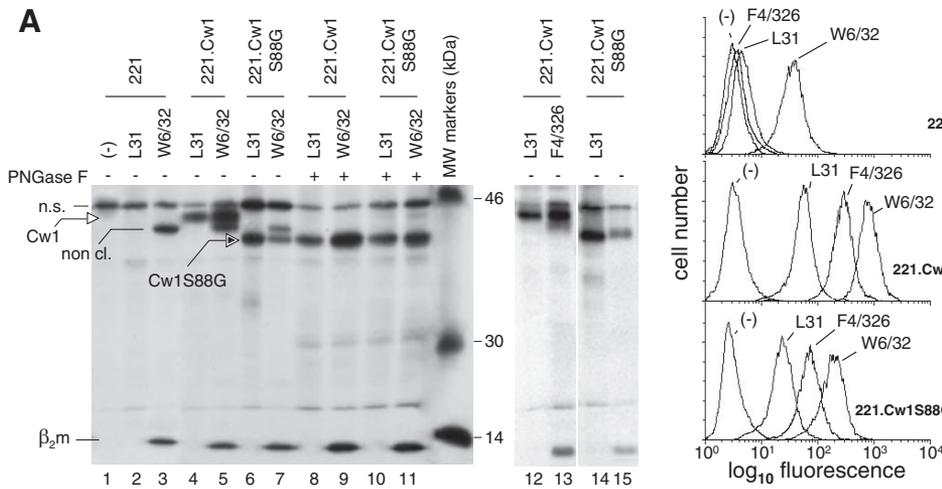
A

B

C

D

FIGURE 1. Comparative analysis of 221.Cw1 and 221.Cw1S88G stable transfectants. *A*, parental 221 cells, as well as 221.Cw1 and 221.Cw1S88G transfectants, were metabolically labeled for 2 h with [^{35}S]methionine (9 MBq/ml), lysed by Nonidet P-40, and immunoprecipitated with either the indicated antibodies or an irrelevant antibody ($-$). Immunoprecipitates were digested with PNGase F where indicated. All samples were run on a SDS-PAGE gel (12.5% acrylamide) under reducing conditions. Nonspecific (*n.s.*) bands, non-classical (*non cl.*) heavy chains, and ^{14}C-labeled molecular mass markers are indicated. *B–D*, cells and transfectants were tested by flow cytometry with the indicated antibodies.
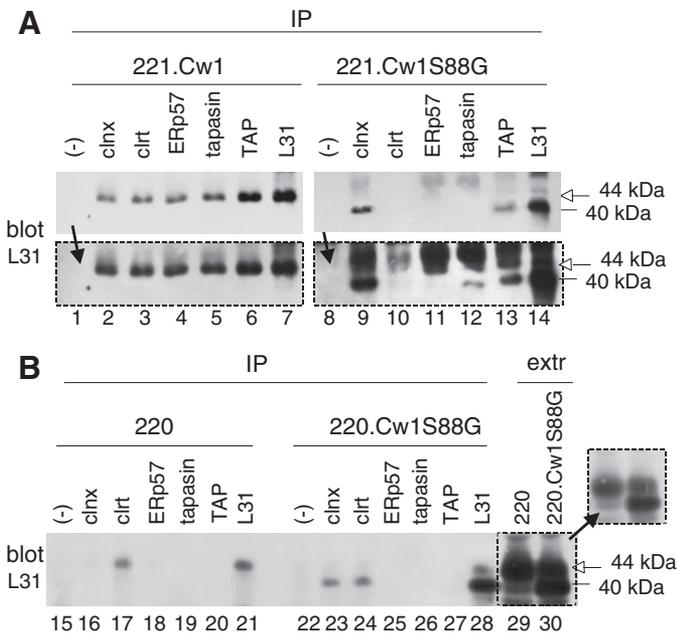
A

B

FIGURE 2. Co-immunoprecipitation of Cw1 and Cw1S88G with chaperones and members of the peptide loading complex. *A*, CHAPS lysates of 221.Cw1 and 221.Cw1S88G cells were immunoprecipitated with antibodies to the indicated chaperones (*clnx*, calnexin; *clrt*, calreticulin) with L31 to Cw1 heavy chains, or in the absence of specific antibodies. Immunoprecipitates were run on a SDS-PAGE slab and blotted with L31 to HLA-C heavy chains. Filters were optimally exposed in the *top panels* and overexposed (*arrows*) in the *bottom panels*. *B*, the same immunoprecipitation experiment as in *A* with 220 and 220.Cw1S88G cells (*lanes 15–28*). CHAPS lysates were electrophoresed and electroblotted in parallel in *lanes 29* and *30* (underexposed in the *inset*).

detected in all the cells but only by the W6/32 antibody (*lanes 3*, *5*, and *7*), known to recognize a widely shared class I epitope (32). In view of its size, presence in untransfected 221 cells, and lack of reactivity with L31 and F4/326, this component corresponds to non-classical class I heavy chains known to be expressed at low levels in 221 cells (see Ref. (33). As also expected, glycosylation-defective Cw1S88G heavy chains

migrated with an electrophoretic mobility very similar to that of enzymatically de-glycosylated HLA-Cw1 and non-classical heavy chains (Fig. 1*A*, *lanes 8–11*).

Removal of the *N*-glycosylation site increased and decreased, respectively, β_2m-free and β_2m-associated heavy chains (Fig. 1*A*, compare *lanes 4* and *6*, *12* and *14*, *5* and *7*, *13* and *15*). Densitometric analysis of heavy chain and β_2m bands, carried out on L31 and F4/326 immunoprecipitates without the interference of non-classical class I molecules, revealed an inversion in the β_2m-free:β_2m-associated heavy chain ratios from ~0.5 in Cw1 to ~3.0 in Cw1S88G (Fig. 1*A*, *lanes 12–13* compared with *lanes 14–15*), whereas heavy chain:β_2m ratios were similar in the two transfectants (3.5 and 3.0 in *lanes 13* and *15*, respectively).

In 15 separate flow cytometry experiments (one of which is shown in Fig. 1*B*), the surface reactivity of 221.Cw1S88G with L31, F4/326, and W6/32 ranged from 30 to 45% the reactivity of the same antibodies with 221.Cw1 (Fig. 1, *C* and *D*). Non-classical heavy chains, detectable in small amounts in parental 221 cells (Fig. 1*B*), are expected to similarly and negligibly contribute to the W6/32-reactive pool in the two transfectants.

Altogether, the results in Fig. 1 demonstrate that removal of the unique Cw1 glycosylation site impairs heavy chain folding without appreciably affecting the stability of β_2m association of the few heavy chains that manage to acquire conformational epitopes. As expected, poor folding results in poor surface expression of all the heavy chain conformers.

Interaction of HLA-Cw1S88G Heavy Chains with Chaperones and Members of the Peptide Loading Complex—Impaired folding of Cw1S88G is likely to reflect deranged interaction with cellular chaperones. To address this issue, wild-type and glycosylation-defective Cw1 heavy chains were solubilized by CHAPS from the 221.Cw1 and 221.Cw1S88G transfectants and either co-immunoprecipitated by antibodies to calnexin, calreticulin, ERp57, tapasin, and TAP1 or directly immunoprecipitated by the control antibody L31, specific for unfolded HLA-C. Upon SDS-PAGE and filter blotting with L31 itself (Fig. 2*A*, short and long exposures of the filters are both displayed), wild-type Cw1 was detectable in association with all the chaperones (*lanes 2–6*), as expected. In contrast, the Cw1S88G mutant (Fig. 2*A*, *lanes 10–12*) lost its ability to interact with calreticulin, ERp57, and possibly tapasin (that was barely detectable in the overexposed panel only) and accumulated in two conformations (calnexin-bound and L31-reactive, Fig. 1*A*, compare *lanes 2* and *7* to *lanes 9* and *14*, respectively) known to be particularly unfolded and partially overlapping (5, 6). Surprisingly, despite these multiple derangements in cellular chaperoning, Cw1S88G retained partial binding to TAP (Fig. 2*A*, *lane 13*).

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These results were reproduced in four additional, independent experiments. The densitometric intensities of the immunoprecipitated and co-immunoprecipitated Cw1S88G heavy chain bands in each experiment were normalized with respect to the intensities of the corresponding Cw1 bands, averaged, and expressed in arbitrary units as mean values ($\pm$ S.D.). –Fold increases in calnexin association and L31 reactivity caused by a lack of glycosylation were estimated to be 1.32 ($\pm$ 0.15) and 2.00 ($\pm$ 0.30), respectively. Residual interaction with TAP amounted to 0.21 ($\pm$ 0.08). Binding to calreticulin and ERp57, as well as tapasin, remained invariably undetectable, indicating that tapasin-Cw1S88G interaction, if any, is too close to the sensitivity threshold of our co-immunoprecipitation assay to be reproducibly observed. It can be concluded that a lack of *N*-linked glycosylation deranges generic chaperoning but reproducibly spares some class I-specific interactions with TAP.

To investigate the role of glycosylation in a cell line that can only provide generic chaperoning, 220 cells were selected in which class I-dedicated chaperoning is selectively lost due to a lack of functional tapasin but residual Cw1 expression (26) and class I-calreticulin interaction (34) are retained. CHAPS extracts from 220 cells and their stable 220.Cw1S88G transfectants were directly blotted with L31 to assess the levels of the two heavy chains. Cw1S88G (overexpressed under the control of a strong promoter) was almost three times more abundant than wild-type Cw1 in untransfected 220 cells run in parallel (Fig. 2*B*, compare *lanes 30* and *29*, see *inset*; densitometric data not shown). Overexpression of Cw1S88G depressed the accumulation of endogenous Cw1 that was five times less abundant than the co-expressed mutant at steady-state (Fig. 2*B*, *lane 30*), most likely due to competition for the translation and/or transcription machinery. Whatever the precise mechanism, Cw1S88G was considerably more unfolded than Cw1, as assessed by its marked accumulation in L31 immunoprecipitates (Fig. 2*B*, *lane 28* compared with *lane 21*). Accordingly, its interaction with calnexin was induced or greatly enhanced (Fig. 2*B*, compare *lanes 23* and *16*). Interaction with calreticulin remained apparently similar (Fig. 2*B*, *lanes 24* and *17*), a finding that, considered in the context of overexpression and extensive unfolding of Cw1S88G, suggests weakened rather than conserved interaction. No TAP, tapasin, or ERp57 interaction with Cw1 or Cw1S88G was detected in either cell line (Fig. 2*B*, *lanes 18–20* and *25–27*).

In summary, the results in Fig. 2 show that a lack of *N*-linked glycosylation: (*a*) preferentially affects interaction with the generic chaperones calnexin and calreticulin, whereas class I-dedicated chaperoning by TAP is less severely affected; (*b*) causes an additional class I folding impairment in a cell line that is already defective in HLA-dedicated (tapasin-dependent) chaperoning; and (*c*) causes a redistribution of heavy chains toward unfolded (L31-reactive and calnexin-bound) conformations. Because a lack of glycosylation and a lack of efficient peptide loading have distinct, complementary, and additive effects, glycosylation-mediated chaperoning of Cw1 may be placed upstream of peptide loading.

Disruption of physiological chaperoning in Fig. 2, *A* and *B*, is due to a folding impairment *in vivo* and not to the absence of the glycan moiety *per se*, because endo-β-*N*-acetylglucosaminidase
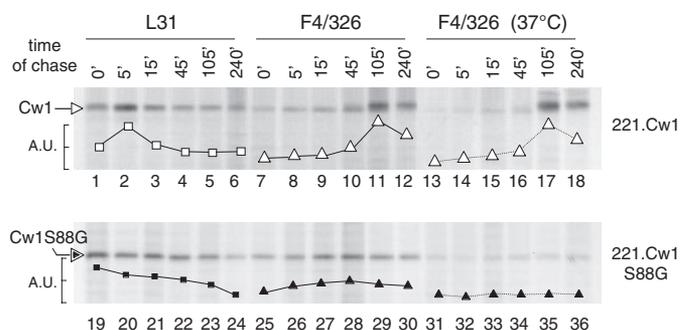

FIGURE 3. **Pulse-chase analysis of 221.Cw1 and 221.Cw1S88G transfectants.** The two stable transfectants were pulsed for 5 min with [^{35}S]methionine (9 MBq/ml), chased for the indicated times, and lysed by Nonidet P-40. Lysates were divided into three aliquots, two of which were kept at 4 °C (*lanes 1–12* and *19–30*). The third aliquot was incubated at 37 °C for 4 h (*lanes 13–18* and *31–36*). Then, class I molecules were immunoprecipitated with the indicated antibodies and resolved by SDS-PAGE as described in the legend to Fig. 1. A densitometric scan (arbitrary units, *A.U.*) of heavy chain bands is shown.

H digestion of calnexin, calreticulin, and TAP co-immunoprecipitates from wild-type 221.Cw1 transfectants did not appreciably disrupt any preformed chaperone interaction (supplemental Fig. S1).

Pulse-Chase Analysis and Thermal Stability of HLA-Cw1 and HLA-Cw1S88G Heavy Chains—Next, we focused on class I folds. Pulse-chase experiments were performed in parallel with 221.Cw1 and 221.Cw1S88G transfectants to compare the appearance and turnover of L31- and F4/326-reactive conformers. To assess the timing of thermal stabilization of the latter, extracts were divided into two aliquots and preincubated for 4 h at either 4 or 37 °C before immunoprecipitation. Fig. 3 shows autoradiography and densitometric scans of Cw1 heavy chain bands. As expected, the two conformers peaked at early and late chase points (5 and 105 min, Fig. 3, *lanes 2* and *11*) with a considerable time lag (100 min) between the peaks. The peak of F4/326 reactivity coincided with the acquisition of thermal stability (Fig. 3, compare *lanes 11* and *12* to *lanes 17* and *18*). In sharp contrast, no clear peaks were evident in the kinetics of accumulation of the two Cw1S88G heavy chain conformers. L31-reactive Cw1S88G heavy chains were most abundant at time 0 min and then slowly and progressively declined (Fig. 3, *lanes 19–24*). F4/326-reactive heavy chains instead displayed a gradual increase until 45 min of chase, mirrored by a subsequent, slow decrease (Fig. 3, *lanes 25–30*) and acquired little thermal stability throughout the chase, as documented by their flat densitometric profile (*lanes 31–36*). In addition, during the three initial chase points, F4/326 conformers were present in slightly greater amounts in 221.Cw1S88G (Fig. 3, *lanes 25–27*) than in 221.Cw1 (*lanes 7–9*) cells, indicating that some Cw1S88G molecules acquired the F4/326 epitope before the physiological time window (45–105 min) of thermal stabilization. Thus, in the absence of glycosylation, we observed abnormal kinetics of conformer accumulation beginning at the earliest (0–5 min) chase points, as well as an altered timing and a compromised efficiency in thermal stabilization.

Peptide Receptivity of Glycosylation-defective HLA-Cw1 Heavy Chains—To obtain additional information on the putative defect in peptide loading caused by a lack of glycosylation, we performed *in vitro* assembly studies on CHAPS lysates of
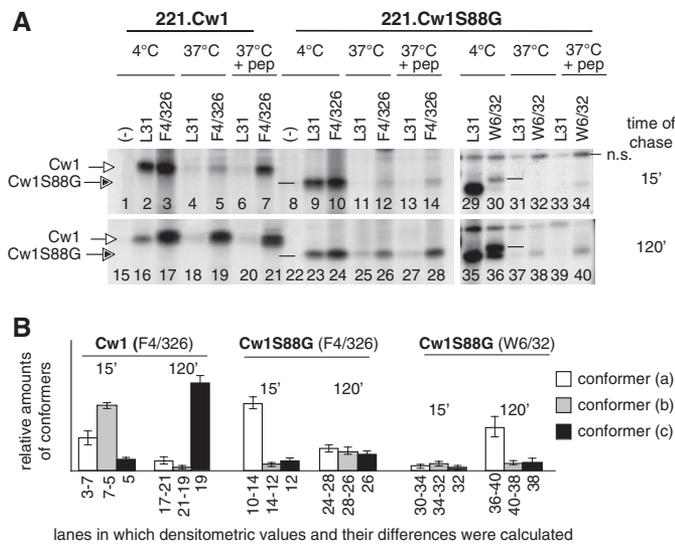
FIGURE 4. *In vitro* assembly of Cw1 and Cw1S88G with a specific Cw1 peptide ligand. *A*, 221.Cw1 and 221.Cw1S88G transfectants were pulsed for 5 min, chased for 15 and 120 min, and lysed by Nonidet P-40. Lysates were divided into three aliquots. The first aliquot was incubated at 4 °C, whereas the remaining two were incubated at 37 °C for 4 h in the presence or absence of a specific Cw1 peptide ligand (NCPERIITL). Each aliquot was immunoprecipitated with the indicated antibodies or in the absence of specific antibody (−) and submitted to SDS-PAGE. *Lanes 29–40* are overexposed to allow the identification of weak bands in some lanes. Non-classical class I heavy chains (*lanes 30* and *36*) and nonspecific (*n.s.*) bands are indicated. *B*, densitometric values of heavy chain bands reactive with F4/326 and W6/32 were obtained in selected lanes of the gels in *A* and in the corresponding lanes of a complete set of replicate experiments. The two sets of values were normalized and averaged. The amounts of conformers (a), (b), and (c) were calculated as described under "Results" by subtracting the densitometric values in the indicated lanes and are displayed as histograms. *Error bars,* ranges of two experiments.

metabolically labeled 221.Cw1 and 221.Cw1S88G at two different, selected (early and late) chase points, preceding (15 min) and following (120 min) the acquisition of thermal stability by wild-type Cw1 heavy chains. Cw1 and Cw1S88G heavy chains were tested for their ability to maintain (at 37 °C) the L31 and F4/326 epitopes in the presence of a known (6) Cw1 ligand (Fig. 4*A*). Under these assay conditions, presence or absence of the *N*-linked glycan, peptide addition, and time of chase had no detectable influence on L31-reactive heavy chains, which were stable at 4 °C (Fig. 4, *lanes 2, 9, 16,* and *23*) but melted at 37 °C in all cases (*lanes 4, 6, 11, 13, 18, 20, 25,* and *27*). More interestingly, F4/326-reactive Cw1 and Cw1S88G heavy chains greatly differed. Wild-type molecules, although thermally unstable (Fig. 4*A*, compare *lanes 3* and *5*), were for the most part peptide-receptive at the early chase point (*lane 7*) and became almost entirely thermally stable at the end of the long chase (compare *lanes 17, 19,* and *21*). This is consistent with an ordered completion of crucial folding and peptide loading steps. Strikingly in contrast, but in agreement with the results shown in Fig. 3, F4/326-reactive Cw1S88G conformers were not only thermally unstable but also peptide-unreceptive at the early chase point (Fig. 4, *lanes 10, 12,* and *14*), and the long chase conferred limited thermal stability, spontaneous as well as peptide-mediated (*lanes 24, 26,* and *28*). When W6/32 was used to probe class I conformation in an identical experiment (Fig. 4, *lanes 29–40*), Cw1S88G was barely detectable at the early chase point (*lane 30*), and its thermal stability (in the presence as well as absence of the specific Cw1 ligand) was even more depressed than in

experiments with F4/326 (compare *lanes 36, 38,* and *40* to *lanes 24, 26,* and *28*).

These results were reproduced in four independent *in vitro* assembly experiments (two with F4/326 and two with W6/32). The densitometric values of selected heavy chain bands in the four experiments were normalized and elaborated (Fig. 4*B*) to assess the accumulation of three F4/326-reactive heavy chain conformers, listed in a decreasing order of thermal stability. Type (c) conformers (Fig. 4*B, black histograms*) are *bona fide* peptide-bound. They correspond to heavy chains displaying spontaneous (no ligand added) stability at 37 °C (Fig. 4*A, lanes 5, 19, 12, 26, 32, 38*). Type (b) conformers (Fig. 4*B, gray histograms*) represent the peptide-receptive fraction of class I molecules. They were estimated indirectly, by subtracting type (c) conformers (above cited lanes) from total heavy chains immunoprecipitated at 37 °C in the presence of a specific, stabilizing ligand (Fig. 4*A, lanes 7, 14, 21, 28, 34,* and *40*). Finally, type (a) conformers (Fig. 4*B, white histograms*) are heavy chains completely refractory to peptide-mediated folding (peptide-unreceptive). They were estimated by subtracting conformers (b) and (c) altogether (Fig. 4*A, lanes 7, 14, 21, 28, 34,* and *40*) from the total heavy chains immunoprecipitated at 4 °C (*lanes 3, 10, 17, 24, 30,* and *36*).

From Fig. 4*B* it is evident that, as compared with wild-type Cw1 heavy chains, newly synthesized Cw1S88G heavy chains were highly enriched in type (a) conformers from the early chase point and then accumulated under the species of all three conformers in approximately equal proportions when reactive with F4/326 and almost exclusively as type (a) conformers when identified by W6/32, an antibody known to be exquisitely conformation-dependent (32). The heterogeneous distribution of two distinct conformational epitopes on non-glycosylated heavy chains is consistent with the generation of abnormal (F4/326$^+$/W6/32$^-$) conformers with mixed (a)/(b)/(c) features.

To compare the thermal stability of Cw1 and Cw1S88G heavy chains expressed on the cell surface, 221.Cw1 and 221.Cw1S88G transfectants were grown at either 37 or 26 °C for 18 h and tested by flow cytometry with L31, F4/326, and W6/32 (Fig. 5). Hypothermia was necessary to slightly and similarly stabilize L31-reactive Cw1 and Cw1S88G (Fig. 5, *C* and *D*), confirming that β_2m-free heavy chains are unfolded regardless of glycosylation. As also expected, wild-type Cw1 displayed thermally stable F4/326 and W6/32 surface epitopes (Fig. 5, *E* and *G*). In contrast, Cw1S88G was unstable, particularly when W6/32-reactive (Fig. 5, *F* and *H*). Estimates of Cw1S88G stability by W6/32 are most likely accurate, because non-classical class I molecules are expected to give minor and similar contributions to the W6/32 pool in 221.Cw1 and 221.Cw1S88G transfectants (see Fig. 1*B*).

DISCUSSION

Disruption of the *N*-glycosylation NXS consensus at position 86–88 of the class I heavy chain has been achieved in previous studies by replacing one or the other conserved residues Asn-86 and Ser-88. The mutants described so far include L^{d}N86K (16, 19), L^{d}N86Q (15), L^{d}S88W (15, 16), D^{d}N86Q (20), D^{d}S88G (21), RT-1A^{a}N86K (23), A2N86M (17), A2S88G (17), A2S88A (35), B7N86G (18), Cw4N86Q, Cw6N86Q, Cw6S88A (36), and
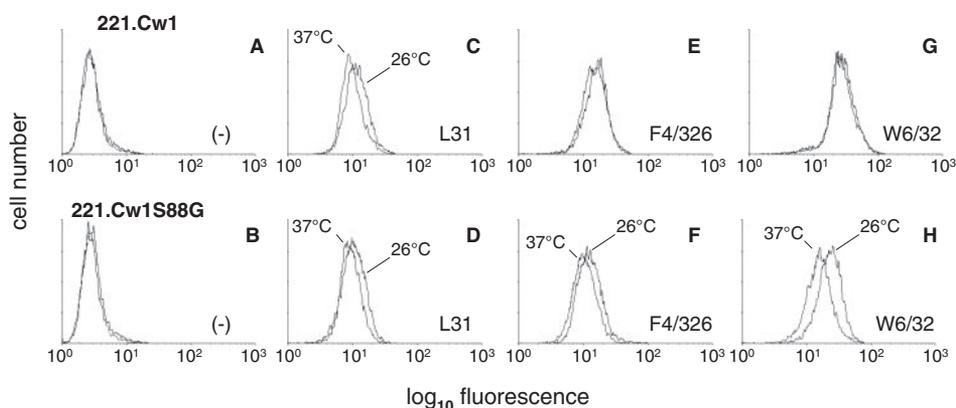
FIGURE 5. **Thermal stability of Cw1 and Cw1S88G heavy chains expressed at the cell surface.** 221.Cw1 and 221.Cw1S88G transfectants (*upper* and *lower panels*, respectively) were grown at either 37 or 26 °C for 18 h and tested by flow cytometry with the indicated antibodies or with an irrelevant antibody (−). Temperatures are indicated where the staining profiles do not coincide.

etB27N86Q (22). Whereas certain amino acid replacements at position 86 (A2N86M and B7N86G) caused an almost complete loss in surface expression, replacements at position 88 moderately and similarly reduced the surface expression of heavy chain mutants, including Cw1S88G (Fig. 1).

The selected S88G mutation was introduced in a class I allele (Cw1) chosen because it shares with other HLA-C alleles poor assembly, slow intracellular transport, and strong association with TAP (Ref. 27, 37, 38 and Fig. 3). In addition, Cw1 binds calreticulin even in the absence of tapasin (Ref. 34 and Fig. 2*B*). These distinctive biochemical features and the tight interaction most likely facilitated the identification of selective perturbations in class I chaperoning and peptide loading, not appreciated in previous studies. Unlike other Asn-86 glycosylation mutants of mouse, rat, or human origin, Cw1 and Cw1S88G were systematically compared upon expression in peptide loading-sufficient 221 cells and peptide loading-deficient 220 cells.

Unique Chaperoning Features of Non-glycosylated Cw1S88G— As shown in Fig. 2, Cw1 binds all the tested class I chaperones in 221 cells but only binds the two major glycosylation-dependent chaperones calnexin and calreticulin in 220 cells. In both cell lines, a lack of the *N*-linked glycan resulted in the redistribution of abnormal (and abundant) heavy chain folds from calreticulin to calnexin, known to bind more immature conformers (Ref. 5 and see Ref. 6). This is in agreement with previous studies demonstrating an increased calnexin binding of mouse L^{d}N86K, L^{d}N86Q, and L^{d}S88W mutants (15, 16), but not with studies on human A2S88A and etB27N86Q mutants in which calnexin binding was found to be weak and conserved (35) or had not been tested (22). Similar to L^d mutants and etB27N86Q (15, 16, 22, 23), Cw1S88G lost its ability to bind calreticulin, ERp57, and tapasin in peptide loading-sufficient cells, but unlike the above mutants (and remarkably) it did retain partial interaction with TAP.

In summary, Cw1 and Cw1S88G both display selective interaction with generic chaperones in peptide loading-defective cells (the former) and with a class I-dedicated chaperone in peptide loading-sufficient cells (the latter). Remarkably, the combination of glycosylation and peptide loading defects (as in 220.Cw1S88G transfectants) results in the most profound chaperoning abnormalities. Altogether, it appears that a lack of glycosylation and a lack of peptide loading have distinct, complementary, and additive effects and that *N*-linked glycosylation is upstream of tapasin-assisted peptide loading, at least in the case of Cw1.

Unique Biochemical Features of Non-glycosylated Cw1S88G Heavy Chains—The poor steady-state accumulation of the glycosylation mutant (Fig. 1) is not due to an early failure to bind β_2m, because β_2m-associated Cw1S88G heavy chains accumulate more than their wild-type counterparts for a long time (45 min) following synthesis (Fig. 3). Rather, the problem appears to be their early decline, beginning before the achievement (at 105 min) of full thermal stability by wild-type Cw1 (Fig. 3). Thus, it appears that folding/β_2m association occurs prematurely and not in synchrony with peptide loading.

In vitro assembly experiments (Fig. 4) are fully consistent with this interpretation, in that HLA-Cw1S88G heavy chains are totally peptide-unreceptive during the physiological time window of peptide loading and mature into a heterogeneous, peculiar mix of class I assemblies, comprising (schematically) three distinct F4/326 conformers. Only one of these (type (c)) was thermally stable under our assay conditions. The remaining two were unstable, *e.g.* peptide-empty/peptide-receptive (type (b)) and peptide-empty/peptide-unreceptive (type (a)), respectively. Interestingly, the relative proportions of the three conformers were shifted toward the deeply aberrant type (a) form, distinctive of glycosylation mutants, when W6/32 was used to probe heavy chain conformation, indicating the presence of a heterogeneous, unprecedented ensemble of misfolded conformers that bear one of two conformational epitopes (F4/326$^+$/W6/32$^-$) and are largely unreceptive or poorly receptive to peptides. These mixed features make a cogent case for dynamic folding-unfolding (1) of glycoproteins during quality control and suggest the accumulation of multiple, abortive heavy chain folds as a result of disparate conformational attempts meeting with limited success. Residual TAP interaction is consistent with Cw1S88G heavy chains being appropriate substrates for folding attempts/retention but inappropriate substrates for efficient peptide loading/stable folding.

The (a-b-c) conformers have not been observed prior to this. However, the glycosylation-defective mutants RT-1A^{a}N86K and etB27N86Q (22, 23) were assembled *in vitro* following bulk isolation from non-ionic detergent cell extracts whereas Cw1S88G was isolated at different, carefully selected chase points, providing the opportunity to observe an initial refractoriness to binding peptides (similar to etB27N86Q) followed by the stabilization of abnormal class I folds (possibly reminiscent of RT-1A^{a}N86K). The present observations may in part reconcile the available, conflicting evidence. The known (39) ability of murine heavy chains to become conformed under extreme conditions (even in the absence of β_2m) may reconcile the remaining differences.
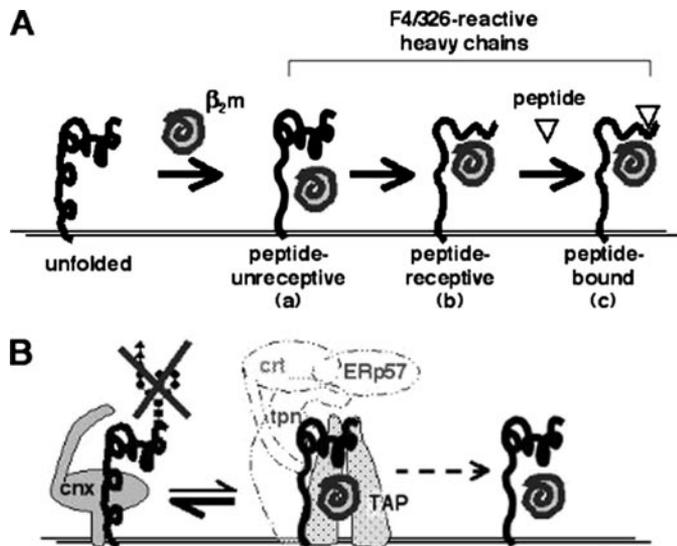
FIGURE 6. **Synopsis of biochemical features and hypothetical chaperoning model of Cw1S88G.** *A*, four different conformers of Cw1 heavy chains are depicted. Unfolded, β_2m-free heavy chains are depicted with unfolded interfaces for both peptide and β_2m. Type (a), (b), and (c) conformers are β_2m-associated intermediates progressively acquiring a folded interface for β_2m and peptide (see "Discussion"). Several observations (see "Discussion") lead us to propose the mechanism of altered Cw1S88G assembly shown in *B*.

The features of the (a-b-c) conformers do not match those of heavy chains synthesized in cells lacking TAP or tapasin, because these are thermally unstable but invariably peptide-receptive (Refs. 25, 26 and extensively referenced in Ref. 27), *e.g.* they behave essentially like (b)-type conformers. Peptide receptivity is also conserved in the T134K HLA-A2 heavy chain, carrying a mutation that impairs TAP and calreticulin binding (11), and in D^b and K^b heavy chains synthesized in calreticulin-defective cells. These heavy chains also retain interaction with TAP, tapasin, and ERp57 (24).

N-Linked Glycosylation and Class I Assembly—In light of the unique features of the (a)-(b)-(c) conformers, we propose that a lack of Asn-86 glycosylation prevents the clearance of a dedicated primary quality control step. In this putative step, an (a)-like intermediate, depicted in Fig. 6*A* as a generic heavy chain fold featuring an at least partially conserved interface for β_2m and an unfolded (and refractory to folding) binding groove, is converted into a (b)-like intermediate with a peptide-receptive interface. Because largely refractory to TAP-assisted peptide loading/folding, (a)-type heavy chains lacking glycosylation are poorly exported from the ER and returned to an exchange-retrieval cycle that involves either calnexin and TAP (Fig. 6*B*) or calnexin and calreticulin (in tapasin-defective cells, not shown in the figure). Failure to clear this step prevents their efficient and timely conversion into (b)-type conformers that represent the obligate intermediates for further conversion into (c)-type molecules, *e.g.* downstream conformational events and productive peptide loading.

Thus, we have shown that primary (glycosylation-dependent) and secondary (peptide loading) quality control events can be resolved and occur in sequence. It follows that class I molecules are able to utilize the integrated functions of the peptide loading complex for the step-by-step achievement of generic and specialized folds. It remains to be determined whether this involves selective engagement with individual chaperones (as in mutant cells lacking functional tapasin) and/or the dynamic exchange of class I conformers between different chaperones within a fully assembled peptide loading complex.

It has been proposed that the secondary quality control of class I molecules has evolved as an adaptation of the calnexin cycle and that modifications of the glycan moiety signal the completion of peptide loading to the members of the peptide loading complex (40). Our results are not in conflict with this proposal. However, the peculiar features of the Cw1 glycosylation-defective mutant show that the Asn-86 glycan of the class I heavy chain enters the scene at an earlier stage.

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