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Immunology

Complete amino acid sequence of murine β_2-microglobulin: Structural evidence for strain-related polymorphism

(histocompatibility antigens/radiosequence analysis)

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ABSTRACT **Primary structural analyses of β_2-microglobulin isolated from the tumor cell lines EL4.BU (derived from a C57BL/6 mouse) and C14 (derived from a BALB/c mouse) have revealed the presence of an amino acid difference at position 85 of this molecule. β_2-Microglobulin isolated from histocompatibility antigens of EL4.BU has alanine at this position, whereas that from C14 has aspartic acid. Determination of the sequence of these molecules has employed radiochemical methodology that was developed in studies of murine histocompatibility antigens. The sequence obtained in this study is: Ile - Gln - Lys - Thr - Pro - Gln - Ile - Gln - Val - Tyr - Ser - Arg - His - Pro - Pro - Glu - Asn - Gly - Lys - Pro - Asn - Ile - Leu - Asn - Cys - Tyr - Val - Thr - Gln - Phe - His - Pro - Pro - His - Ile - Glu - Ile - Gln - Met - Leu - Lys - Asn - Gly - Lys - Lys - Ile - Pro - Lys - Val - Glu - Met - Ser - Asp - Met - Ser - Phe - Ser - Lys - Asp - Trp - Ser - Phe - Tyr - Ile - Leu - Ala - His - Thr - Glu - Phe - Thr - Pro - Thr - Glu - Thr - Asp - Thr -Tyr - Ala - Cys - Arg - Val - Lys - His - Ala/Asp - Ser - Met - Ala - Glu - Pro - Lys - Thr - Val - Tyr - Trp - Asp - Arg - Asp - Met. Comparison of the sequence of murine β_2-microglobulin to the sequences reported for the homologues from man, rabbit, and guinea pig indicate identities of 68%, 66%, and 61%, respectively.**

β_2-Microglobulin (β_2-M) (molecular weight 11,800) was discovered in human urine (1), and has been found to be noncovalently associated with several membrane antigens (2), including the major histocompatibility antigens of man (HLA), mouse (H-2), and other species (3–5). Despite its known attachment to various cell surface proteins, the function of β_2-M remains unclear, although there is some evidence that the molecule may stabilize the tertiary structure of associated proteins (6). The complete amino acid sequence has been determined for the human molecule (7), as well as for the rabbit (8) and guinea pig (9) homologues. A partial sequence of the first 40 residues of mouse β_2-M has been reported (10).

In the course of establishing the primary structure of the H-2^d and H-2^b alloantigens of the mouse by radiosequence techniques (11–14), significant amounts of radiolabeled β_2-M have been copurified. The present work describes the determination of the primary structure of murine β_2-M entirely by radiosequence methodology and documents the discovery of a polymorphism in the structures of the β_2-M isolated from tumor cell lines derived from two different mouse strains.

MATERIALS AND METHODS

Preparation of Radiolabeled Murine β_2-M. Radiolabeled amino acids were incorporated into murine tumor cell lines EL4.BU(H-2^b), a lymphoblastoid cell line derived from C57BL/6 mice, and C14 (H-2^d), an Abelson virus-induced cell line derived from BALB/c mice (12). The H-2K^b, H-2K^d, and H-2D^d alloantigens were isolated by specific immunoprecipitation as described (11–13). The associated β_2-M was separated by chromatography on a column (190 × 2 cm) of Sephadex G-75 in 1 M formic acid (11–13).

Cleavage with CNBr. Radiolabeled β_2-M was dissolved in 4 m of 70% (vol/vol) formic acid and allowed to react with 1.1 g of CNBr (Eastman Kodak) in the presence of 40 mg of sperm whale myoglobin (Sigma) as described (11, 12). Resulting peptides are indicated by C.

Citraconylation. Radiolabeled β_2-M was dissolved in 2 ml of 2 M guanidine hydrochloride (Gdn·HCl)/50 mM sodium borate, pH 8.3, together with 2 mg of horse heart cytochrome *c* (Sigma). A total of 50 μl of citraconic anhydride (Eastman Kodak) was added in 10 aliquots, while the pH was maintained between 8 and 9 with 2 M NaOH. Thirty minutes after the last addition, the citraconylated material was desalted on a column of Sephadex G-10 (30 × 2 cm) equilibrated with 20 mM NH_4HCO_3, pH 8.0, and lyophilized. Citraconyl groups were removed by incubating peptides in 50% (vol/vol) acetic acid for 4 hr at room temperature, followed by dilution to 10% acetic acid and lyophilization.

Reduction and Alkylation. Lyophilized C1 peptides containing 2–4 mg of carrier protein (horse heart cytochrome *c*) were dissolved in 2 ml of 6 M Gdn·HCl/0.8 M Tris·HCl, pH 8.2/10 mM EDTA and flushed with N_2 for 5 min. After the addition of 0.4 ml of 0.1 M dithiothreitol, the mixture was stirred for 1 hr at room temperature. After the addition of 17.4 mg of iodoacetic acid (Sigma), the reaction mixture was stirred in the dark for 20 min prior to gel filtration on a column (0.9 × 200 cm) of Sephadex G-50 superfine equilibrated in 2 M formic acid.

Lyophilized T1T1 peptides were dissolved in 3 ml of 6 M Gdn·HCl/0.2 M *N*-methylmorpholine, pH 8.6, and flushed with N_2. Thirty microliters of 0.1 M dithiothreitol was added and the reaction mixture was incubated at 50°C for 30 min under N_2. A total of 0.15 ml of 1 M *N*-(iodoethyl)trifluoroacetamide (Aminoethyl-8, Pierce) in methanol was added in two aliquots over a period of 2 hr. Aminoethylated peptides were desalted on a column (1.5 × 25 cm) of Sephadex G-25 in 2 M formic acid and lyophilized.

Enzymatic Digestion of Peptides. Peptides C1A and T1 were further digested by using 1% trypsin [treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; Worthington] for 2 hr at 37°C, whereas C1B peptides were digested with 1% *Staphylococcus aureus* V8 protease (V8) (Miles) for 24 hr at 37°C in 25 mM NH_4HCO_3, pH 8.1. The citraconylated β_2-M and the aminoethylated T1T1 peptides were digested by using 2% trypsin for 4 hr at 37°C in 25 mM NH_4HCO_3, pH 8.1.

Desalting of Peptides. All material pooled from columns equilibrated in 6 M Gdn·HCl was desalted on a Sephadex G-10

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Abbreviations: β_2-M, β_2-microglobulin; HPLC, high-pressure liquid chromatograph; Gdn·HCl, guanidine hydrochloride; V8, *Staphylococcus aureus* V8 protease.

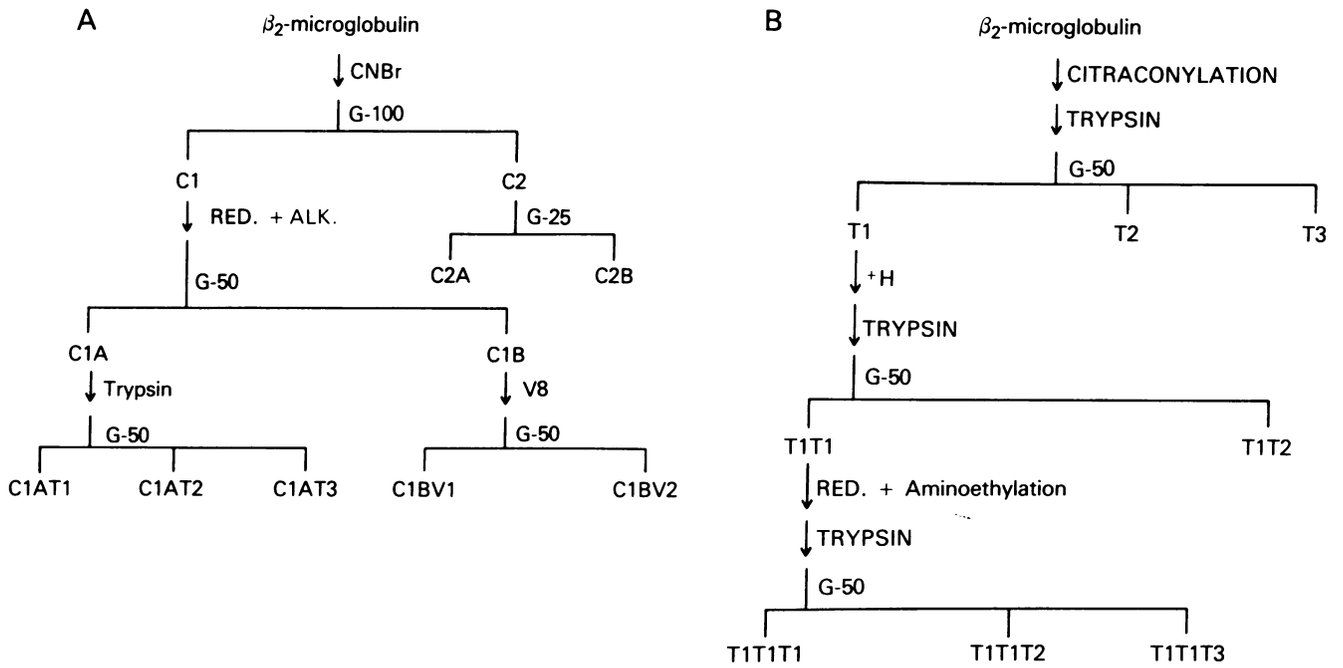

FIG. 1. Flow diagram for the purification of overlapping peptides from β_2-M. (*A*) Production of CNBr-derived peptides. (*B*) Production of tryptic peptides. Enzymatic digestions and chemical modifications—reduction (Red.), alkylation (Alk.), and aminoethylation—are described in *Materials and Methods*. G-100, G-50, and G-25 refer to Sephadex gel filtration chromatography as described in *Results*.

column (2 × 30 cm) in 25 mM NH_4HCO_3 and subsequently lyophilized.

Automated Sequence Analysis. Details of the radiochemical amino acid sequencing methodology have been published (13, 14). Radioactive phenylthiohydantoin amino acid derivatives were identified by cochromatography with unlabeled standard phenylthiohydantoin amino acid derivatives on a high-pressure liquid chromatograph (HPLC), using the isocratic elution conditions of Gates *et al.* (8).

RESULTS

Isolation of Radiolabeled Murine β_2-M. Two murine tumor cell lines, C14 and EL4.BU, were cultured in the presence of various groups of radiolabeled amino acids. The H-2K^b, H-2K^d, and H-2D^d antigens were isolated from a membrane glycoprotein pool by specific immunoprecipitation, and the associated radiolabeled β_2-M was purified by gel chromatography. Amino-terminal radiosequence analysis of the isolated material dem-

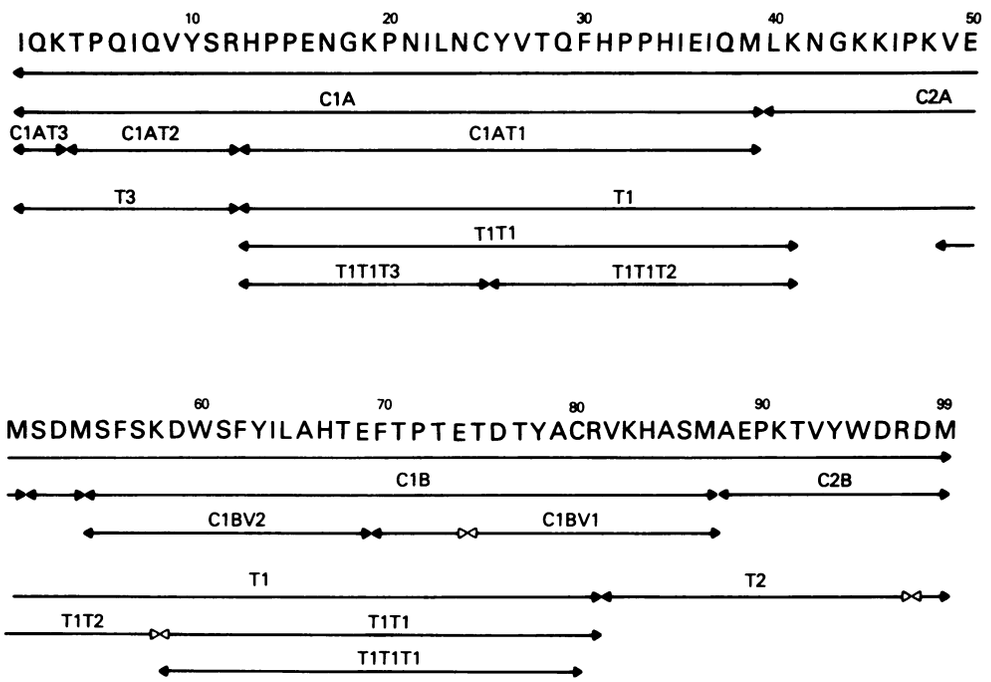

FIG. 2. Summary of amino acid sequence information for murine β_2-M. Amino acid residues are listed by the single-letter code (15). Assignment of residues in peptides is given in Table 1. Residue 85 is an alanine in EL4.BU cells, an aspartic acid in C14 cells. Open arrows indicate partial cleavage sites.

Table 1. Identification of amino acid residues in peptides

Residue no.	Amino acid*	Peptide†	Labeling groups‡	Identification§	Residue no.	Amino acid*	Peptide†	Labeling groups‡	Identification§
1	I	N	E2, C1, C2	LC	51	M	C2A/T1T2	EC2/EC5	LC
2	Q	N	E6	LC	52	S	T1T2	EC5	LC
3	K	N/C1A	E1, E7, C1/E12	LC	53				
4	T	N/C1AT2	E2, C2/C3	LC	54	M	T1T2	EC5, C7	LC
5	P	N	E1, E3, E10	LC	55	S	C1B/T1T2	E11, EC1/EC5	LC
6	Q	N	E6	LC	56	F	C1B/C1BV2	C3/E14, C3	LC
7	I	N/C1AT2	E2, C1, C2/C3	LC	57	S	C1B/T1T2	E11, EC1/EC5	LC
8	Q	N	E6	LC	58	K	C1B/C1BV2/T1T2	E12/E14/C6	LC
9	V	N/C1AT2	E2, C2/C3	LC	59				
10	Y	N	E1, C1	LC	60	W	C1B/C1BV2	EC1, C3/C3	LC
11	S	N	E8	^{3}H	61	S	C1B/T1T1T1	E11, EC1/EC5	LC
12	R	N/C1A	C1/E12	LC	62	F	C1B/C1BV2	C3/E14, C3	LC
13	H	N/C1AT1	E2, C2/EC1, C3	LC	63	Y	C1BV2	E14	LC
14	P	N/C1AT1	E1, E3, E6, E10/EC2	LC	64	I	C1B/C1BV2	EC1, C3/C3	LC
15	P	N/C1AT1	E1, E3, E6, E10/EC2	LC	65	L	C1BV2	E14	LC
16	E	N/C1AT1	E3, E6/EC2	LC	66	A	C1BV2	E14	LC
17	N	N/C1AT1	E9/E9	LC	67	H	C1B/C1BV2	EC1, C3/C3	LC
18	G	N/C1AT1	E4/EC1	LC	68	T	C1B/C1BV2	EC1, C3/C3	LC
19	K	N/C1A	E1, E7, C1/E12	LC	69	E	C1BV2/T1T1T1	EC2/C8	LC
20	P	N/C1AT1	E1, E3, E10/EC2	LC	70	F	C1B/C1BV1	C3/EC2, E14, C3	LC
21	N	N/C1AT1	E9/E9	LC	71	T	C1B/C1BV1	EC1, C3/C3	LC
22	I	N/C1AT1	E2, C1, C2/EC1, C3	LC	72	P	C1BV1	EC2, E14	LC
23	L	N/C1AT1	E1, C1/EC2	LC	73	T	C1B/C1BV1	EC1, C3/C3	LC
24	N	N/C1AT1	E9/E9	LC	74	E	C1BV1	EC2	LC
25	C	N/C1AT1	E1, E5, E11	^{35}S	75	T	C1B/C1BV1	EC1, C3/C3	LC
26	Y	N	E1, C1	LC	76				
27	V	N/C1AT1	C2/EC1, C3	LC	77	T	C1B/C1BV1	EC1, C3/C3	LC
28	T	N/C1AT1	C2/EC1, C3	LC	78	Y	C1BV1	E14	LC
29	Q	N/C1AT1	E6/C4	LC	79	A	C1BV1	E14	LC
30	F	N/C1AT1	E1, C1/EC2, C3	LC	80	C	C1B	E11	^{35}S
31	H	N/C1AT1	E2, C2/EC1, C3	LC	81	R	C1B/C1BV1	E12/E14	LC
32	P	N/C1AT1	E10/EC2	LC	82	V	C1B/C1BV1/T2	EC1, C3/C3/EC3	LC
33	P	N/C1AT1	E10/EC2	LC	83	K	C1B/C1BV1/T2	E12/EC2, E14/EC4, C6	LC
34	H	N/C1AT1	C2/C3	LC	84	H	C1B/C1BV1/T2	EC1, C3/C3/EC3	LC
35	I	N/C1AT1	C2/C3	LC	85	A	T2	EC4, E15	LC
36	E	C1AT1	EC2, C4	LC	86	S	T2	EC5	LC
37	I	N/C1AT1	C2/C3	LC	87	M	C1BV1/T2	EC2/EC3, EC5, C7	LC
38	Q	C1AT1	C4	LC	88	A	C2B/T2	E14/EC4, E15, C5	LC
39	M	N	E7	^{35}S	89	E	C2B/T2	EC2/C6	LC
40	L	C2A	EC2, E14, C6	LC	90	P	C2B	E14, EC2	LC
41	K	N/C2A	E7/EC2, E14, E12, C6	LC	91	K	C2B/T2	E14, EC2, E12/EC4, C6	LC
42	N	N/C2A	E9/E9, C6	LC	92	T	C2B/T2	EC1, E13, C3/EC3	LC
43	G	N/C2A	E4/EC1	LC	93	V	C2B/T2	EC1, E13, C3/EC3	LC
44	K	C2A	EC2, E14, E12, C6	LC	94	Y	C2B/T2	E14, EC2/EC4, C6	LC
45	K	C2A	EC2, E14, E12, C6	LC	95	W	C2B/T2	EC1, E13, C3/EC3	LC
46	I	N/C2A	C2/E13, C3	LC	96				
47	P	C2A	EC2, E14	LC	97	R	C2B/T2	E14, E12/C6	LC
48	K	C2A	EC2, E14, E12, C6	LC	98				
49	V	C2A	EC1, E13, C3	LC	99	M	C2B/T2	EC2/EC3, C7	LC
50	E	C2A	EC2	LC					

* Amino acids are identified by single-letter code (15). Positions that are blank (53, 59, 76, 96, 99, and position 85 in β_2-M from C14 cells) did not radiolabel and are assigned to be aspartic acid residues.

† N refers to NH_2-terminal sequence analysis. Other peptides are as described in Figs. 1 and 2.

‡ Labeling groups are, for EL4.BU cells: E1, [^{3}H]- FALKPY and [^{35}S]C; E2, [^{3}H]- SIGHTVW; E3, [^{3}H]- EP; E4, [^{3}H]- GS; E5, [^{3}H]A and [^{35}S]C; E6, [^{3}H]- QEP; E7, [^{3}H]K and [^{35}S]M; E8, [^{3}H]S; E9, [^{3}H]N; E10, [^{3}H]P; E11, [^{3}H]S and [^{35}S]C; E12, [^{3}H]- RK; E13, [^{3}H]- HITVW and [^{35}S]C; E14, [^{3}H]- FALKPYR and [^{35}S]C; E15, [^{3}H]A; EC1, [^{3}H]- HITVW; EC2, [^{3}H]- FLKY and [^{35}S]M; EC3, [^{3}H]- HITVW and [^{35}S]M; EC4, [^{3}H]- FLAQKY; EC5, [^{35}S]M. For C14 cells they are: C1, [^{3}H]- FRYKIL; C2, [^{3}H]- HITVW; C3, [^{3}H]- HITVWF; C4, [^{3}H]- QE; C5, [^{3}H]A; C6, [^{3}H]- LYRNKQ and [^{35}S]C; C7, [^{35}S]M; C8, [^{3}H]E; EC1, [^{3}H]G; EC2, [^{3}H]- EP; EC3, [^{3}H]- HITVWF; EC4, [^{3}H]- GA; EC5, [^{3}H]S.

§ Identification of residues: LC denotes HPLC; ^{3}H and ^{35}S denote single label.

onstrated that it was homogeneous and that it corresponded in sequence to the murine β_2-M isolated by Appella *et al.* (10). Murine β_2-M could be radiolabeled at levels sufficient for radiosequence analysis with any of the common amino acids except aspartic acid. H-2 antigen preparations labeled with $^{32}PO_4$, [^{3}H]fucose, or [^{3}H]glucosamine did not contain radioactivity in the β_2-M fraction.

Isolation of Peptides. The general scheme for the preparation of the overlapping peptides used to determine the amino acid sequence of murine β_2-M is outlined in Fig. 1. The CNBr

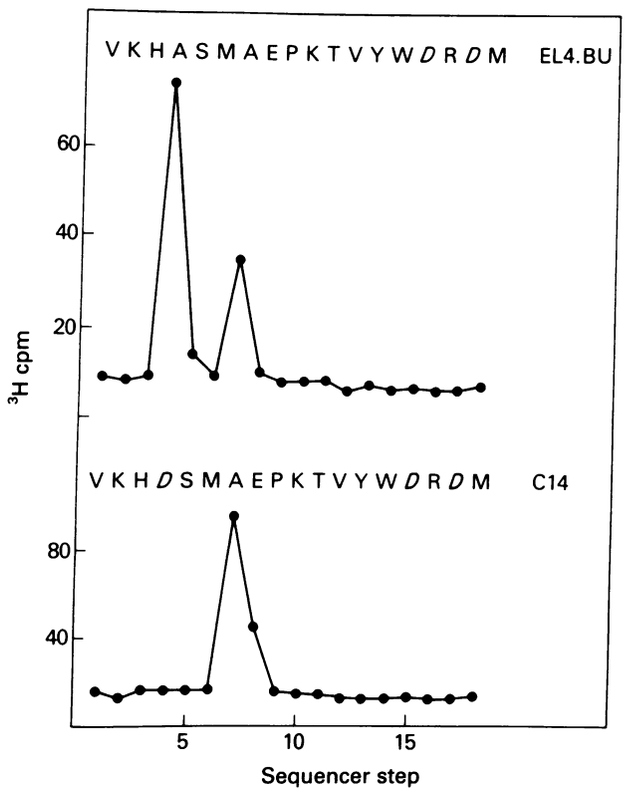

FIG. 3. Radiosequence analysis of [^{3}H]alanine-labeled T2 peptides derived from EL4.BU and C14 cell lines. Automated Edman degradation was performed on T2 peptides labeled with [^{3}H]alanine, and the radioactivity in the fraction obtained from each cycle of degradation was determined in a liquid scintillation counter. (*Upper*) T2 from EL4.BU cells showing [^{3}H]alanine at positions 4 and 7. (*Lower*) T2 from C14 cells showing [^{3}H]alanine only at step 7. Identification of [^{3}H]alanine was confirmed by HPLC analysis.

digest of β_2-M (Fig. 1*A*) yielded two pools, C1 and C2, when chromatographed on a column (1.5 × 145 cm) of Sephadex G-100 in 6 M Gdn·HCl. Pool C2 contained two small peptides which were easily separated on a column (1.5 × 200 cm) of Sephadex G-25 superfine in 1 M formic acid. Upon reduction and alkylation of pool C1, two peptides, C1A and C1B, were obtained which were resolved by chromatography on a column (0.9 × 200 cm) of Sephadex G-50 superfine in 2 M formic acid. Tryptic and V8 enzymatic digests of these two peptides were chromatographed on the same Sephadex G-50 column.

The tryptic digest of citraconylated β_2-M (Fig. 1*B*) yielded three peptides, T1, T2, and T3, when fractionated on a column (1.5 × 145 cm) of Sephadex G-50 in 6 M Gdn·HCl/25 mM Tris·HCl, pH 8.3. After removal of the citraconyl groups with acid, the largest peptide (T1) was digested with trypsin and chromatographed on the same Sephadex G-50 column, yielding peptides T1T1 and T1T2. Peptide T1T1 was subjected to reduction and aminoethylation followed by digestion with trypsin, and the resulting peptides were purified using the same column.

Identification of Residues. As shown in Table 1 and Fig. 2, 94 of the 99 residues of murine β_2-M could be labeled by using radioactive amino acids and identified by HPLC analysis after automated sequence analysis of the whole molecule or its constituent peptides. Of these 94 identifications, 83 were established with β_2-M from EL4.BU cells, and 76 were established with β_2-M from C14 cells. The remaining five positions (residues 53, 59, 76, 96, and 98) were not radiolabeled in any of the labeling groups of Table 1 and have been assigned to be aspartic acid, which has not been used successfully as an intrinsic label in radiosequence analysis (16). These indirectly assigned residues are homologous to aspartic acid residues in β_2-M from other species (7–9).

Secondary Structure of Murine β_2-M. The largest CNBr peptide, C1, can be resolved into two cysteine-containing peptides, C1A and C1B, only after reduction and alkylation. This restriction implies that the β_2-M isolated from H-2 antigens contains an intrachain disulfide bond between residues 25 and 80. Similar results have been obtained in the studies of β_2-M from other species (7–9).

Polymorphism of Murine β_2-M. Although the chromatographic behavior of peptides derived from the β_2-M of either C14 or EL4.BU tumor cells was always similar on gel filtration columns, sequence analysis of the T2 peptides indicated a structural difference. When the two cell lines were intrinsically labeled with [^{3}H]alanine, the resulting T2 peptides gave distinctly different patterns on radiosequence analysis (Fig. 3). HPLC

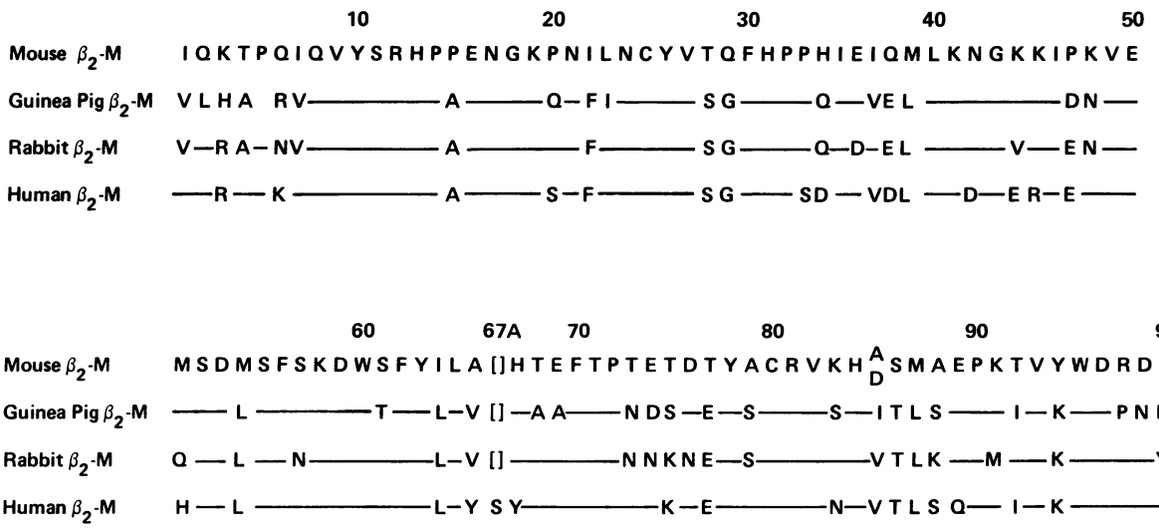

FIG. 4. Comparison of β_2-M sequence data. Brackets at position 67a of the mouse, rabbit, and guinea pig sequences represent an insertion introduced to maximize identity with the human sequence. Only complete sequences have been used for comparison. Mouse β_2-M, this paper; rabbit β_2-M ref. 8; guinea pig β_2-M, ref. 9; human β_2-M, ref. 7. Lines indicate identity with the mouse sequence. Position 85 of the mouse sequence is alanine in EL4.BU cells and aspartic acid in C14 cells.

analysis of the radioactive sequencer steps demonstrated that the T2 peptide from EL4.BU cells (from H-2K^b-associated β_2-M) contained alanine at positions 4 and 7. However, the same peptide isolated from C14 cells (from either H-2K^d-associated or H-2D^d-associated β_2-M) was labeled by [^{3}H]alanine only at position 7. Position 4 in the T2 peptide from C14 material was not labeled in any of the other labeling groups (Table 1), nor was position 16 of the C1BV1 peptide, indicating that this position is occupied by aspartic acid in C14 cells. Although an alanine to aspartic acid conversion is not a conservative amino acid interchange, it may represent a single base change (GCN $\rightarrow$ GA^T_C) in the corresponding codon. As shown in Fig. 3, the remaining 17 residues of peptide T2 from C14 cells are identical to those from EL4.BU cells.

Comparison to Other β_2-M. The amino acid sequence of murine β_2-M is compared in Fig. 4 to the complete sequences established for β_2-M of other species. The mouse sequence is most like the human sequence (68% identity) and slightly less like the rabbit (66%) and guinea pig (61%) sequences. These amino acid differences represent a minimum of 43 nucleotide changes from the human DNA sequence, 44 from the rabbit, and 46 from the guinea pig. Nonconservative amino acid changes, multiple base changes, and multiple amino acid differences between species exist throughout the sequences of these molecules.

DISCUSSION

In the course of purifying intrinsically labeled H-2^d and H-2^b alloantigens, sufficient amounts of the associated murine β_2-M have been copurified to enable radiochemical sequence analysis. The present report documents the determination of the primary structure of murine β_2-M by radiochemical sequence analyses of overlapping CNBr and tryptic peptides. The results are consistent with most of the residues assigned by amino-terminal sequence analysis of β_2-M from spleens and livers of A/J mice (10), except that glutamine has been identified at positions 29 and 38 instead of glutamic acid and aspartic acid, respectively, and methionine was found at position 39 instead of leucine.

The primary structures of the β_2-M from mouse, rabbit, man, and guinea pig are similar, although a number of amino acid interchanges occur throughout the molecule (Fig. 4). Position 6 has a different amino acid in all four β_2-M homologues, and there are 14 other positions in which there are three different amino acid residues. The mouse sequence, like that of the rabbit and guinea pig, is one amino acid shorter than the human sequence, maximal identity being maintained by introducing an insertion between positions 66 and 67. It has been suggested that the serine residue at position 67 of the human sequence is in fact an erroneous assignment (9).

In comparing the amino acid sequence of mouse β_2-M to the sequence for the H-2K^b antigen (17), maximal homology is to the second disulfide loop of the heavy chain, just as has been observed in the comparison of human β_2-M with HLA-B7 (18). Although only 25 residues of murine β_2-M are identical to amino acids in positions 179 to 278 of H-2K^b, 22 of these positions are also conserved in the human, rabbit, and guinea pig sequences of β_2-M. In combination with a large number of conservative amino acid interchanges, this limited identity suggests that β_2-M and the region of the second disulfide loop of histocompatibility antigens may have a common evolutionary origin. The functional significance of this homology must await an analysis of the biological role and three-dimensional structure of the histocompatibility antigens.

Genetic variants of β_2-M have long been sought to aid in the determination of its biological activity, its interactions with cell surface molecules, and the linkage analysis of the structural gene. Two forms of guinea pig β_2-M originally detected electrophoretically and by amino acid composition analysis (19) may arise by action of an exopeptidase rather than result from a genetic polymorphism (9). Thus reports of polymorphism that are based on isoelectric focusing patterns (20, 21) and electrophoretic mobility differences (22) may not describe structural gene differences but may instead describe postsynthetic modification. On the other hand, the present discovery of an alanine–aspartic acid interchange between the β_2-M from two mouse tumor cell lines might merely represent a mutational event in one of the cell lines. However, the β_2-Ms isolated from EL4.BU and C14 cells show differences in electrophoretic mobility (unpublished results) that correspond to similar differences noted for material obtained from various mouse strains (22). Thus we believe that the structural differences observed here represent a true genetic polymorphism between the parent C57BL/6 and BALB/c strains. This polymorphism may thus be used for linkage analysis of the genes encoding murine β_2-M.

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