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Competition for Access to the Rat Major Histocompatibility Complex Class I Peptide-loading Complex Reveals Optimization of Peptide Cargo in the Absence of Transporter Associated with Antigen Processing (TAP) Association*

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Major histocompatibility complex (MHC) class I molecules load peptides in the endoplasmic reticulum in a process during which the peptide cargo is normally optimized in favor of stable MHC-peptide interactions. A dynamic multimolecular assembly termed the peptide-loading complex (PLC) participates in this process and is composed of MHC class I molecules, calreticulin, ERp57, and tapasin bound to the transporter associated with antigen processing (TAP) peptide transporter. We have exploited the observation that the rat MHC class I allele RT1-A^a, when expressed in the rat C58 thymoma cell line, effectively competes and prevents the endogenous RT1-A^u molecule from associating with TAP. However, stable RT1-A^u molecules are assembled efficiently in competition with RT1-A^a, demonstrating that cargo optimization can occur in the absence of TAP association. Defined mutants of RT1-A^a, which do not allow formation of the PLC, fail to become thermostable in C58 cells. Wild-type RT1-A^a, which does allow PLC formation, also fails to become thermostable in this cell line, which carries the rat TAPB transporter that supplies peptides incompatible for RT1-A^a binding. Full optimization of RT1-A^a requires the presence of the TAP2A allele, which is capable of supplying suitable peptides. Thus, formation of the PLC alone is not sufficient for optimization of the MHC class I peptide cargo.

Major histocompatibility complex (MHC)[1] class I molecules are composed of two subunits, a heavy chain and a β2-microglobulin (β2m), which fold such that the α1 and α2 domains of the heavy chain form a binding groove capable of accepting short peptides of between 8–10 amino acids in length. These peptides, usually derived by cytosolic proteasomal degradation (1), are translocated into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (2, 3). Peptide-loaded MHC class I molecules traffic to the cell surface where they can be recognized by $CD8^+$ T lymphocytes, potentially leading to the activation of an immune response.

During assembly in the lumen of the ER, MHC class I molecules form the peptide-loading complex (PLC), a multi-molecular complex comprising the MHC class I heavy chain and β2m, the chaperone calreticulin, the oxidoreductase ERp57, the class I-specific accessory molecule tapasin, and the peptide transporter TAP (4). Thus, assembly of MHC class I molecules is a complex process involving multiple interactions with ER-resident chaperones and accessory polypeptides.

Each of the components of the PLC is likely to perform one or more specific roles. Tapasin, for example, not only forms a bridge between the MHC class I molecule and TAP but also stabilizes TAP in an as yet unknown manner (5–7). It is also involved in the process of editing, or optimization of peptides loaded into MHC class I (8). This process leads to high-affinity peptides being loaded onto MHC class I molecules in the ER. Although peptide optimization is thought to be highly dependent upon tapasin for many MHC class I alleles, it is not yet clear if TAP association is a prerequisite for this process to occur.

The oxidoreductase ERp57 may be involved in multiple stages of MHC class I assembly, including the early stages of calnexin association, and later, in the PLC (9–13). As part of the PLC, one of the two thioredoxin-like redox active sites of ERp57 (Cys-Gly-His-Cys) seems to be involved in a disulfide interaction with an unpaired cysteine residue on tapasin (14). The role of the other redox site during MHC class I assembly remains to be established.

Calreticulin and calnexin are lectins which assist in polypeptide folding by binding to *N*-linked glycans, in particular monoglucosylated ($Glc_1Man_{5-9}GlcNAc_2$) structures that exist transiently during assembly in the ER. Both calnexin and calreticulin possess an extended P-domain arm (15, 16), which contains a binding site for ERp57 (17). A quality control mechanism seems to exist in which cycles of folding and disulfide formation or isomerization occur during association of calnexin- or calreticulin-ERp57 complexes, controlled by the addition or removal of the terminal glucose residue on the glycan.

MHC class I molecules contain between one and three *N*-linked glycans, attached to Asn^{86} in the α1 domain, Asn^{176} in the α2 domain, and Asn^{256} in the α3 domain. The number and distribution of these glycans differs between species and sometimes between alleles within species. Human MHC class I molecules contain glycans only on Asn^{86}, whereas for mouse MHC class I molecules, glycans are found on Asn^{86} and Asn^{176}, and additionally in some alleles (for example H-$2L^d$ and H-$2D^b$) on Asn^{256}. Most rat MHC class I molecules possess a different

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[1] The abbreviations used are: MHC, major histocompatibility complex; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; β2m, β2-microglobulin; PLC, peptide-loading complex; IFN, interferon; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate.

distribution of glycans; they are found at Asn^{86} and Asn^{256}. It has not been established whether the role of these glycans in the rat is analogous to those in human and mouse, wherein the Asn^{86}-linked glycan seems to be important for association of calreticulin (18, 19). As part of our ongoing studies into the control of the assembly of rat MHC class I molecules (12, 20, 21), we studied the formation of the rat PLC. We show that formation of the PLC does not lead to the rapid optimization of the peptide cargo of RT1-A^a in C58 cells, most likely as a consequence of improper peptide supply. Conversely, utilizing competition between RT1-A^a and -A^u for TAP binding in these cells (22), we also show that RT1-A^u can optimize its peptide cargo in the absence of TAP association.

MATERIALS AND METHODS

Cell Lines and Antibodies—The C58(NT)D rat thymic lymphoma cell line (23) was cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal calf serum (Invitrogen). Transfectants were generated by electroporation at 975 μF and 160–180 V, followed by selection in medium containing 1 mg/ml G418 (PAA Laboratories). The previously generated line C58.A^a.TAP2A clone B5 (22, 24) was cultured in medium containing G418 and HAZA (Sigma). Human 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfection of 293 cells was performed with Fugene 6 (Roche Applied Science) using 4 μl of Fugene and 2 μg of plasmid DNA per well of a six-well plate. Cells were stimulated with 200 units/ml human IFN-γ (Sigma) as required.

Monoclonal antibody (mAb) MAC30 recognizes RT1-A^a, and mAbs NR5/10 and GN7/5.B11 recognize RT1-A^u. mAb W6/32 recognizes HLA class I molecules. Rabbit antiserum 116 recognizes rat TAP2. Anti-calreticulin (SPA600) was purchased from Stressgen. Anti-murine tapasin antiserum was a gift from Ted Hansen (Washington Univ. School of Medicine, St. Louis, MO). Anti-human tapasin antiserum was a gift from Tim Elliott (University of Southampton, Southampton, UK). Anti-ERp57 antiserum was a gift from Neil Bulleid (University of Manchester, Manchester, UK).

PCR-mediated Site-directed Mutagenesis—Wild-type RT1-A^a was subcloned into the pCR3 mammalian expression vector (Invitrogen) by excision of the full-length cDNA with EcoR1 from the pHBneo vector (25), and orientation was confirmed by sequencing. Site-directed mutagenesis was performed with QuikChange methodology (Stratagene) using the following primers (mutated position underlined, but complementary sequences not listed): N86K 5′-CGCGGCTACTACAAGCAGAGCGAGGGC-3′; N256K 5′-GGGAAGGAGCAGAAGTACACATGCCGT-3′; B27.H114D 5′-CTCCGCGGGTACGACCAGGACGCCTACGACGGC-3′. The double mutant N86K/N256K was created in a second round of mutagenesis of the N86K mutant. All mutants were verified by sequencing of the full-length cDNA insert.

FACS Analysis—Cells were stained with 50 μl of relevant tissue culture supernatant for 30 min at 4 °C, then washed three times in PFN (phosphate-buffered saline, 2% fetal calf serum, 0.1% sodium azide), stained with FITC conjugated anti-mouse IgG (Sigma), washed three more times and fixed with 1% formaldehyde in phosphate-buffered saline. Staining of HLA-B27 on 293 cells was performed directly with FITC-coupled anti-HLA-B27 (BD Biosciences). Analysis was performed using CellQuest software on a FACSCalibur flow cytometer (BD Biosciences).

Immunoprecipitations and Western Blotting—For metabolic-labeling experiments, cells were preincubated in methionine and cysteine-free RPMI 1640 medium (Sigma) for 30 min and then labeled for 15 min with 7.2 MBq Trans-label (ICN). Cells were then resuspended in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide) containing 1% digitonin (Sigma) or 1% Nonidet P-40 (Sigma), as required. Nuclei and insoluble material were removed by centrifugation at 20,000 × g. For peptide stabilization assays, lysates were supplemented with 5 μM or 50 μM of the RT1-A^a binding peptide SMFPVSENR (26), incubated for 1 h on ice, heated at 37 °C for 1 h, and followed by immunoprecipitation with MAC30. Thermostability assays were performed based on the protocol of Williams *et al.* (8). Lysates of metabolically labeled cells were incubated at the indicated temperatures for 30 min, followed by immunoprecipitation with MAC30 or GN7/5.B11.

For immunoprecipitations of the PLC, aliquots of digitonin lysates of metabolically labeled cells were immunoprecipitated with antisera to calreticulin, tapasin, and TAP. Control lysates were immunoprecipitated with MAC30 and NR5/10.

Pulse-chase analysis was performed on metabolically labeled cells returned to culture at 37 °C. Samples were removed, lysed at the indicated times of chase, and immunoprecipitated with MAC30 or GN7/5.B11. Immunoprecipitates were then digested with 5 milli-units of endoglycosidase H (Roche Applied Science) for 1 h at 37 °C.

Western blotting of *N*-ethylmaleimide-treated lysates of 293 cells was performed as described previously (27).

RESULTS

Expression of RT1-A^a and N-linked Glycosylation-site Mutants in C58 Cells—Using site-directed mutagenesis, we constructed cDNAs of RT1-A^a devoid of its Asn^{86} and Asn^{256} glycosylation sites by mutation of the relevant Asn residues to Lys (Fig. 1*a*). Rat C58 thymoma cells were transfected with the mutated constructs lacking Asn^{86} (denoted N86K), Asn^{256} (N256K), and the double-mutant (N86K/N256K). FACS analysis with MAC30 confirmed that all three mutants were expressed at the cell surface (Fig. 1*b*) and at levels similar to wild-type RT1-A^a. To further verify that each mutant retained the ability to form a peptide-binding structure, we incubated Nonidet P-40 lysates of metabolically radiolabeled cells with or without the specific RT1-A^a binding peptide SMFPVSENR. Lysates were then heated at 37 °C to induce dissociation of molecules devoid of high-affinity peptides, and then followed by immunoprecipitation with MAC30. All three mutants of RT1-A^a were stabilized by the presence of peptide, indicating correct folding of the MHC class I molecule by the criterion of peptide-receptiveness (Fig. 1*c*).

Immunoprecipitation of the RT1-A^a mutants also revealed a small difference in the relative molecular mass of the N86K and N256K products (Fig. 1*d*), with mutant N256K resolving with slightly higher mobility than N86K. Digestions with endo H and N-glycosidase F, the latter to remove completely the glycan unit, suggested that the difference was due to glycosylation (not shown). This supposition was further supported by pulse-chase experiments in which the molecular mass change occurred at the onset of glycan maturation (Fig. 2*d*). Taken together, these data suggest that the *N*-linked glycans situated on the α1 and α3 domains are subjected to differential glycan modification.

Glycosylation at Asn^{86} Controls Association of RT1-A^a with the Peptide-loading Complex—Next, we determined whether the removal of glycosylation sites from RT1-A^a disrupted the formation of the PLC. Digitonin lysates of radiolabeled N86K and N256K cells were immunoprecipitated with antibodies recognizing calreticulin, tapasin, and TAP. In addition, to distinguish the association of mutant RT1-A^a molecules from that of the endogenous RT1-A^u class I allele expressed in C58 cells, lysates were also immunoprecipitated with antibodies specifically recognizing RT1-A^a (MAC30) and RT1-A^u (NR5/10). RT1-A^u can be resolved from RT1-A^a by its slightly different gel mobility. Fig. 2*a* shows the region of the gel containing the MHC class I heavy chains. Removal of the α1 domain glycan (N86K) abolished the association of RT1-A^a with the PLC, in contrast to the removal of the α3 domain glycan (N256K), which still allowed PLC formation (Fig. 2*a*). The association of RT1-A^u in the N86K line can be seen very faintly in this image, but can readily be detected upon longer exposure of the autoradiograph. We conclude, therefore, that it is the α1 domain glycan of RT1-A^a which is crucial for the formation of the PLC.

The absence of PLC interactions has been linked to a failure in quality control and peptide cargo optimization of some MHC class I molecules (28). MHC class I molecules which have been optimized and contain high-affinity peptides are more resistant to heat treatment (8). Therefore, we tested the thermostability of the mutant RT1-A^a molecules by heating lysates of radiolabeled cells at 37 °C and 42 °C for 30 min, followed by immunoprecipitation with MAC30. However, both the wild-type and the mutant RT1-A^a molecules displayed poor thermostability (not shown). We reasoned that this result may have occurred because of the

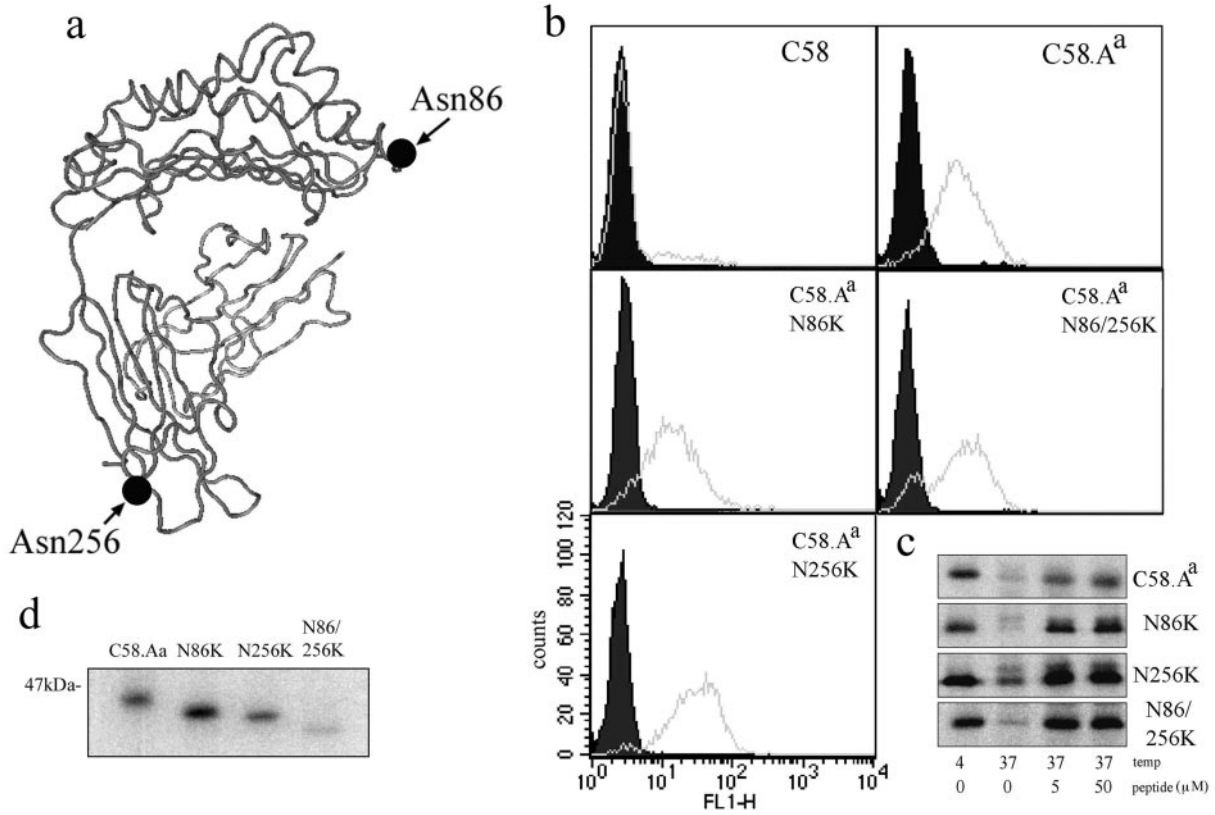

FIG. 1. **Expression of RT1-A^a glycosylation site mutants in C58 cells.** *a*, location of *N*-linked glycosylation sites in the α1 and α3 domains of RT1-A^a. Asn^{86} and Asn^{256} are identified with *black dots*. The RT1-A^a structure (Protein Data Bank accession number 1ED3; Ref. 45) was analyzed using Cn3D software. *b*, C58 cells transfected with wild-type RT1-A^a, mutants N86K and N256K, and the double-mutant N86K/N256K cDNAs were immunostained with MAC30 (*gray line*). Staining with second-stage FITC anti-mouse IgG alone is shown in *solid black*. All mutants are expressed at the cell surface. *c*, peptide stabilization in detergent lysates. Radiolabeled detergent lysates were supplemented with the indicated amount of RT1-A^a binding peptide SMFPVSENR, heated at 37 °C, and immunoprecipitation with MAC30 followed. All mutants retained the capacity to bind and be stabilized by peptide. *d*, MAC30 immunoprecipitation of mutant RT1-A^a molecules indicates higher SDS-PAGE mobility of N256K molecules compared with N86K.

inability of the TAPB allele of the rat TAP transporter expressed in C58 cells to supply suitable peptides, which for RT1-A^a would normally require the presence of the rat TAPA allele (24, 29). Therefore, we repeated the thermostability experiment, but this time, we included cells co-expressing RT1-A^a and the rat TAP2A allele (which in combination with rat TAP1 forms the TAPA transporter). Poor thermostability of RT1-A^a was again observed, except in the presence of the TAP2A allele, which displayed good thermostability even after treatment at 42 °C (Fig. 2*b*). We next tested if optimization increased with the passage of time. Wild-type and mutant N256K displayed some limited acquisition of thermostability after a 1 h chase, whereas the N86K mutant failed to become thermostable, indicating that formation of the PLC can have some effect on RT1-A^a in the context of TAPB (Fig. 2*c*). However, our data clearly demonstrate that the rapid acquisition of thermostability is dependent upon the presence of an adequate supply of relevant peptides suitable for MHC class I binding, and that formation of the PLC alone is not enough to ensure optimization.

Mutations which affect the formation of the PLC often influence the kinetics of MHC class I molecule export from the ER to the cell surface (28). Therefore, we performed pulse-chase analysis to investigate the export of the RT1-A^a mutants. Wild-type C58.A^a and mutant N86K, N256K, N86K/N256K cells were pulse-radiolabeled and subsequently lysed in detergent buffer after 30, 60, and 120 min of chase in ordinary medium. MAC30 immunoprecipitates of the resulting lysates were digested with endoglycosidase H to determine the acquisition of resistance, which acts as a readout of exit from the ER. As shown in Fig. 2*d*, N86K cells displayed more rapid exit of RT1-A^a from the ER in comparison to wild-type and N256K cells, with full acquisition of endo H resistance by 120-min chase. This phenotype of more rapid exit from the ER is in keeping with other mutations in class I molecules, such as T134K, which fail to form interactions with the PLC (28, 30). With no glycans present in the N86K/N256K mutant, no maturation was observed.

RT1-A^u Achieves Optimization in the Absence of TAP Association—It has been reported previously that expression of RT1-A^a in C58 cells results in retention of a pool of RT1-A^a molecules in the ER and accumulation in association with the TAPB transporter allele expressed by this cell line (22). The net effect is to prevent RT1-A^u molecules from associating detectably with TAP, an observation that we confirm in Fig. 3*a* by immunoprecipitation of TAP from digitonin lysates of C58, C58.A^a, and C58.A^a.N86K cells. In the latter cells, the inability of N86K to form a PLC allows the restoration of RT1-A^u association with TAP.

We decided, therefore, to determine whether preventing the association of RT1-A^u with TAP resulted in defects in its ability to optimize peptide cargo. N86K cells provided a control cell line in which RT1-A^a is present, but as a non-TAP-competing variant.

FACS analysis of the three cell lines revealed similar expression levels of RT1-A^u on the cell surface (Fig. 3*b*). In addition, the pulse-chase kinetics of ER-exit of RT1-A^u was not significantly altered in the three lines (Fig. 3*c*). RT1-A^u molecules from all three lines were similarly thermostable after the initial labeling period, matching the data for RT1-A^a in the presence of TAP2A (Fig. 3*d*). We conclude from this data that RT1-A^u is capable of loading and optimizing its peptide cargo despite being prevented from associating with TAP.

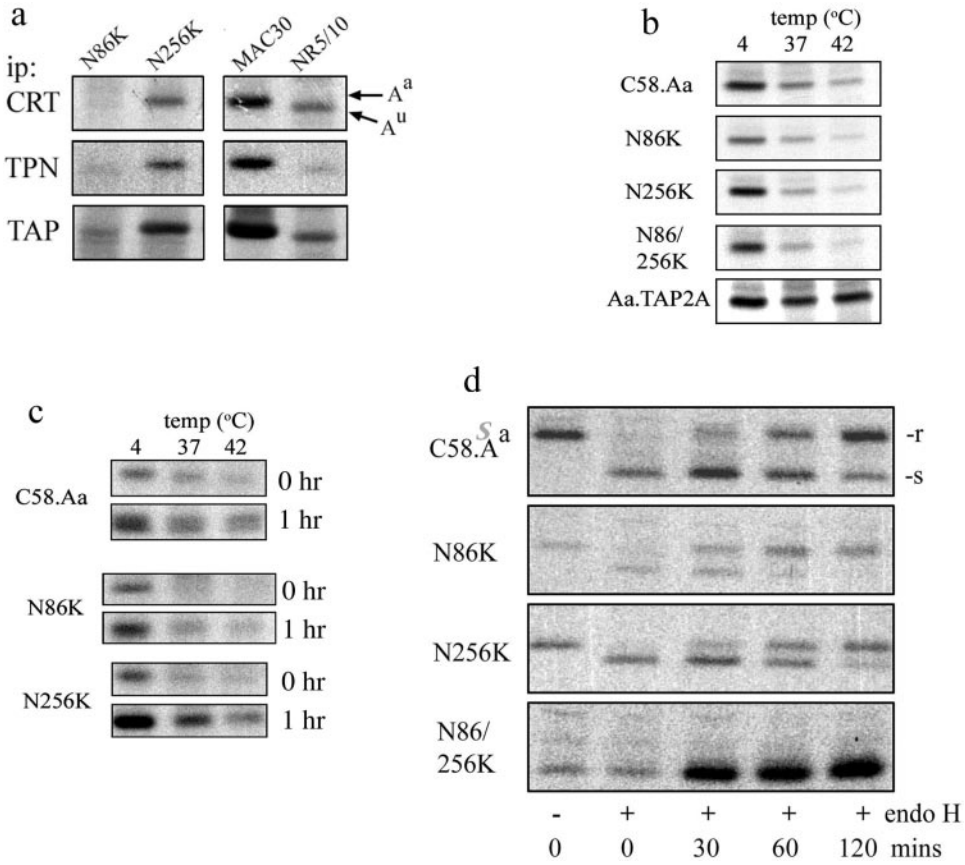

FIG. 2. **Formation of the peptide-loading complex and thermostability of RT1-A^a mutants.** *a*, digitonin lysates of radiolabeled C58.A^a.N86K and C58.A^a.N256K cells were immunoprecipitated with antisera to calreticulin (*CRT*), tapasin (*TPN*), and TAP (*left panel*). Control lysates were immunoprecipitated with anti-RT1-A^a mAb MAC30 or anti-RT1-A^u mAb NR5/10 (*right panel*). N86K failed to form an interaction with the PLC. *b*, radiolabeled lysates of the mutants cells and C58.A^a.TAP2A cells were heated at the indicated temperatures and then immunoprecipitated with MAC30. RT1-A^a displays thermostability only in the presence of TAP2A. *c*, the experiment shown in *b* was repeated with samples analyzed for thermostability immediately after labeling (0 h) and after a chase period (1 h). *d*, pulse-chase phenotype of mutant RT1-A^a molecules. Mutant N86K displayed a more rapid ER exit than wild-type or N256K molecules. Endo H resistant (*r*) and endo H sensitive (*s*) molecules are indicated. N86K/N256K molecules displayed no acquisition of endo-H resistance, consistent with their expected glycan-free status.

We next determined whether RT1-A^u represented a tapasin-independent allele, which may explain its ability to optimize in competition with RT1-A^a. Both RT1-A^u and -A^a possess Arg^{114} and Asp^{116} residues in the F-pocket region of the peptide-binding groove, a pattern similar to that of HLA-B*2705, which permits a tapasin-independent phenotype (31). Repeated attempts to transfect the tapasin-deficient cell line .220 with RT1-A^u and -A^a were unsuccessful. Therefore, we made use of the human cell line 293 which expresses little, if any, tapasin in the absence of stimulation with IFN-γ. As shown in Fig. 4*a*, tapasin and the tapasin-ERp57 disulfide-linked complex (14, 27) were only detected by Western blot after IFN-γ stimulation. FACS analysis with W6/32 showed that IFN-γ treatment increased endogenous MHC class I expression. To test the system, we transiently expressed wild-type HLA-B*2705 (tapasin-independent) and also a mutant of B*2705, in which residue His^{114} had been altered to Asp^{114} (denoted H114D) which, based on the data of Park *et al.* (31), should induce a tapasin-dependent phenotype into B27. Wild-type B*2705 was expressed on the cell surface of unstimulated 293 cells, with up-regulation in the presence of IFN-γ (Fig. 4*b*). In marked contrast, B27.H114D was only detected at the cell surface after IFN-γ treatment. Expression of RT1-A^u and -A^a constructs at the cell surface in the presence and absence of IFN-γ treatment suggested that both behave as tapasin-independent alleles (Fig. 4*c*).

DISCUSSION

Removal of the α1 domain glycan located at Asn^{86} prevents the association of class I molecules, such as human HLA-B27 (32) and mouse H2-L^d (33), with the PLC. In contrast, removal of Asn^{176} in the α2 domain of L^d, leaves the PLC intact (18), demonstrating that Asn^{86} bears the crucial glycan unit. Our data now extend this observation to a rat MHC class I molecule, with the observation that removal of Asn^{86} from RT1-A^a prevents formation of the PLC. The function of this glycan is most likely to provide a site of interaction for calreticulin. In the absence of Asn^{86}, calreticulin is not found in contact with MHC class I molecules, whereas, in the absence of tapasin, calreticulin can still associate with MHC class I (19). This latter observation suggests that calreticulin plays a critical role in the formation of the normal PLC. However, it has also been reported that tapasin, ERp57, and MHC class I heavy chain can be found associated with TAP in calreticulin-deficient cells (34), which may argue against such a role. A possible explanation for this apparent discrepancy may reside in the observation that tapasin forms a disulfide-bonded partnership with ERp57 (14). In β2m-deficient Daudi cells, tapasin and ERp57 can be detected in a disulfide-conjugate (27). ERp57 and tapasin can also be found in association with TAP in Daudi cells (35). Although unfolded MHC class I molecules cannot associate with this tapasin-ERp57-TAP complex in Daudi cells, partially folded MHC class I/B2m molecules may be able to form this association in calreticulin-deficient cells. Nevertheless, such complexes in calreticulin-deficient cells do not complete normal assembly and are loaded with a suboptimal peptide cargo, indicating that calreticulin is indeed an important partner in the PLC (34).

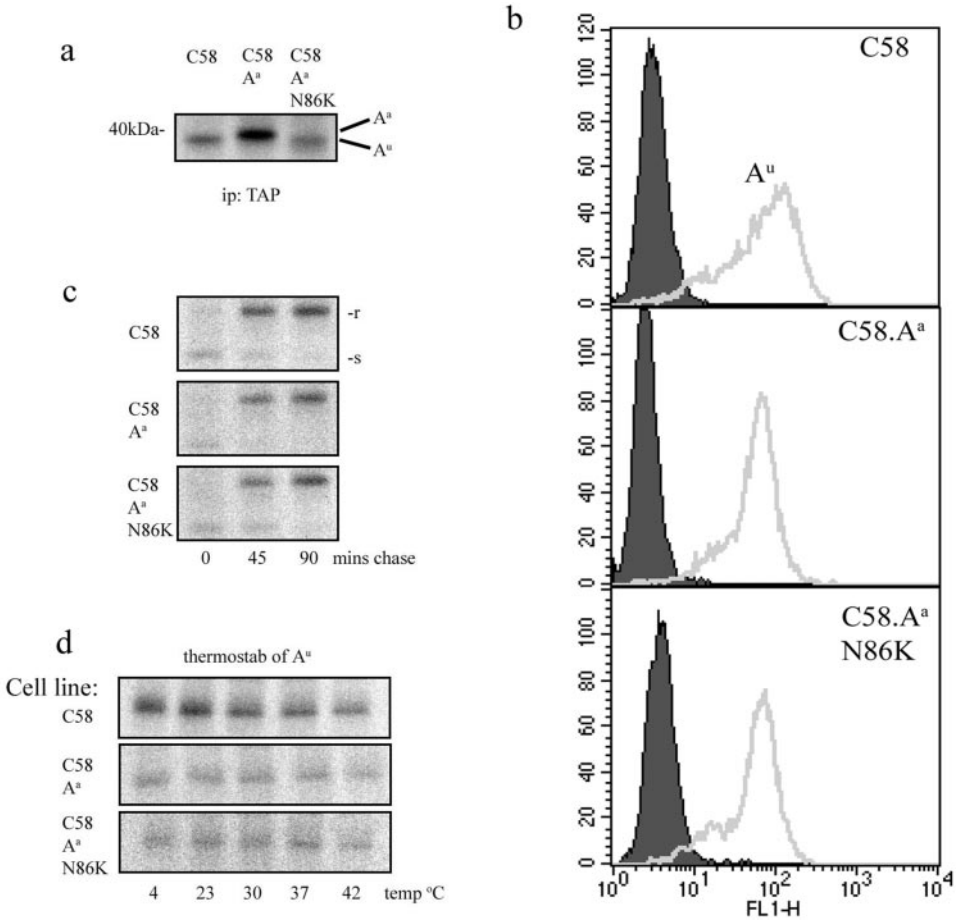

FIG. 3. **Competition for TAP association and cell surface expression of RT1-A^u.** *a*, digitonin lysates of radiolabeled C58, C58.A^a, and C58.A^a.N86K cells were lysed and immunoprecipitated for TAP. The gel shows only the MHC class I heavy chain region. *b*, the same cells as in *a* were immunostained with anti-RT1-A^u mAb NR5/10 (*gray line*). Second-stage FITC-labeled reagent alone is shown in *solid black*. *c*, C58, C58.A^a, and C58.A^a.N86K cells were pulse-labeled, and samples were lysed and immunoprecipitated with anti-RT1-A^u mAb GN7/5.B11 at the indicated times. Samples were also endo H digested. *d*, the same cells were radiolabeled, and lysates were heat-treated at the indicated temperatures for 30 mins before immunoprecipitation with GN7/5.B11.

Clear roles for the glycans situated on Asn^{176} or Asn^{256} of MHC class I molecules have not been elucidated. In the case of mouse MHC class I bearing Asn^{176} glycans, it is possible that this site permits the co-operative binding of calnexin, which has similar lectin specificity to calreticulin. Indeed, the engineering of an Asn^{176} site into a human class I allele resulted in increased calnexin association (36). However, removal of Asn^{176} from mouse L^d did not prevent calnexin binding (18). Evidence for a role for glycans at Asn^{256} is also rare. H2-D^b molecules may require this glycan to permit stable assembly and cell surface expression in the absence of B2m (37). There is currently no evidence in the rat that a similar situation may occur for RT1-A^a or any other rat allele. However, our data do indicate that the processing MHC class I glycans received during assembly in the ER and/or transit to the cell surface differs for the α1 and α3 domain glycans, confirming data previously observed that compared the three glycan sites on H-2D^k (38). The difference observed in electrophoretic mobility (Figs. 1*d* and 2*d*) was also detectable in transient transfections of HeLa cells (not shown) and is, therefore, not a specific effect of expression in C58 thymoma cells. The physical location of the two different glycans may permit easier access to Asn^{86} compared with Asn^{256} (the latter being juxtaposed to the cell surface, as is evident from Fig. 1*a*), with the difference, therefore, representing steric hindrance to the accessibility of glycan-modifying enzymes.

The emerging picture of the PLC is that of a complex which has evolved to perform the optimization of peptide cargo, leading to the production of stable cell surface MHC class I molecules. By expressing glycosylation-site mutants of RT1-A^a in the C58 cell line, we have been able to separate the formation of the PLC from its ability to perform peptide optimization. C58 cells express the rat TAPB transporter allele, which is highly inefficient in supplying suitable peptides for the loading and assembly of RT1-A^a. This mismatching of rat TAP allele and rat MHC class I is known as the class I modification (cim) phenomenon and is due to functional polymorphism in rat TAP2 (24, 39–42). TAP complexes which contain the rat TAP2A allele (and are therefore called TAPA) transport a wider range of peptides than TAP2B containing complexes (TAPB complexes) (43) including, crucially, a pool of peptides with arginine at the COOH terminus, which is a preferred anchor residue of RT1-A^a (29). In the results described here, wild-type RT1-A^a and its N256K mutant formed a PLC, whereas N86K did not. However, even in the presence of the PLC, heat treatment of lysates revealed suboptimal peptide loading, with only partial recovery over time. Full, rapid optimization was only achieved in the additional presence of the TAP2A allele. This is a useful confirmation of the model that optimal stabilization of a class I molecule requires both the correct formation of the PLC and the presence of a suitable pool of peptides for loading.

Williams *et al.* (8) recently studied the optimization of various HLA class I alleles in .220 and tapasin-restored .220 cells,

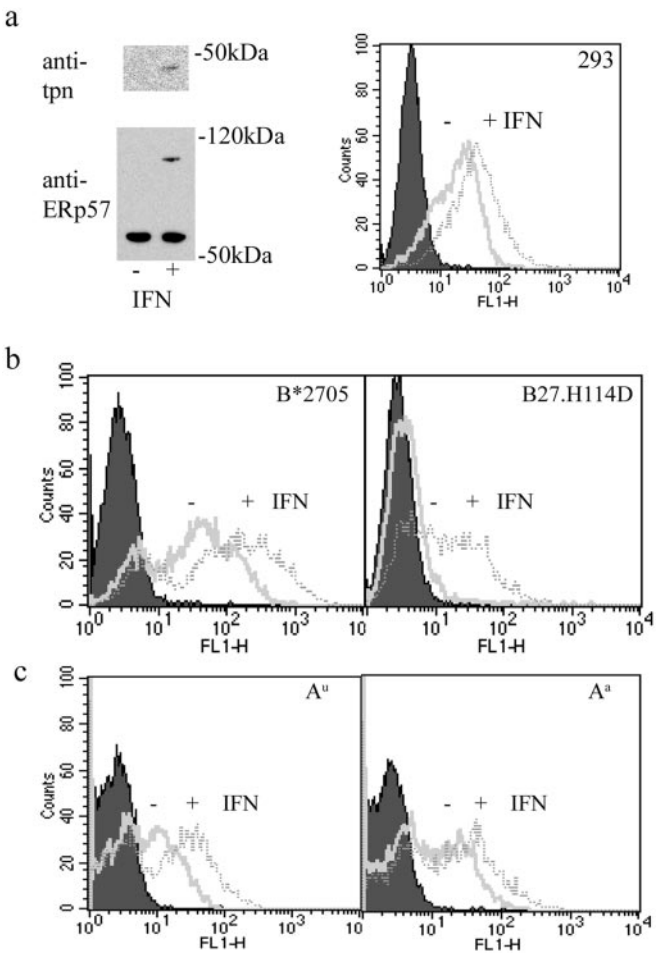

FIG. 4. **Tapasin-independent phenotype of RT1-A^a and A^u.** *a*, reduced lysates of 293 cells treated with or without IFN-γ were probed with anti-human tapasin antiserum (*top panel*). Non-reduced *N*-ethylmaleimide-treated lysates were probed with anti-ERp57 antiserum (*bottom panel*). FACS staining of cells incubated in the absence (−) or presence (+) of IFN-γ was performed with W6/32. *b*, 293 cells transiently transfected with HLA-B*2705 or B27.H114D plasmids were immunostained with FITC anti-B27. Treatment with or without IFN-γ is indicated. *c*, 293 cells transiently transfected with RT1-A^u or -A^a were immunostained with NR5/10 and MAC30, respectively. Treatment with or without IFN-γ is indicated.

noting that, for some alleles such as B2705, there may be a route of optimization without the need for TAP interaction. We have been able to address this question directly utilizing the ER competition for TAP association between RT1-A^a and RT1-A^u (22). Our data indicate that loading and rapid optimization of RT1-A^u molecules can occur without TAP association. It remains to be determined in more detail what the relative dependence on tapasin may be for both RT1-A^a and -A^u alleles. We describe here a useful model system using 293 cells, in which tapasin expression is very low in the absence of IFN-γ stimulation. Such a system may prove useful for rapid screening of mutants for tapasin-dependent expression. However, it is possible that there may be subtle differences in the tapasin-MHC class I interaction between human and rodent. For example, expression of mouse tapasin in .220 cells fails to result in peptide cargo optimization of the tapasin-dependent HLA class I allele B*4402 (44). Co-expression of rat tapasin with various RT1-A alleles in a suitable host cell line will be required to address this issue fully.

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