The Cytoplasmic Domain of the H-2L\textsuperscript{d} Class I Major Histocompatibility Complex Molecule Is Differentially Accessible to Immunological and Biochemical Probes during Transport to the Cell Surface\*  

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An antiserum was generated against a synthetic peptide corresponding to a portion of the cytoplasmic domains of the H-2L\textsuperscript{d} and H-2D\textsuperscript{b} class I major histocompatibility complex molecules of the mouse. This antibody, R4, binds exclusively to endoglycosidase H-resistant H-2L\textsuperscript{d}/H-2Db molecules which are not associated with \(\beta_2\)-microglobulin. Interestingly, acquisition of resistance to endoglycosidase H precedes acquisition of R4 reactivity by 30 min. R4-reactive H-2L\textsuperscript{d} and H-2D\textsuperscript{b} molecules occur on the cell surface and are phosphorylated in vivo. Other studies show that the tyrosine in the cytoplasmic domain is accessible to radioiodination only on a subset of H-2L\textsuperscript{d} molecules, and that the two-dimensional electrophoretic profiles of phosphorylated H-2L\textsuperscript{d}/H-2Db molecules, of R4-reactive molecules, and of H-2L\textsuperscript{d} molecules radiolabeled on this cytoplasmic domain tyrosine are virtually identical. R4-reactive H-2L\textsuperscript{d} molecules do not undergo the peptide- and \(\beta_2\)-microglobulin-induced conformational changes characteristic of free class I major histocompatibility complex heavy chains. The accessibility of the H-2L\textsuperscript{d} cytoplasmic domain to R4 and to radioiodination late in biosynthesis and its biological significance are discussed.

Class I major histocompatibility complex (MHC)\textsuperscript{a} molecules are highly polymorphic cell-surface glycoproteins that bind and present endogenously derived antigenic peptides to T lymphocytes (1–3). The antigen-presenting complex is a noncovalently associated trimolecular assembly consisting of the class I MHC molecule, \(\beta_2\)-microglobulin (\(\beta_2\)-m) light chain, and antigenic peptide (4–6). Evidence from a number of studies supports a model in which this trimolecular complex is assembled in the endoplasmic reticulum soon after the class I MHC molecule is synthesized (7–9). The assembled complex is then transported through the Golgi apparatus (10, 11) en route to the cell surface where it serves as a membrane-bound ligand for T lymphocytes. Studies on the highly conserved cytoplasmic domain showed that modifications of this region of the H-2L\textsuperscript{d} molecule of the mouse result in slower transport through the Golgi apparatus (12).\textsuperscript{2} Hence, it seemed possible that the cytoplasmic domain facilitates the transport of the class I MHC molecule through the cell. To explore this possibility, we generated a polyclonal antibody against a synthetic peptide corresponding to a 13-amino acid portion of the cytoplasmic domain of the H-2L\textsuperscript{d} molecule. The resulting polyclonal antibody, R4, immunoprecipitates H-2L\textsuperscript{d} and H-2D\textsuperscript{b} class I molecules from cell lysates. Three major features characterize R4-reactive H-2L\textsuperscript{d}/H-2Db molecules: They are not associated with the \(\beta_2\)-m light chain, they are endoglycosidase H (Endo H)-resistant, and, if not all, are phosphorylated. The latter two characteristics show that R4 binds H-2L\textsuperscript{d}/H-2Db molecules which have traversed the medial Golgi apparatus. R4-reactive H-2L\textsuperscript{d}/H-2Db molecules also are found on the cell surface. Hence, it appears that the cytoplasmic domains of the H-2L\textsuperscript{d}/H-2Db molecules are inaccessible to R4 until late in biosynthesis.

In an independent assessment of the accessibility of the H-2L\textsuperscript{d} cytoplasmic domain, we ascertained whether a tyrosine in this region of the molecule can be radioiodinated. The H-2L\textsuperscript{d} molecules which undergo radioiodination of the cytoplasmic domain are electrophoretically homogeneous, whereas a more heterogeneous population of H-2L\textsuperscript{d} molecules is detected by radioiodination of tyrosines on the external domains. The two-dimensional gel profiles of H-2L\textsuperscript{d} molecules which undergo radioiodination on the cytoplasmic domain, those precipitable by R4, and phosphorylated H-2L\textsuperscript{d}/H-2Db molecules are highly similar. Other studies show that R4-reactive H-2L\textsuperscript{d} molecules do not associate with exogenously supplied antigenic peptide and/or \(\beta_2\)-m and do not undergo the peptide- and \(\beta_2\)-m-induced conformational changes characteristic of free class I MHC molecules (8, 9). We discuss these findings both in terms of potential conformational changes in the H-2L\textsuperscript{d}/H-2Db cytoplasmic domain (13) and the effects of cellular factors which may bind to and shield the H-2L\textsuperscript{d}/H-2Db cytoplasmic domains early in biosynthesis.

MATERIALS AND METHODS

Cell Lines—EL\textsuperscript{3}3 cells, L cell transfectants 27.5.27, 911-7D6, 2.2.1 DD5, and HCT-L/\(\beta_2\)-m cells are described elsewhere (12, 14–16).\textsuperscript{3} The EL\textsuperscript{3}3 and HCT-L/L/\(\beta_2\)-m cell lines were grown in RPMI 1640 supplemented with Serum Plus\textsuperscript{®} (a non-serum, defined medium supplement). L cell transfectants were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Synthetic Peptides and Human \(\beta_2\)-m—The synthetic peptide H-2L13 (GSQSSEMILRDCQ), which is shown enclosed in the box in Fig. 1A, was the kind gift of Susanna Horvath. Antigenic peptides previously

1 The abbreviations used are: MHC, major histocompatibility complex; \(\beta_2\)-m, \(\beta_2\)-microglobulin; Endo H, endoglycosidase H; ER, endoplasmic reticulum; KLI, keyhole limpet hemocyanin; SMCC, succinimidyl 4-(N-maleimidomethyl)cyclhexane-1-carboxylate; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; bisacrylamide, N,N'-methylenebisacrylamide; TEMED, N,N,N',N'-tetramethylethylenediamine; mAb, monoclonal antibody.

2 G. G. Capps and M. C. Zúñiga, unpublished observations.

3 M. C. Zúñiga, H. Wang, G. G. Capps, and B. R. Robinson, submitted for publication.
shown to bind and be presented by H-2Ld (amino acids 168-176 of murine cytomegalovirus immediate early protein pp89) (17), by H-2Kb (amino acids 345-360 of influenza nucleoprotein, but with an amino-terminal tyrosine not normally found in this protein) (18), and by H-2Dd (amino acids 50-63 of influenza nucleoprotein) (18) were purchased from Applied Biosystems. These peptides are referred to as Ld peptide, Kd peptide, and Dd peptide, respectively, in this paper.

Preparation of Synthetic Peptide Immunogen.—The H-2L13 peptide was synthesized and conjugated to keyhole limpet hemocyanin (KLH) using succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) as a cross-linker. 60 nmol of KLH in 1 ml of phosphate-buffered saline (PBS, pH 7.5), was incubated with 2.5 mmol of SMCC dissolved in dimethyl formamide (DMF) for 30 min at room temperature. 10 pmol of synthetic peptide in 300 ml of 4 % guanidine hydrochloride (made in PBS, henceforth called Gu-PBS) was brought to pH 3-4 with 17% trifluoroacetic acid (TFA) and then brought to 0.15 M dithiothreitol, and incubated for 10 min at room temperature. The KLH-SMCC and peptide solutions were aliquoted sequentially to a 2-ml Sephadex G-10 column equilibrated with 4 M urea-PBS, pH 7.5. 200-μl fractions were collected. Fractons containing peptide-KLH conjugate were pooled and brought to pH 7.0 with NaOH.

Antibodies—Rabbits were immunized three times over a 2-month period with 108 μg of the H-2L13 peptide-KLH conjugate in complete Freund’s adjuvant. Purified, anti-serum were injected i.v. with H-2L13 conjugated to ovalbumin. The positive antibody preparation thus obtained is called R4 herein. Monoclonal antibodies (mAbs) 30.5.7, 28.14.8, and 64.3.7 have been described previously (8, 19, 21).

Subcellular Fractionation—6 x 10^9 EL-3 cells were biosynthetically labeled for 90 min at 100 μCi/ml [35S]methionine as described below. Cells were then washed once in 15 ml of PBS, once in 4 ml of cavitation buffer (0.245 M sucrose, 10 mM isocitrate, 5 mM Hepes, pH 7.0), suspended in 1 ml of cavitation buffer, and loaded into a Parr model 4713 cavitation bomb (Parr Instrument Co., Moline, IL) for nitrogen cavitation (700 psi, for 2 min on ice). The cavitation was centrifuged at 1500 rpm for 5 min to remove the cell nuclei, and the clarified cavitated was loaded onto the top of a step gradient consisting of 2 g each of 24%, 20%, 16%, 12%, 8%, and 4% (all w/w) Nycodenz (Robbins Scientific Corp., Sunnyvale, CA) dissolved in cavitation buffer. The gradient was centrifuged for 17 h at 4°C at 25,000 rpm in a Beckman SW41 swinging bucket rotor, and 0.9-ml fractions were collected, lysed as described below, and subjected to immunoprecipitation analysis.

35S Methionine Labeling of Cells, Immunoprecipitation of Proteins, and Endo H Digestion of Immunoprecipitates—[35S]Methionine biosynthetic labeling, pulse-chase studies, double labeling with [35S]methionine and [32P]orthophosphate, immunoprecipitation from cell lysates, and Endo H digestions of immunoprecipitates were performed as described previously (12, 16, 22). Proteins were eluted from protein A-Sepharose with 0.2% SDS, 0.05 M Tris-HCl, pH 7.5. In the immunoprecipitation experiment described in Fig. 3, lysates were incubated with 5 pmol of Db peptide, 5 μM Dd peptide, and/or 0.5 μM P2-m as indicated in Fig. 9. 28.14.8 precipitate the same population of molecules is present in both the R4 and mAb 28.14.8 precipitates (Fig. 2).

RESULTS

The Anti-cyttoplasmic Domain-Specific Antibody, R4, Binds the Cytoplasmic Domains of H-2Ld/Dd Class I MHC Molecules —A polyclonal antiserum (R4) against a synthetic peptide identical with 13 amino acids of the mouse H-2Ld cytoplasmic domain (Fig. 1A) was tested for its ability to immunoprecipitate class I MHC molecules. Because the amino acid sequences of the cytoplasmic domains of H-2Ld and H-2Dd are identical (25, 26), R4 is expected to bind both of these class I MHC molecules. EL-4 cells (H-2Ld+ and H-2Db+) were surface-radioiodinated and lysed, and the lysates were immunoprecipitated with either R4 or with mAb 28.14.8 which binds the terminal tyrosine not normally found in this protein) (18), and by H-2Db (amino acids 168-176 of murine cytomegalovirus immediate early protein pp89) (17), by H-2Ld molecules were then immunoprecipitated with mAbs 30.5.7, mAb 28.14.8, or R4. Immunoprecipitates containing H-2Ld, and immunoprecipitated proteins were eluted as described above. Peptide co-precipitating with H-2Ld molecules was determined by counting eluates in a γ counter. See Fig. 8 for further details. 10^9 EL-3 cells were labeled biosynthetically for 90 min and lysed as described above, and lysates were incubated with 5 μM Ld peptide, 5 μM Dd peptide, and/or 0.5 μM P2-m as indicated in Fig. 9. H-2Ld and H-2Dd molecules were then immunoprecipitated with mAbs 30.5.7, 28.14.8, 64.3.7, and R4 as indicated in Fig. 9. Class I MHC molecules were eluted as described previously (12) and above. Endo H digestion products were analyzed by SDS-PAGE.

SDS-PAGE and Two-dimensional Electrophoresis—Polyacrylamide gels used for analysis of immunoprecipitates consisted of 5% acrylamide, 0.133% N,N,N,N”-methylenebisacrylamide (bisacrylamide), 0.1 mM NaPO₄, pH 6.6, 0.1% SDS, 0.1% ammonium persulfate, and 0.05% N,N,N’’-tetramethylethylenediamine (TEMED), topped by a 3% stacking gel (3.5% acrylamide, 0.093% bisacrylamide, 0.1% NaPO₄, pH 6.6, 1% SDS, 0.1% ammonium persulfate, and 0.25% TEMED). The gel used to resolve cyagen bromide digestion products is a discontinuous gel system run in Tris glycine, pH 8.3, buffer in which the running gel is 15% acrylamide cross-linked with 0.43% bisacrylamide and has been described previously (12). Two-dimensional gel electrophoresis of immunoprecipitated proteins was performed according to published procedures (24) under reducing conditions in both dimensions. Electrophoretically resolved proteins were analyzed by autoradiography of dried gels (12, 22) or transferred to membranes as described above.

Cyanogen Bromide Digestion—Radiolabeled proteins were purified by transfer to Immobilon membranes (Millipore) and treated with cyanogen bromide (CNBr) as described previously (16). CNBr digestion products were resolved by SDS-PAGE, as described above.

CONCLUSIONS

To investigate its specificity further, R4 was examined for its ability to precipitate H-2Ld molecules having either altered or deleted cytoplasmic domains. In these studies, L cells expressing the wild type H-2Ld molecule (27,27), a tail-less H-2Ld molecule (2.2.1.1DD), or an H-2Ld molecule which has undergone deletion of all but seven amino acids of the cytoplasmic domain (911-7 D6), were labeled biosynthetically and immunoprecipitated with R4, mAb 30.5.7, or mAb 28.14.8. The latter two mAbs are external domain-specific, and their binding to H-2Ld is not influenced by the structure of the H-2Ld cytoplasmic domain of both H-2Ld and H-2Db (20)). The molecules immunoprecipitated by R4 and mAb 28.14.8 have identical mobilities on SDS-PAGE (Fig. 1B). Further evidence that R4 and mAb 28.14.8 precipitate the same population of molecules is provided by CNBr digestion analysis of the 45-kDa components of the two immunoprecipitates (Fig. 1C).

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sequence
Panel C, D
noprecipitated from cell lysates with mAb 28.14.8. Cells were cell-surface radioiodinated and lysed. Proteins were immunoprecipitated from cell lysates with mAb 28.14.8 and resolved by SDS-PAGE. The mobilities of the 90-kDa H-2Ld/Db dimer, the 45-kDa H-2Ld/Db*, and 12-kDa β2-m are indicated.

FIG. 1. Detection of cell-surface H-2Ld and H-2Dd class I molecules by R4 and mAb 28.14.8. Panel A, sequence in the single-letter code of the cytoplasmic domain of H-2Ld and H-2Dd. The boxed-in sequence corresponds to the peptide used for immunization. Panel B, ELd3 cells were cell-surface radioiodinated and lysed. Proteins were immunoprecipitated from cell lysates with mAb 28.14.8 (lane 1) or antibody R4 (lane 2) and resolved by SDS-PAGE. The mobilities of the 90-kDa H-2Ld/Db dimer, the 45-kDa H-2Ld/Db*, and 12-kDa β2-m are indicated. Panel C, the 45-kDa H-2Ld/Db* band in each of the immunoprecipitates was eluted, subjected to CNBr digestion, and resolved by SDS-PAGE.

in Fig. 1, these data show that R4 specifically binds to cytoplasmic domain sequences expressed by H-2Ld and H-2Dd molecules.

R4-reactive H-2Ld and H-2Dd Molecules Arise Late in Their Biosynthesis and Are Not Associated with the β2-m Light Chain—SDS-PAGE analysis of R4 immunoprecipitates from surfaceraidiodinated ELd3 cells (Fig. 1B) and overexposure of the autoradiogram in Fig. 2 (data not shown) demonstrate that, unlike mAb 30.5.7 and mAb 28.14.8, R4 binds H-2Ld/Db heavy chains which are not associated with β2-m. Since H-2Ld molecules occur as both β2-m-associated heterodimers and as free heavy chains both early in biosynthesis and at the cell surface (8, 12, 16), it was of interest to determine when the H-2Ld/Db molecules become R4-reactive. ELd3 cells were pulsed with 35S)methionine for 30 min and chased for the times indicated in Fig. 3 prior to immunoprecipitation of H-2Ld/Db molecules with R4, mAb 30.5.7, or mAb 28.14.8. SDS-PAGE resolution of Endo H-digested immunoprecipitates shows that the R4-reactive H-2Ld and H-2Dd molecules appear only after a 60-min chase, are entirely Endo H-resistant, and are not associated with β2-m (Fig. 3, lanes 1–5). In contrast, H-2Ld and H-2Ld/Db class I molecules bound by mAbs 30.5.7 and 28.14.8 occur throughout the various chase periods, exist in both Endo H-sensitive and Endo H-resistant forms, and are associated with β2-m (Fig. 3, lanes 6–15). Approximately 50% of the biosynthetically labeled H-2Ld and H-2Dd molecules in ELd3 cells are Endo H-resistant after a 30-min chase period (Fig. 3, lanes 8 and 13). However, R4-reactive H-2Ld and H-2Dd molecules appear only after a 60-min chase period (Fig. 3, lane 4), and R4 reactivity is greater after 90 min (Fig. 3, lane 5). Thus, H-2Ld/Db* molecules become Endo H-resistant prior to becoming reactive to R4. Similar results were obtained from pulse-chase experiments using 27.5.27 L cell transfectants (data not shown).

Not All β2-m* H-2Ld Molecules Are Reactive with R4—mAb 64.3.7 binds H-2Ld molecules which are not associated with β2-m (8). Unlike R4, however, mAb 64.3.7 binds both Endo H-sensitive and Endo H-resistant H-2Ld molecules (8). To compare the intracellular locations of mAb 64.3.7-reactive and R4-reactive molecules directly, biosynthetically labeled ELd3 cells were disrupted by nitrogen cavitation, and subcellular organelles were separated by density centrifugation as described under “Materials and Methods.” Class I MHC molecules were...
immunoprecipitated from gradient fractions with mAb 64.3.7 or with R4, digested with Endo H, and resolved by SDS-PAGE. The vast majority of the mAb 64.3.7-reactive H-ZLd molecules are Endo H-sensitive and occur in denser gradient fractions (Fig. 4A, lanes 5–8). Lesser amounts of mAb 64.3.7-reactive H-2Ld molecules are Endo H-resistant and occur in the less dense fractions of the gradient (Fig. 4A, lanes 2–4). In contrast to mAb 64.3.7, R4 precipitates only Endo H-resistant H-2Ld/Db class I molecules which occur in less dense fractions (Fig. 4B). These R4-reactive H-2Ld/Db molecules occur in fractions which include the plasma membrane (data not shown). Similar results have been obtained with cells which express only H-2Ld and not H-2Dd (data not shown).

**R4 Binds Phosphorylated H-2Ld/Db Class I Molecules**—Class I MHC molecules undergo phosphorylation in vivo at serine residues in the cytoplasmic domain (25). Phosphorylation of class I MHC molecules has been shown to be a post-endoplasmic reticulum, and presumably a cell surface, event (27). Since H-2Ld/Db molecules acquire R4 reactivity late in biosynthesis, we were interested in determining if R4-reactive molecules are phosphorylated in vivo. EL3 cells were labeled biosynthetically for 90 min, then mAb 30.5.7, or mAb 28.14.8. Immunoprecipitated proteins were digested with Endo H, then resolved by SDS-PAGE under nonreducing conditions. Fig. 5A is an autoradiogram resulting from a direct exposure of the x-ray film. Fig. 5B was generated by placing a piece of paper between the dried gel and the x-ray film, thereby preventing exposure of the film by 35S emissions (22). This analysis shows that all three antibodies precipitate phosphorylated H-2Ld molecules which are Endo H-resistant (compare lanes 2 and 3 of Fig. 5A to lanes 2 and 3 of Fig. 5B). Since mAb 30.5.7 precipitates exclusively β2-m-associated H-2Ld molecules from β2-m+ cells (28, 29), and since mAb 30.5.7-reactive H-2Ld molecules are phosphorylated (Fig. 5B, lane 2), phosphorylation of the H-2Ld heavy chain does not require loss of β2-m. In addition to phosphorylated H-2Ld/Db heavy chains, mAb 28.14.8 and R4 also bind phosphorylated H-2Ld/Db heavy chain dimers (Fig. 5B, lanes 1 and 3), which have been shown to arise late in biosynthesis in β2-m+ cells (16).

35S/32P-labeled proteins immunoprecipitated by mAbs 30.5.7 and 28.14.8 and by R4 were analyzed further by two-dimensional gel electrophoresis. (Heavy chain dimers are not seen on these gels because the two-dimensional gels were run under reducing conditions.) H-2Ld molecules precipitated from 35S/32P-labeled EL3 cells by mAb 30.5.7 (Fig. 6A) display marked heterogeneity. However, the autoradiogram generated by placing a piece of paper between the dried gel and the x-ray film shows that only a subset of these molecules is phosphorylated (Fig. 6B). Indeed, mAb 30.5.7-precipitable 32P-labeled H-2Ld molecules bound by Endo H prior to SDS-PAGE resolution. Panel A is a direct exposure of the dried gel. Panel B was generated by placing a piece of paper between the gel used to generate panel A and the x-ray film, thereby preventing exposure by 35S emissions. Mobilities of Endo H-resistant H-2Ld molecules are shown in panel A. H-2Ld molecules bound by Endo H-resistant H-2Ld/Db dimers (H-2Dimer) are Endo H-resistant H-2Ld/Db heavy chains (H-2 EH'), Endo H-cleaved H-2Ld/Db heavy chains (H-2 EH'), β2-m, and free 32PO4 are indicated.

The Cytoplasmic Domain Tyrrosine of Only a Subset of H-2Ld Molecules Is Accessible to Radioiodination—We next used radioiodination of membrane vesicles to determine if the inaccessibility of Endo H-sensitive H-2Ld molecules to R4 is unique to this probe or reflects a general inaccessibility of the cytoplasmic tail. Membranous vesicles generated by gentle cavitation of EL3 cells were either radioiodinated and lysed or radioiodinated. H-2Ld molecules then were precipitated with mAb 30.5.7. Under the cavitation conditions used, the external domains of class I molecules remain inside the lumena of the vesicles generated by cavitation and thus are protected from radioiodination, while their cytoplasmic domains extrude from the vesicles and are available to radioiodination (Fig. 7A). Two-dimensional gel electrophoretic resolution of these samples showed that the β2-m co-precipitated with H-2Ld molecules is not radioiodinated (Fig. 7C). In contrast, radioiodinated β2-m occurs in mAb 30.5.7 immunoprecipitates of H-2Ld molecules from radioiodinated lysates (Fig. 7B).
peptide from these cell lysates (Fig. 8, panel 3). Furthermore, precipitation of H-2L<sup>d</sup> molecules by R4 does not deplete L<sup>d</sup> peptide-binding H-2L<sup>d</sup> molecules reactive with mAb 30.5.7 (Fig. 8, panel 4).

Based on their studies of the cytoplasmic domain of H-2K<sup>β</sup>, Smith and Barber (13) suggested that dissociation of a class I MHC heavy chain from β<sub>2</sub>-m induces a conformational change in the cytoplasmic domain, rendering it accessible to antibodies specific for exon 7-encoded sequences. Other studies have shown that addition of antigenic peptide and β<sub>2</sub>-m induces a conformational change in the class I MHC molecule (8, 18, 28–31). Of particular interest here was the demonstration by Hansen and colleagues (8) that addition of peptide to cell lysates results in a depletion of Endo H-sensitive, mAb 64.3.7-reactive H-2L<sup>d</sup> molecules and a concomitant increase in Endo H-sensitive, mAb 30.5.7-reactive H-2L<sup>d</sup> molecules. To determine if R4-reactive H-2L<sup>d</sup> molecules could be similarly affected by specific peptide and β<sub>2</sub>-m, EL<sup>d</sup>/3 cells were labeled biosynthetically for 2 h and lysed, and the lysates were supplemented with the peptide and β<sub>2</sub>-m additions indicated in the legend to Fig. 9. Under the labeling conditions employed, both Endo H-sensitive and Endo H-resistant H-2L<sup>d</sup> and H-2D<sup>β</sup> molecules are biosynthetically labeled, as detected by mAbs 30.5.7 and 64.3.7 (H-2L<sup>d</sup>, Fig. 9, lanes 1–4) and mAb 28.14.8 (H-2L<sup>d</sup> and H-2D<sup>β</sup>, Fig. 9, lanes 5–6). Addition of L<sup>d</sup> peptide and β<sub>2</sub>-m to cell lysates prior to immunoprecipitation causes an increase in Endo H-sensitive H-2L<sup>d</sup> in mAb 30.5.7 immunoprecipitates and a decrease in Endo H-sensitive H-2L<sup>d</sup> in mAb 64.3.7 immunoprecipitates, but does not affect the Endo H-resistant H-2L<sup>d</sup> (Fig. 9, lanes 2 and 4). Similarly, addition of L<sup>d</sup> peptide, D<sup>β</sup>-peptide, and β<sub>2</sub>-m to cell lysates results in an increase of Endo H-sensitive H-2L<sup>d</sup>D<sup>β</sup> precipitated by mAb 28.14.8, again without affecting the Endo H-resistant H-2L<sup>d</sup>D<sup>β</sup> molecules (Fig. 9, lane 6). Only Endo H-resistant H-2L<sup>d</sup> is observed in R4 immunoprecipitates, and its quantity is unchanged by the addition of L<sup>d</sup> peptide, D<sup>β</sup>-peptide, and β<sub>2</sub>-m (Fig. 9, lanes 7 and 8). These results show that H-2L<sup>d</sup>D<sup>β</sup> molecules bound by R4 are unable to bind peptide and to undergo a peptide- and β<sub>2</sub>-m-induced conformational change in vitro.

**DISCUSSION**

In this paper we have examined the accessibility of the cytoplasmic domain of H-2L<sup>d</sup> and H-2D<sup>β</sup> class I MHC molecules to immunological and biochemical probes. We show that the cytoplasmic domain-specific antibody R4 detects H-2L<sup>d</sup>D<sup>β</sup> molecules which are not associated with β<sub>2</sub>-m. Previous studies showed that a relatively large pool of free H-2L<sup>d</sup> heavy chains occurs in β<sub>2</sub>-m<sup>+</sup> cells (8). However, R4 immunoprecipitates only a small proportion of the free H-2L<sup>d</sup> heavy chains in a cell.

**Cellular Location and Genesis of R4-reactive H-2L<sup>d</sup>/D<sup>β</sup> Molecules—**R4-reactive molecules are characteristically Endo H-resistant and electrophoretically homogeneous. Consistent with a postmedial Golgi localization, R4-reactive H-2L<sup>d</sup>D<sup>β</sup> class I molecules occur in less dense fractions (which include the plasma membrane) on density gradients. R4-reactive H-2L<sup>d</sup>D<sup>β</sup> molecules occurring at the cell surface exist both as free heavy chains and as heavy chain dimers. While H-2L<sup>d</sup> and H-2D<sup>β</sup> have been shown to be transported to the cell surface in a β<sub>2</sub>-m<sup>+</sup> form, we believe that R4-reactive molecules arise from H-2L<sup>d</sup>β<sub>2</sub>-m heterodimers after the dissociation of β<sub>2</sub>-m. This conclusion stems from two observations. First, R4-reactive molecules arise late in the biosynthesis of H-2L<sup>d</sup> even though free H-2L<sup>d</sup> heavy chains occur early in biosynthesis. Secondly, H-2L<sup>d</sup> molecules synthesized in β<sub>2</sub>-m<sup>+</sup> cells do not become phosphorylated even though they are expressed at the cell surface.  

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**Inaccessibility of the Class I MHC Cytoplasmic Domain**

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**Fig. 6. Two-dimensional gel analysis of H-2L<sup>d</sup>/D<sup>β</sup> phosphoproteins precipitated by R4 and mAb 28.14.8.** mAb 30.5.7 (panels A and B), mAb 28.14.8 (panel C), and R4 immunoprecipitates (panel D) from [35S]methionine- and [32P]Pi-labeled EL/3 cell lysates were resolved first by non-equilibrium pH gel electrophoresis (NEPHGE) under reducing conditions and then by SDS-PAGE under reducing conditions. A was generated by a direct exposure of the x-ray film, while B-D were generated by placing a piece of paper between the dried gels and the x-ray film. The mobilities of β<sub>2</sub>-m and the H-2L<sup>d</sup>/D<sup>β</sup> proteins are indicated.

**Fig. 7.** The lack of association of R4-reactive H-2L<sup>d</sup>/D<sup>β</sup> molecules with P2-m occurs late in the biosynthesis of H-2L<sup>d</sup> even though free H-2L<sup>d</sup> heavy chains occur in β<sub>2</sub>-m<sup>+</sup> cells (8). However, R4 immunoprecipitates only a small proportion of the free H-2L<sup>d</sup> heavy chains in a cell.
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Since both R4-reactive free H-2L\(^d\) heavy chains and \(\beta_2\)-m-associated (mAb 30.5.7-reactive) H-2L\(^d\) molecules are phosphorylated, R4-reactive H-2L\(^d\) molecules may arise from mAb 30.5.7-reactive H-2L\(^d\) molecules which have dissociated from peptide and \(\beta_2\)-m. H-2L\(^d\) molecules are likely to become R4-reactive at the cell surface since there is a delay between acquisition of resistance to Endo H by H-2L\(^d\) molecules and their reactivity to R4 (as shown by comparison of R4 and mAb 30.5.7/mAb 28.14.8 immunoprecipitates in Fig. 3).

The Nature of the Cytoplasmic Domain Inaccessibility—A highly conserved tyrosine occurs in the cytoplasmic domains of most class I MHC molecules (26). This tyrosine is separated from the epitope recognized by R4 (peptide H-13) by only four amino acids (Fig. 1A). The studies shown in Fig. 7C demonstrate that when membrane vesicle preparations are radioiodinated only a subset of H-2L\(^d\) molecules is labeled at the cytoplasmic domain tyrosine. The two-dimensional gel profile of these molecules is similar to that of R4-reactive H-2L\(^d\) molecules (Fig. 6 and data not shown).

What then might influence accessibility of the H-2L\(^d\) cytoplasmic domain to R4 and to \(^{125}\)Iiodine? The portion of the cytoplasmic domain of H-2L\(^d\)/Db which is recognized by R4 corresponds to the same epitope recognized by an antibody specific for the cytoplasmic domain of H-2K\(^b\) (13). This particular H-2K\(^b\)-specific antibody also binds free H-2K\(^b\) heavy chains but not \(\beta_2\)-m-associated H-2K\(^b\) molecules. It was suggested that association-disassociation with \(\beta_2\)-m causes a conformational change in the cytoplasmic domain of the H-2K\(^b\) molecule which influences its reactivity with this anticytoplasmic domain antibody (13). With this model in mind, we examined the ability of R4-reactive H-2L\(^d\) and H-2D\(^b\) molecules and of free H-2L\(^d\) molecules to bind specific peptides and \(\beta_2\)-m. Addition of H-2L\(^d\)- and H-2D\(^b\)-specific antigenic peptides and exogenous \(\beta_2\)-m affects the assembly of virtually all of the Endo H-sensitive, mAb 64.3.7-reactive, free H-2L\(^d\) heavy chains into mAb 30.5.7-reactive complexes (Fig. 9, lanes 1–4). In contrast, peptide and \(\beta_2\)-m have no effect on the H-2L\(^d\)/Db molecules precipitable by R4 or on the Endo H-resistant H-2L\(^d\) molecules precipitable by mAbs 64.3.7, 30.5.7, and 28.14.8. These studies do not exclude the possibility that R4-reactive molecules fail to

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**Fig. 7. Immunoprecipitation analysis of H-2L\(^d\) molecules radioiodinated in membrane vesicles.** Unlabeled EL3 cells were disrupted by nitrogen cavitation, and the cavitate was either lysed prior to radioiodination (panel B) or radioiodinated prior to lysis (panel C). A schematic for this experiment is shown in panel A in which class I molecules in cell cavitates are shown to have only their cytoplasmic domains exposed and to be accessible to radioiodination, while all portions of the class I molecule are accessible to radioiodination in cell lysates. The starbursts (*) represent potential radioiodinated tyrosines (26). The unknown modification of the H-2L\(^d\) cytoplasmic domain which renders it inaccessible to radioiodination is indicated by the question mark covering the cytoplasmic portion of the molecule. mAb 30.5.7 immunoprecipitates of EL3-derived H-2L\(^d\) molecules precipitated from radioiodinated lysates (panel B) and radioiodinated cavitates (panel C) were resolved first by non-equilibrium pH gel electrophoresis (NEPHGE) under reducing conditions, then SDS-PAGE under reducing conditions (as in Fig. 6). Mobilities of the H-2L\(^d\) heavy chain and \(\beta_2\)-m are indicated.
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Association of the external domains with peptide and $\beta_2$-m. Another argument against this particular conformational change model is the finding that only a small fraction of free H-2Ld heavy chains in a cell is R4-reactive (Figs. 4 and 9).4 The amount of R4-reactive H-2Ld/Db does not increase during a prolonged label.2 It is therefore tempting to speculate that R4 reactivity occurs soon before degradation of dysfunctional H-2Ld/Db molecules. Ongoing studies in our laboratory are directed toward investigation of this possibility.

Phosphorylation of the cytoplasmic domain serines on the H-2Ld molecule might also influence R4 reactivity, especially since the immunogenic peptide bound by R4 includes serines which potentially can be phosphorylated (25), and some, if not all, R4-reactive H-2Ld molecules are phosphorylated. Because the immunogenic peptide was not phosphorylated prior to immunization, it is unlikely that phosphorylation per se determines R4 reactivity. However, phosphorylation of the cytoplasmic domain could influence R4 binding by affecting the conformation of the cytoplasmic domain or altering interactions of the cytoplasmic domain with other proteins. An obvious way to distinguish between these possibilities is to determine the R4 reactivity of native and denatured H-2Ld/Db molecules. Accordingly, Western blot analysis of R4-reactive molecules is in progress.

Implications for Intracellular Transport of Class I MHC Molecules and Other Integral Membrane Proteins—The inaccessibility of the cytoplasmic domain of the H-2Ld molecule to antibody probes and radiolabeling until late in biosynthesis is reminiscent of the reduced accessibility of the cytoplasmic domain of vesicular stomatitis virus G protein to proteases early in biosynthesis (32). These observations are interesting in light of previous studies which demonstrated that H-2Ld molecules with full-length cytoplasmic domains are transported through the cell more rapidly than are H-2Ld molecules with truncated cytoplasmic tails (12). The facilitation of intracellular transport by the cytoplasmic domain is not unique to H-2Ld; in fact, it has been reported for a number of integral membrane proteins (33–37). However, there is no similarity in cytoplasmic tail length or sequence of the various proteins examined in these studies (12, 32–37). It is tempting to propose that facilitation of intracellular transport by the cytoplasmic domains of integral membrane proteins destined for the cell surface is due to their association with intracellular components and that cytoplasmic domain accessibility to biochemical and immunological probes late in biosynthesis is due to dissociation from these components. Ongoing studies are directed at determining the relative contributions of conformation, phosphorylation, and association with cellular proteins to differential cytoplasmic domain accessibility during intracellular transport.

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REFERENCES

1. Zinkernagel, R. M., and Doherty, P. C. (1978) Adv. Immunol. 27, 51–177
2. Wallny, H. J., and Rammeese, H. G. (1990) Nature 343, 275–278
3. Townsend, A. R. M., Gotch, F. M., and Davve, J. (1985) Cell 42, 457–467
4. Fremont, D. H., Matsuzuma, M., Sturgis, R. A., Peterson, P. A., and Wilson, I. A. (1992) Science 257, 919–927
5. Bjorkman, P. J., Strominger, J. L., and Wiley, D. C. (1985) J. Mol. Biol. 188, 205–210
6. Deveron, E. V., Gow, I. R., Coadwell, W. J., Monaco, J. D., Butcher, G. W., and Howard, J. C. (1990) Nature 348, 738–741
7. Degen, J., Cohen, D. M., and Williams, D. B. (1992) J. Exp. Med. 175, 1653–1661
8. Smith, J. D., Lie, W. R., Gorka, J., Kindele, C. S., Myers, N. B., and Hansen, T. H. (1992) J. Exp. Med. 176, 191–202

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9. Townsend, A., Ohlen, C., Foster, L., Bastin, J., Ljunggren, H. G., and Karre, K. (1989) Cold Spring Harbor Symp. Quant. Biol. 1, 299–308
10. Ploegh, H. L., Orr, H. T., and Strominger, J. L. (1981) Cell 24, 287–299
11. Owen, M. J., Kissocergia, A., M., and Lodish, H. F. (1986) J. Biol. Chem. 355, 9678–9684
12. Zübita, M. C., and Hood, L. E. (1986) J. Cell Biol. 102, 1–10
13. Smith, M. H., and Barber, B. H. (1990) Mol. Immunol. 27, 169–180
14. Zübita, M. C., Malissen, B., McMillan, M., Brayton, P. R., Clark, S. S., Forman, J., and Hood, L. (1983) Cell 34, 53–54
15. Capps, G. G., Van Kampen, M., Ward, C. L., and Zübita, M. C. (1989) J. Cell Biol. 108, 1317–1329
16. Capps, G. G., Robinson, B. E., Lewis, K. D., and Zübita, M. C. (1993) J. Immunol. 151, 159–169
17. Reddehase, M. J., Rothbard, J. B., and Koszinowski, U. H. (1989) Nature 337, 651–653
18. Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B., and Tse, A. (1990) Cell 62, 285–296
19. Moore, M. W., Carbone, F. R., and Bevan, M. J. (1988) Cell 54, 777–785
20. Evans, G. A., Margules, D. H., Shykind, B., Seidman, J. G., and Ozato, K. (1983) Nature 300, 765–767
21. Shiraiishi, T., Evans, G. A., Appella, E., and Ozato, K. (1985) J. Immunol. 134, 623–629
22. Capps, G. G., and Zübita, M. C. (1990) Biotechniques 8, 62–69
23. Keški-Oj, J., Mosher, D. F., and Vaheri, A. (1977) Biochim. Biophys. Res. Commun. R74, 699–706
24. Jones, P. A. (1980) in Selected Methods in Cellular Immunology (Mishell, D., and Shiigi, S., eds) pp. 398–440, W. H. Freeman and Co., San Francisco
25. McCluskey, J., Boyd, L. F., Malory, W. L., Coligan, J. E., and Margulies, D. H. (1986) EMBO J. 5, 2477–2483
26. Pullen, J. K., Horton, R. M., Cal, Z. L., and Pease, L. R. (1992) J. Immunol. 148, 953–957
27. Lippe, R., Luhe, E., Khua, Y. T., Lomas, C., and Jeffries, W. A. (1991) J. Exp. Med. 174, 1159–1166
28. Lee, W. R., Myers, N. B., Gorka, J., Rubocki, R. J., Connolly, J. M., and Hansen, T. H. (1990) Nature 344, 439–441
29. Lie, W. R., Myers, N. B., Connolly, J. M., Gorka, J., Lee, D. R., and Hansen, T. H. (1991) J. Exp. Med. 173, 449–459
30. Myers, N. B., Lee, W. R., Nett, M., Rubocki, R. J., and Hansen, T. H. (1989) J. Immunol. 142, 2751–2758
31. Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H. G., Foster, L., and Karre, K. (1989) Nature 340, 443–448
32. Mack, D., Kluxen, B., and Kruppa, J. (1989) J. Cell Biol. 109, 2057–2065
33. Doyle, C., Roth, M. G., Sambrook, J., and Gething, M. J. (1985) J. Cell Biol. 100, 704–714
34. Doyle, C., Sambrook, J., and Gething, M. J. (1986) J. Cell Biol. 103, 1199–1204
35. Parks, G. D., and Lamb, R. A. (1990) J. Virol. 64, 3605–3616
36. Raviprakash, K., Rasile, L., Gheas, K., and Ghokh, H. P. (1989) J. Biol. Chem. 266, 1777–1782
37. Doma, R. W., Rueda, A., Machamer, C., Helenius, J., Helenius, A., and Rose, J. K. (1988) J. Cell Biol. 107, 89–99