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The Murine Liver-Specific Nonclassical MHC Class I Molecule Q10 Binds a Classical Peptide Repertoire

Francesca Zappacosta, Piotr Tabaczewski, Kenneth C. Parker, John E. Coligan, and Iwona Stroynowski

The biological properties of the nonclassical class I MHC molecules secreted into blood and tissue fluids are not currently understood. To address this issue, we studied the murine Q10 molecule, one of the most abundant, soluble class Ib molecules. Mass spectrometry analyses of hybrid Q10 polypeptides revealed that α1α2 domains of Q10 associate with 8–9 long peptides similar to the classical class I MHC ligands. Several of the sequenced peptides matched intracellularly synthesized murine proteins. This finding and the observation that the Q10 hybrid assembly is TAP2-dependent supports the notion that Q10 groove is loaded by the classical class I Ag presentation pathway. Peptides eluted from Q10 displayed a binding motif typical of H-2K, D, and L ligands. They carried conserved residues at P2 (Gly), P6 (Leu), and P9 (Phe/Leu). The role of these residues as anchors/auxiliary anchors was confirmed by Ala substitution experiments. The Q10 peptide repertoire was heterogeneous, with 75% of the groove occupied by a multitude of diverse peptides; however, 25% of the molecules bound a single peptide identical to a region of a TCR V β-chain. Since this peptide did not display enhanced binding affinity for Q10 nor does its origin and sequence suggest that it is functionally significant, we propose that the nonclassical class I groove of Q10 resembles H-2K, D, and L grooves more than the highly specialized clefs of nonclassical class I Ags such as Qa-1, HLA-E, and M3.

Molecular and biochemical analyses of class I MHC molecules led to the identification of two major subgroups of these proteins. The classical class I (class Ia) Ags are highly polymorphic, nearly ubiquitously expressed polypeptides that associate with self- and nonself 8–10 residue-long peptides (1, 2). They play key roles in T cell and NK cell-mediated elimination of virally infected and/or malignant transformed cells. The nonclassical class I (class Ib) Ags are a heterogeneous group of β2m-microglobulin (β2m)-associated proteins that display little polymorphism and frequently exhibit low level expression and/or unique tissue distributions (3, 4). Furthermore, many of the class Ib molecules exist in soluble forms that are secreted into the serum and body fluids (5, 6).

Recent studies of rodent and human members of class Ib families revealed remarkable diversity of their ligands, Ag-presenting capacities, and immune as well as nonimmune functions (7–12). Some of the membrane-bound class Ib proteins are dedicated to presentation of structurally unique forms of ligands. For example, M3 Ag widely expressed on murine tissues, binds selectively N-formylated peptides of mostly prokaryotic origin (13). This property allows M3 to be recognized as a restriction element during CD8+ T cell-mediated clearance of bacterial infections. Another ubiquitously expressed murine Ag, Qa-1, as well as its proposed human homologue HLA-E, associate preferentially with a limited set of hydrophobic leader peptides from class I MHC Ags (14, 15). The resulting class Ib complexes serve as targets for alloreactive cytotoxic T cells, as shown for Qa-1 (16), and as recognition elements for NK receptors (17–19).

Not all of the known class Ib proteins bind structurally unique ligands. Some, such as murine Qa-2 and human HLA-G, associate with diverse repertoires of peptides reminiscent of class Ia peptides (20, 21). The biological significance of these types of classes Ib complexes is still poorly understood (22). Additionally, very little is currently known about Ag-presenting properties or function(s) of soluble class Ia or Ib molecules reported to exist in a wide range of species, including mouse (23) and human (24, 25).

To address these issues, we performed analysis of ligands associated with the soluble Q10 class Ib protein. This 38- to 40-kDa β2m-associated molecule is detectable in serum as a multivalent complex of 200–300 kDa, at concentrations ranging from 20 to 60 μg/ml, depending on the mouse strain (26, 27). The Q10 proteins are encoded in the Q region of the H-2 complex, which also contains Qa-2 genes (3), and a cluster of several other class Ib sequences. In common with other Q region class Ib genes, Q10 shows >80% homology with the classical H-2K, D, and L loci (28). Structurally, the protein is truncated at the C terminus and

Abbreviations used in this paper: β2m, β2-microglobulin; GPI, glycosylphosphatidylinositol; CAD, collision-activated dissociation; MS, mass spectrometry; PI-PLC, phosphatidylinositol-specific phospholipase C; ESI, electrospray ionization; m/z, mass:charge ratio.

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carries several substitutions in the hydrophobic region correspond-
ing to the transmembrane segments of class Ia heavy chains. These
features account for the inability of Q10 to insert into the plasma
membrane and explain why Q10 is secreted (26, 28).

The Q10 locus exhibits two hallmarks of class I b genes: it is
well conserved, with >99.4% homology between different se-
quenced alleles (28), and it is expressed in tissue-specific fashion.

In adult mice, the protein is synthesized mainly by liver and, in
trace amounts, by kidney and stomach (26, 29). During early de-
velopment, Q10 transcripts are detectable in major organs of fetal
hematopoiesis: visceral yolk sac and fetal liver (30). This expres-
sion pattern led to the speculation that Q10 participates in the
induction of T cell tolerance and/or regulation of embryonic
hematopoiesis. We demonstrate here that the peptide-binding (α1α2)
domains of Q10 associate with eight and nine residue-long self-
peptides similar to the class Ia ligands and discuss this finding in
the context of potential T cell and NK cell recognition.

Materials and Methods

Cloning of Q10 cDNA and construction of hybrid genes

The nonpolymorphic Q10 cDNA was isolated from the NODLt1 (H-2r)
cDNA library derived by Girgis et al. (31). The cDNA fragment
encoding the N-terminal portion of Q10 (exons 1–3) was amplified by
PCR, subcloned into pC20H plasmid (American Type Culture Collection
(ATCC), Manassas, VA), and sequenced. It was found to be identical to the
genomic sequence of Q10 from C3H mouse (32) and cDNAs amplified from
C57BL/6 and C57BL/10 mice (data not shown).

We designed two hybrid Q10/Q7s molecules. The first, MQ10, en-
codes the N-terminal portion of Q10 (leader peptide, a1 and a2) and the
C-terminal portion of Qa-2 (α3 and the glycosylphosphatidylinositol (GPI)
moiety linking Qa-2 product to the cell surface). MQ10 is membrane
bound. The second hybrid molecule, SQ10, consists of the same N-terminal
domains of Q10 linked to the α3 domain of the soluble form of Qa-2,
followed by six additional histidines (6xHis-tag), and is secreted. The C-
terminal domains of MQ10 and SQ10 were derived from different isoforms
of Qa-2 genes, Q9m and Q7s, respectively (5).

The following pair of primers were used to amplify hybrid Q2 mole-
cules: for the α1α2 region of Q10, (P1) 5'-AACCGGTGACGATC
CCAGATGGGGGCGATGCCG-3' (signal peptide sequence in bold,
SulI site underlined), and (P2) 5'-AACCCGAGTGGCAGCACG
CAGGCTCT3' (C-terminal part of α2 domain in bold, BglI site underlined);
for the a3 region of Q9m, (P3) 5'-GCGCACGATCT
CCCCAAAAGGCACATGTGACCCATC-3' (Q9m a3 N-terminal region
in bold, BamHI site underlined), and (P4) 5'-CTGGCAGTCCTGAG
CATGCTGGAGTGGAACGATCCCC3' (Q9m C terminus in bold, EcoRI site underlined).
The Q9m and Q7s fragments were cloned into plasmid pIC20H and the Q10
transcripts are detectable in major organs of fetal development, Q10 transcripts are detectable in major organs of fetal
hematopoiesis led to the speculation that Q10 participates in the
induction of T cell tolerance and/or regulation of embryonic
hematopoiesis. We demonstrate here that the peptide-binding (α1α2)
domains of Q10 associate with eight and nine residue-long self-
peptides similar to the class Ia ligands and discuss this finding in
the context of potential T cell and NK cell recognition.

Flow cytometry

Cells from subconfluent cultures were stained by indirect immunofluores-
cence using FITC-conjugated goat-anti mouse IgG as the secondary Ab
(Cappel, Durham, NC). The acquisition was performed by FACSscan (Be-
ton Dickion, Mountain View, CA). Data were analyzed with the Lysis
program (Becton Dickinson). Dead cells were excluded by a combination
of gates set on forward/side scatter and by exclusion of cells staining
positive with propidium iodide dye.

Radiolabeling and immunoprecipitation

Radiolabeling and immunoprecipitations were conducted by a modification
of a standard method described previously (5, 36). Briefly, RNA transfect-
tants (103 cells) were harvested at the logarithmic phase of growth (8 ×
105/ml), washed twice in ice-cold PBS, and resuspended in labeling me-
dium: 1 ml of methionine/cysteine-deficient RPMI 1640 medium (ICN
Pharmaceuticals, Costa Mesa, CA) supplemented with 10% dialyzed FBS and
0.5 mM of [35S]methionine and cysteine (Trans [35S]-label, ICN Phar-
maceuticals). For phosphatidylinositol-specific phospholipase C (PI-PLC)
treatment, tissue culture media were supplemented with 0.3 U of PI-PLC
(American Radiolabeled Chemicals, St. Louis, MO). After incubation for
4 h at 37°C, cell supernatants were harvested and precleared with 50 µl of
normal rabbit serum. Recombinant Q10 and control proteins were precip-
itated with saturated amounts of mAb 46 Ab. Ag-mAb 46 complexes were
identifiable by the silver staining method (Sigma-Cell, St. Louis, MO), washed six times with PBS,
denatured, reduced, and analyzed by one-dimensional SDS-PAGE. Gels
were stained with Coomassie blue. Radioactively labeled proteins were
detected by autoradiography.

Measurement of expression levels and stability of Q10 molecules by ELISA

RMA, RMA-S cells, and their transfectants were grown to a density of 8 ×
105 cells/ml. Caps of tissue culture flasks were tightened, and cells were
incubated overnight at room temperature. Cells were harvested and washed
three times with ice-cold PBS. Pellets were lysed with 0.5% nonionic de-
tergent Nonidet P-40 (Sigma) in 0.2 M phosphate buffer (pH 7.05) and in
the presence of proteinase inhibitors: pepstatin A, 5 µg/ml; benzamidine, 2.5 mM; soybean trypsine inhibitor, 20 µg/ml;
PMSE, 100 µM; and EDTA, 4 mM. Cell nuclei were pelleted by centrif-
gulation. The protein concentrations were measured using the bicinchoninic
acid protein assay (Pierce, Rockford, IL). Where appropriate, adjustments
were made to standardize protein concentrations of lysates. Supernatants
containing class I complexes were stored on ice until needed (no more than
16 h). To measure MHC levels, we used a modified semiquantitative
two-Ab sandwich ELISA assay (41). For MQ10 and SQ10 measurements,
mAb 46 (anti-α3 of Qa-2) was used as primary Ab and biotinylated mAb
S19.8 (anti-β3) as secondary Ab. The assay for Qa-2 was performed using
the same protocol on cell lysates of MQa-2 transfectants (41). The assay
for H-2Kw was performed similarly with mAb 20-8-4 as a primary Ab
and biotinylated mAb Y3 as a secondary Ab (41).

Isolation of endogenous peptides from Q10 complexes

MQi0 and SQ10 complexes and their ligands were purified using two
different methods. Endogenous peptides bound to MQ10 molecules were

Cell lines and tissue culture

The murine cell lines RMA and its TAP2-deficient mutant, RMA-S (37),
were transfected with linearized Q10 constructs and pHEKneo vector
(G418 resistance marker) by electroporation as described previously (38).
Transfectants expressing the highest levels of MQ10 were selected by flow
cytometry with mAb 46. Clones secreting the highest levels of SQ10 were
identified by a two-Ab sandwich ELISA (see below) and further charac-
terized by immunoprecipitation with mAb 46. Transfectants were prop-
gated in the presence of 0.15 mg/ml of active G418 (Fisher Scientific, Pitts-
burgh, PA). Large scale cultures of RMA/MQ10-positive cells were grown in the Laborator of Cellular and Developmental Biology, National Insti-
tute of Diabetes and Digestive and Kidney Diseases, National Institutes
of Health (Rockville, MD) under the direction of Dr. J. Shiloach. Large scale
cultures of RMA/SQ10-positive cells were grown to saturation (2 × 109
ml) in Frewal Lifecell TC Flasks of 3-liter capacity (Baxter Scientific Pro-
ducts, McGaw Park, IL) at the University of Texas Southwestern Medical
Center, Dallas. The rat YB1/0 (39) and human CIR (40) cell lines were
obtained from ATCC (ATCC CRL 1662) and Dr. J. Forman (University of
Texas Southwestern Medical Center, Dallas), respectively. The RMA and
RMA-S transfectants expressing MQua-2 and Sqqa-2 were described else-
where (38, 41).

Antibodies

The mAbs 46 (anti-α3 of Qa-2) (34) and S19.8 (anti-mouse β3m) (35) used in Q10-affinity purification and ELISA were purified from mouse ascites fluid
by glass fiber affinity chromatography on protein-A-sepharose (Cl-4B, Pharmacia, Piscatway, NJ) using standard protocols (36). Secondary
mAbs used in ELISA were biotinylated with N-hydroxysuccinimidotriol
(Sigma, St. Louis, MO) as described previously (36).
isolated by a modification of a method previously described for other membrane-bound class I complexes (42). A total of 10^9 MQ10-transfected RMA cells were lysed in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% [3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 0.25% sodium deoxycholate, 1 mM PMSF, 100 mM iodoacetamide, 5 μg/ml apro- tinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 mM EDTA, and 0.04% sodium azide. After centrifugation, the cell lysate was loaded onto a column of inactivated Sepharose CL-4B and subsequently onto a Sepharose CL-4B column to which mAb 46 had been coupled (36). After extensive washing, MQ10 complexes were eluted with 10% acetic acid. The released peptides were isolated by centrifugation through a hollow fiber cartridge with a 30-kDa cutoff (model UFP-3-C-5; A/G Technology, Needham, MA). The concentrate was spun for 2 h and filtered through a 0.2-μm membrane. Tris-HCl was added to a final concentration of 0.1 M, and the pH was adjusted to 8.0. SQ10 proteins containing the 6xHis-tag were purified by metal affinity chromatography. Briefly, 30 ml of Ni-NTA Sepharose beads (Qiagen, CA) was stirred gently overnight at 4°C with the concentrated sample containing recombinant SQ10. Sepharose beads suspension was transferred to a chromatography column and extensively washed. The SQ10 was eluted from the column with 2 column volumes of PBS and 250 mM imidazole. To achieve prompt neutralization, fractions (5 ml) were collected in tubes containing 0.5 ml of 1 M Tris-HCl (pH 7.0). Fractions containing SQ10 were identified by sandwich ELISA with mAb 46 and S19.8 Abs as described above in “Measurement of expression levels and stability of Q10 molecules by ELISA”.

Results

Construction of cell lines expressing hybrid Q10 proteins

To perform direct biochemical analysis of endogenously synthesized Q10-binding ligands, it is necessary to isolate sufficient quantities of the relevant class I complexes. Since there are currently no known mAbs that would allow purification of wild-type Q10 from serum, we cloned Q10 cDNA and expressed Q10 molecules as class I hybrid proteins in transfected tissue-cultured cell lines (Fig. 1). The two putative ligand-binding domains (α1 and α2) of Q10 were fused to the α3 domain and C-terminal portion of another Q region protein, Qa-2 (44–46). The Qa-2 proteins exist in two isoforms: membrane-bound Qa-2 attached to cell surface via GPI moiety (MqA-2) and soluble Qa-2 derived from the same gene by alternative splicing (5, 46) (SAQ-2). The choice of the α3 domain in the Q10 hybrids was dictated by the high homology between the Q10 and Qa-2 sequences (28, 45), by availability of multiple mAbs recognizing unique Qa-2 epitopes on the α3 domain (34, 35, 38), and by previous studies showing that the shuffling of Qa-2 domains with other class I domains does not disturb the conformation of the α1α2 portion of hybrid complexes (47, 48).

Two forms of Q10 hybrid genes were constructed: SQ10, encoding soluble form of Q10, and MQ10, encoding membrane-bound, GPI-linked Q10 protein. The structure of the predicted hybrid Q10 genes and proteins is depicted in Fig. 1. The hybrid constructs were transfected into lymphoid-derived cell lines: murine (RMA), rat (YB2/0), and human (CIR) cell lines. High levels of Q10 proteins were detected in every case, suggesting that assembly of these complexes is not limited by the lack of appropriate ligands or chaperones (data not shown and see below). We were unable to perform any studies with transfected liver cell lines because these cells could not be grown to the densities necessary for biochemical characterization of the hybrid molecules (46).

To verify the integrity of Qa-2 conformational epitopes on the α3 domain of MQ10, the transfected murine RMA cells were stained with six anti-α3 Qa-2 mAbs (data not shown). All reacted with MQ10 as well as with control wild-type Qa-2-positive cells. As expected, anti-α1α2 Qa-2 Abs did not react with MQ10.
The m.w. of MQ10 and SQ10 hybrids, their association with βm, and GPI attachment of MQ10 were tested as follows. RNA cells transfected with hybrid Q10 constructs and control Qa-2 genes were biosynthetically labeled with [35S]methionine and cysteine, and supernatants collected from an equal number of cells were analyzed by immunoprecipitation with mAb 46. The precipitated molecules in lanes 1–10 originated from supernatants of the following cells: lanes 1 and 2, two independent RNA transfants of SQ10 (P29-4.4 and P29-4.1); lane 3, RMA cells transfected with soluble Qa-2 carrying the 6xHis-tag at the C terminus (P29-3.4); lane 4, RMA cells synthesizing soluble Qa-2 without the 6xHis-tag (PI-6.60); lane 5, PI-PLC-treated RMA cells expressing MQ10 (M15-3); lane 6, RMA cells expressing MQ10 (M15-3) that were not treated with PI-PLC; lane 7, PLC-treated RNA transfants expressing membrane-bound Qa-2 (P5-5.6); lane 8, RMA transfants expressing membrane-bound Qa-2 (P5-5.6) that were not treated with PI-PLC; and lanes 9 and 10, control untransfected RMA cells treated (9) and untreated (10) with PI-PLC. B, Coomassie blue-stained and SDS-PAGE-resolved purified SQ10. The heavy chain and βm are indicated by arrows. The additional bands correspond to heavy and light Ig chains which were also released from the mAb 46 immunoaffinity column. Coomassie blue-stained βm may include murine and bovine species, whereas radioactively labeled βm in Fig. 2A corresponds to endogenously synthesized murine βm only.

Partial TAP dependence of MQ10 membrane expression

Multiple studies have demonstrated that mutations in the TAP genes, that direct synthesis of the peptide transporter molecules in the class I Ag presentation pathway, lead to reduced levels of classical class I Ags on the cell surface (37, 50). This phenotype is thought to result from limiting quantities of peptide ligands delivered to the endoplasmic reticulum in TAP-negative mutants and the resulting instability of “empty” class I-βm complexes. In most cases, the decrease in membrane expression can be reversed by low temperature (~26°C), which stabilizes peptide-free class I complexes that reach the cell surface in TAP-negative cells.

To address the question of whether MQ10 associates with TAP-delivered peptides, we introduced MQ10 into TAP-negative RMA-S cells and compared its expression to the parental RMA cells by FACS staining (Fig. 3). The control H-2Kb Ag expressed on MQ10 transfants displayed classical TAP-dependent behavior: ~4-fold reduced expression in RMA-S vs RMA cells at 37°C and 42°C and ~8-fold induction of H-2Kb levels in RMA-S cells at 26°C (38). Qa-2 Ag showed a more drastic reduction of surface levels in RMA-S compared with RMA cells (12–14-fold) at 37°C and 42°C (38). This expression was enhanced only weakly at 26°C, in agreement with our previous data showing that most of the empty Qa-2 fail to reach the cell surface in TAP-negative cells and accumulate intracellularly (41). MQ10 expression showed a TAP2-dependent phenotype intermediate between H-2Kb and Qa-2. Surface MQ10 levels were reduced (~5-fold in RMA-S vs RMA cells) and were only weakly inducible at 26°C. Thus, compared with wild-type Qa-2, MQ10 contains a somewhat larger fraction (~20%) of heat-stable molecules that reach the cell surface in a TAP2-independent fashion in RMA-S cells. This phenotype is most likely controlled by the structural properties of the α1 and α2 domains of Q10.

To confirm that the majority of MQ10 complexes in TAP2-negative cells remain intracellular and behave as heat-unstable empty molecules, we analyzed MQ10 from lysates of RMA-S transfants using conformation-dependent ELISA. The data in Fig. 4 show that almost all MQ10 molecules, as well as the control H-2Kb and Qa-2 molecules released from lysates of transfected RMA-S cells, lose conformational epitopes upon incubation at 42°C for 80 min, whereas the majority of RMA-expressed complexes are stable under the same conditions. The partial loss of conformational epitopes (in ~30% of the RMA-derived complexes) may be indicative of empty molecules, which accumulate intracellularly because they have not been loaded with peptides or, alternatively, may reflect the fact that some molecules associate with peptides of low affinity that are released upon heat shock.

Taken together, these observations are consistent with the notion that the majority (~80%) of mature MQ10 molecules require a functional TAP pathway for cell surface expression. This property suggests that α1α2 of MQ10 molecules are peptide loaded.
Isolation and sequencing of peptides associated with membrane-bond and soluble Q10 proteins

The MQ10 and SQ10 complexes expressed in RMA cells were purified by immunoaffinity chromatography and the sequences of several endogenously bound peptides were determined by tandem mass spectrometry (MS/MS).

Because of the different properties of the secreted and membrane-bound class I Ags, the two Q10 complexes were purified using slightly different approaches (see Materials and Methods). The SQ10 complexes were purified from tissue culture medium using metal affinity chromatography followed by immunoaffinity chromatography using the anti-α3-specific mAb 46. In our previous studies with human MHC class I molecules, we routinely quantitated the amount of purified complex by measuring the concentration of β2m that was retained by the ultrafiltration membrane used to separate the peptides from intact proteins. The intact proteins retained in the high m.w. fraction were separated by RP-HPLC, and the amount of β2m present was estimated by both Edman degradation and absorbance at 280 nm. In the SQ10 preparation both the β2m and the SQ10 heavy chain were readily detected, allowing us to estimate that about 4 nmol of complex had been purified. Electrospray ionization mass spectrometry (ESI/MS) analysis of the fraction containing the SQ10 heavy chain yielded a molecular mass of 38,874 Da, about 4850 mass units greater than expected for the unglycosylated molecule (molecular mass, 34,026 Da). This mass difference is most likely due to N-linked carbohydrate moieties at Asn 86 and Asn 256, and it could be accounted for by two triantennary carbohydrate structures. After digestion with N-glycosidase F, in fact, two components with molecular masses of 36,518 and 34,170 Da, respectively, were detected, most likely corresponding to a partially and a completely deglycosylated form of the protein. When the fraction containing β2m was analyzed by Edman sequencing, a mixed sequence was obtained, indicating that about 20% murine β2m and 80% bovine β2m was present; ESI/MS analysis detected only bovine β2m (molecular mass, 11,632). The preferential association of the SQ10 heavy chain with bovine β2m is consistent with the observation that murine β2m undergoes exchange with other species of β2m present in the medium (Fig. 2). The long period of incubation of the SQ10 complex in the FCS-supplemented tissue culture supernatants before purification may account for the observed high proportion of bovine β2m in SQ10 complexes.

MQ10 hybrid molecules were immunoaffinity-purified using mAb 46 specific for the α3 domain of Qa-2. In this preparation, to our surprise, no β2m or MQ10 heavy chain was recovered from the ultrafiltration membrane, making it impossible to quantify the amount of class I complexes that had been purified, even though peptides could easily be detected (see below).

The peptides associated with both MQ10 and SQ10 were acid extracted and separated by narrow-bore HPLC. The HPLC profile of the MQ10-associated peptides is shown in Fig. 5A. An enlarged view of the region containing the majority of the eluted peptides is shown in Fig. 5B. The anticipated peptide-containing fractions were analyzed by ESI/MS. The ESI/MS analysis showed the presence of at least 50 peptides for MQ10 and 110 peptides for SQ10 (data not shown) whose molecular mass fell into the mass range appropriate for 8–11-mer peptides, many of which were present in both samples. The larger number of peptide signals detected for SQ10 may reflect the ability of the soluble form to bind a larger number of peptides. Alternatively, because we were not able to quantify the amount of MQ10 complexes purified, it might simply indicate that a larger quantity of purified SQ10 complex was available for analysis.

CAD analysis (51) performed on selected ions present in both the MQ10- and SQ10-purified material defined plausible amino acid sequences for six peptides (Table I). A representative CAD
spectrum is shown in Fig. 6 for the peptide with mass:charge ratio \((m/z)\) of 920.2 (peptide 5, Table I). By this means, complete sequences were obtained for several peptides. For peptide QGVQXXDF (peptide 5) assignment of Q vs K (which are of nearly identical mass) was made by MS analysis following acetylation. This derivatization resulted in a single 42 mass unit shift, leading us to conclude that the N-terminal amino group is the only amino group present in the peptide. Four of the six peptides were eight amino acids long, and two were nine amino acids long, suggesting that depending on the sequence, Q10 preferentially binds octamers but occasionally nonamers, similar to H-2K\(\beta\) and HLA-B8 (52). All six of the peptides contained Gly at P2, five of the six peptides contained Lxx at P6, and at P0 all peptides contained a hydrophobic residue: either Phe (in four sequences) or Lxx. The other positions of the peptides were more variable.

One of the largest peaks in the absorbance trace, at 34 min (Fig. 5), contained peptide TGTETXYF. Unlike most of the other peaks (some of the larger of which were present both in the nonspecific material eluted from glycine-Sepharose and in the Q10 mAb eluate), the peak at 34 min had an UV spectrum with a maximum at 278 nm, typical for peptides containing tyrosine residues, leading to the conclusion that the large absorbance of the material in this peak is largely due to peptide TGTETXYF and not to unrelated molecules. On the basis of absorbance and Edman degradation data (which matched the MS/MS sequence with Leu at P6), we concluded that there was about 250–300 pmol of peptide TGTETLYF in the MQ10 preparation and about 1 nmol in the SQ10 preparation; all other peptides were 60- to 100-fold less abundant. These estimates suggest that the TGTETLYF peptide may constitute as much as ~25% of the total Q10 ligand pool.

Synthetic peptides corresponding to the sequences of the constitutively bound peptides form complexes with MQ10 in vitro

When gene and protein sequence databases were searched for possible parent proteins of the Q10-specific peptides, four peptides from Table I matched murine protein sequences. Interestingly, all of these putative proteins correspond to fairly abundant polyproteins. Peptide 1 is identical to an octameric sequence present within two distinct proteasome subunits: constitutively expressed PSMB5 (53) and IFN-\(\gamma\) regulated LMP7 (54). Peptide 2 is homologous to ribophorin (accession number D31717.1) and peptide 6 to cytochrome c oxidase (accession number P43024). The putative
source protein of the most abundant peptide (peptide 3) corresponds to TCR V\(\beta\)-chain (55) and is the only source protein which would not be normally expressed in liver cells. Its presence in the Q10-transfected RMA cells is consistent with the lymphoma phenotype of the parental line. All of the putative source proteins are expressed intracellularly, suggesting that their peptide components were introduced into the Q10 grooves by the class I Ag presentation pathway and were not incorporated into the complexes during the purification procedure from extracellular sources such as tissue culture medium. The alignment of peptide sequences 1, 2, and 6 with their putative sources allowed us also to assign Leu and Ile for these three peptides.

Peptides corresponding to the six sequences reported in Table I were synthesized using Leu as the default amino acid in positions where Leu could not be distinguished from Ile (reported as X in Table I). In each case, CAD fragmentation spectra were identical to the spectra derived from the Q10-associated peptides, confirming that the deduced sequences were concordant by this criteria. However, when the RP-HPLC retention times of the six synthetic peptides were compared with those obtained for the endogenous Q10 peptides, peptides 4 and 5 showed a higher retention time than expected, likely due to the presence of Ile instead of Leu at some positions. Due to a large number of potential permutations of Leu and Ile in peptides 4 and 5, we have not synthesized additional candidate peptides.

To verify that the sequences obtained in this study represent genuine endogenous peptides that can associate specifically with Q10, the synthetic homologues were tested in an in vitro peptide-binding assay. The assay measured the peptide-dependent stabilization of MQ10 epitopes on the a3 domain (recognized by mAb 46) and murine \(\beta_m\) (recognized by mAb S19.8) by sandwich ELISA. The signal:background ratio of this assay is lower than the one observed with MQa-2 (data not shown). This effect may be explained by preferential displacement of murine \(\beta_m\) from MQ10 heavy chain by bovine \(\beta_m\) and/or by higher background of “temperature-resistant” MQ10 complexes formed in transfected RMA-S cells. Five of the six synthetic peptides stabilized the MQ10/\(\beta_m\) complexes over a wide range of peptide concentrations: 100 ng to 100 \(\mu\)g, as shown in Figs. 7 and 8. Although the peptide stabilization assay cannot be regarded as a rigorous measurement of peptide affinity, the half-maximal and maximal points

### Table I. Sequences of Q10-binding peptides are homologues to endogenous murine proteins

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<thead>
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<th>Peptide</th>
<th>m/z</th>
<th>Position</th>
<th>Protein of Origin</th>
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<td>3</td>
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<td>TCR V(\beta) chain</td>
</tr>
<tr>
<td>4</td>
<td>843.1</td>
<td>X G A A X X G D</td>
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<tr>
<td>5</td>
<td>920.2</td>
<td>Q G V V Q X X D F</td>
<td></td>
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<td>6</td>
<td>966.2</td>
<td>V G V S M L N V F</td>
<td>Cytochrome C oxidase</td>
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</table>

* X indicates Leu or Ile residues which are not distinguishable by tandem MS.

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**FIGURE 6.** CAD mass spectrum of peptide ions at m/z 920.2 (peptide 5). Predicted masses for fragment ions of types b and y (51) are shown above and below the deduced sequence, respectively. Ions observed in the spectrum are underlined. Interpretation of CAD spectra is fully explained elsewhere (51). Because Ile and Leu are of identical mass, they cannot be differentiated on the triple quadrupole instrument and are specified here as Lxx.

**FIGURE 7.** Stabilization of MQ10 conformational epitopes with Q10 synthetic peptides. Group A peptides correspond to synthetic homologues of Q10 peptides listed in Table I. B is a poly(A) nonamer AAAAAAAAA, group C peptides are (left to right): RYWAIRTRS, FRYNGLIHL, RYWATRSGG, and GRIDKPIILK. All peptides have been used at saturating concentrations of 20 \(\mu\)g/ml. The values shown correspond to the mean of triplicate experiments and are expressed in arbitrary units relative to the internal ELISA standard.
of concentration curves in Fig. 8 do not give any indication that the dominant TGTETLYF peptide binds Q10 better than other titered peptides. Hence, we conclude that the peptide-binding affinity of TGTETLYF is comparable to other peptides tested by ELISA approach. Peptide 4, LGAALLGDL, was consistently negative in our assay (comparable to negative controls in Fig. 7). This peptide contains four Leu residues synthesized as default amino acids in positions in which Leu could not be distinguished from Ile in the endogenous Q10 sequence.

Q10-associated peptides display classical peptide-binding motif

MS sequencing of MQ10- and SQ10-eluted peptides suggested that these molecules bind a heterogeneous mixture of diverse, endogenously synthesized ligands, which occupy as much as ~75% of all Q10 receptors. The remaining ~25% of Q10 molecules are filled with a single peptide species TGTETLYF. This dual affinity of Q10 molecules prompted us to examine sequence requirements of the Q10 ligands for binding to Q10 groove. We reasoned that a groove that is severely biased toward accepting peptides with defined sequences will be less efficient in associating with mutant peptides. Hence, we conclude that the peptide-binding affinity of TGTETLYF (homologous to TCR V β-chain), would be present in liver cells and is identical to TGTETLYF in five of eight positions. A series of synthetic peptides substituted by Ala or Ser at each of the positions was synthesized (see legend to Fig. 9), and the peptides were used in the ELISA sandwich peptide-binding assay. The results of the binding experiments indicated that only three peptide residues could not tolerate being replaced with Ala for efficient binding to MQ10: Gly at P2, Leu at P6, and Phe at P8. Substitutions of these residues with Ala led to either reduction or loss of binding comparable to negative control peptides VSV, L19, and NP (Fig. 9). The three anchor residues correspond to the conserved residues determined from the MS sequencing (Table I). Thus, we conclude that the majority of the peptides associating with the Q10 groove display a classical peptide-binding motif similar to the diverse repertoire of ligands that occupy the H-2K, H-2D, or HLA-A and -B grooves.

Discussion

Recent research into the functions of the nonclassical class Ib MHC Ags led to the recognition of their diversity and heterogeneous properties; however, despite the fact that much has been learned about immunological features of murine M3, Qa-1, and human CD1, HLA-E, and HLA-G Ags, the great majority of the class Ib molecules remain uncharacterized. One such molecule is the soluble, liver-specific Q10 protein that is expressed in a wide variety of inbred and wild mouse strains.

In an attempt to learn about Ag-presenting functions of Q10 proteins, we analyzed peptide ligands constitutively associated with the Q10 α1α2 domains. Because of the technical limitations imposed by the necessity to produce large amounts of this protein, we expressed and analyzed hybrid Q10/Qa-2 molecules in lymphoid-derived cells. The results of the MS sequencing of the Q10-associated ligands revealed that they are very similar to the processed protein fragments eluted from classical class I Ags. As is the case with H-2Kα or H-2Kβ peptides (2), the majority of the Q10 ligands are octameric (although nonamers were also detected). The Q10 peptides carry a peptide-binding motif typical of the class Ia motifs. The conserved residues include a hydrophobic (Phe or Leu) dominant anchor at Pω and two additional invariant residues: Gly.
at P2 and Leu/Val at P6. All three of these positions influence binding of Q10 synthetic peptide homologues to Q10 groove. The residues found at P2, P6, and P9 on Q10 ligands have been reported to serve as anchors in peptides eluted from other class I MHC Ags (2). This is not surprising considering the fact that the predicted geometry of the Q10 groove is very similar to HLA-A2. Although Q10 α1α2 domains contain a number of unique substitutions that are not commonly found in other class I MHC proteins (at positions 24, 75, 89, 90, 102, 109, 137, 162, and 176), only one of them, Ile 24, is located at a position predicted to face the peptide-binding groove.

One unusual feature of peptide Q10 is that the invariant Gly at P2 is followed by a variant amino acid at P3. The only other class I molecules for which Gly has been deduced to be critical for binding are H-2Dd, where Gly at P2 is nearly invariably paired with Pro at P3 (57) and HLA-B51, where Gly at P2 is almost always paired with an aromatic residue at P3 (2). Because Gly has no side chain and therefore cannot function as an anchor residue directly, but instead promotes local flexibility and destabilization of the peptide binding, it is possible that all four amino acids found at P3 (Ala, Ile, Val, and Thr) play an important role in anchoring of peptides to Q10 groove. The Gly anchor residue at P2 would be expected to correlate with large side chains in the B pocket of the peptide-binding cleft. The only unusual B pocket residue is Ile-24, which is only found in Q8 (45), whose motif has not been determined, and in Qa-1, which is occupied predominantly with a single peptide species carrying Met at P2 (14).

The nature of the putative source proteins giving rise to Q10 peptides warrants some discussion. All identified sequences matched intracellular murine proteins: the LMP7 and PSMB5 proteasomal subunits, ribophorin, cytosome c oxidase, and TCR V β-chain. This is in agreement with the notion that the peptides bound to Q10 groove originated from cytoplasmic proteins and were delivered to the complex by components of classical class I MHC Ag presentation pathway. Consistent with this interpretation we found that mutation of the TAP2 gene led to significant reduction of heat-resistant, peptide-filled Q10 molecules expressed on the surface of RMA-S cells. The small proportion of thermally stable MQ10 on RMA-S cells was comparable to H-2Kb expressed in the same background and may correspond to MHC complexes loaded by TAP1/TAP1 homodimers (58).

The identification of the degraded product of TCR V β-chain as the most abundant peptide in the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the most abundant peptide in the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide TGTETLYF occupied as much of the Q10 groove in RMA cells was the unexpected finding of this study. Edman degradation and absorbance data suggested that peptide


