Journal of Supramolecular Structure
and Cellular Biochemistry 16:167–177 (1981)
Cellular Recognition 189–199

Biochemical Analysis of an MHC-Linked Hematopoietic Cell Surface Antigen, Qa-2

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The region of the murine 17th chromosome telomeric to H-2D encodes a group of serologically defined cell surface antigens termed Qa-1-5. These antigens are of interest because their expression is restricted to hematopoietic cells. In addition, the molecular weight and subunit structure (ie, association with β-2 microglobulin) of Qa-2 molecules are similar to H-2 and TL antigens. In the present studies, we have prepared isotopically labeled Qa-2 and H-2 molecules from mitogen-stimulated C57BL/6 spleen cells. Comparative peptide mapping of tryptic peptides from Qa-2 and H-2 molecules (K^b, D^bK^k, D^d) reveal that Qa-2 has a unique primary structure. However, considerable homology is indicated since 30–40% of the Qa-2 peptides cochromatograph with peptides derived from H-2K^b, H-2D^b, H-2K^k, and H-2D^d. Studies by other investigators have demonstrated that similar levels of structural homology are observed when H-2K, H-2D, and H-2L tryptic peptides are analyzed. We conclude from these studies that the Qa-2 alloantigen is structurally related to a class of cell surface molecules (ie, H-2) that play critical roles in immune recognition processes. These data further suggest that the genes encoding Qa-2 and H-2 molecules have arisen from a common primordial gene.

Key words: gene duplication, H-2 alloantigen, Qa-2 alloantigen

The molecular basis of cell-cell communication is a central problem of biology. Its resolution is crucial for understanding such diverse phenomona as embryonic development, organization of the central nervous system, and immune function. In recent years the murine 17th chromosome has been the focus of

Abbreviations used: MHC, major histocompatibility complex; B6, C57B6/J mice; RAMIg, rabbit anti-mouse immunoglobulin; GAMIg, goat antimouse immunoglobulin; NMS, normal mouse serum; Con A, concanavalin A; LPS, lipopolysaccharide; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; TBS, Tris-buffered saline; NP-40, nonidet P-40; S aureus, Cowan I strain of Staphylococcus aureus; SDS, sodium dodecyl sulfate; HGG, human gamma globulin; PA, pyridine acetate; SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Received April 6, 1981; accepted May 20, 1981.

0275-3723/81/1602-0167$03.50

attention since it has been shown to contain two regions, the T/t complex and the major histocompatibility complex (MHC), which control functions involved in embryogenesis and immune processes respectively [1, 2]. These two regions have been shown to encode a variety of cell surface structures involved in cell-cell interactions. For example, the H-2K, H-2D, and H-2L loci of the MHC have been shown to encode distinct 44,000 cell surface glycoproteins that are noncovalently associated with β-2 microglobulin [3]. Functionally, these antigens may be involved in cytotoxic T cell mediated immune mechanisms [4, 5]. In addition, the I region of the MHC encodes several two chained glycoprotein molecules (Ia antigens) which have been implicated in cell-cell interactions critical for immune responsiveness [3, 6]. The gene products of the T/t complex are much less understood. However, analysis of T/t mutations found in wild mouse populations have been interpreted as suggesting that the T/t encoded molecules effect cell-cell interactions at various stages of embryogenesis [1, 7]. Serological and biochemical studies have demonstrated that T/t complex controlled antigens can be found on embryonic tissue, sperm, and testicular cells [8, 9].

Recently, a third region, the Tla region, located telomeric to H-2D, has been described which controls several serologically defined lymphoid cell-surface alloantigens [10]. These antigens have been termed TL and Qa 1-5 antigens. Analysis of the cell subpopulation that expresses these antigens has shown them to be distributed on a wide variety of hematopoietic cells. For example, Qa-2 can be demonstrated to be expressed on B cell subsets [10–14], T cells [10–13], NK cells [15], myeloid progenitor cells [14], and pluripotential stem cells [14]. Biochemical analysis has demonstrated that antisera against TL, Qa-1, or Qa-2 specifically recognize a 44,000-dalton glycoprotein noncovalently associated with β-2 microglobulin [16–18]. It appears therefore that some Tla region gene products may be biochemically related to gene products of the MHC.

In this communication we report the biochemical characterization of the Qa-2 alloantigens. Evidence will be presented that the Qa-2 alloantigen is encoded by a loci separate from those encoding H-2 alloantigens. In addition, considerable homology between Qa-2 and H-2 alloantigens is observed at the primary structural level. These results indicate that Qa-2 is perhaps functionally and evolutionarily related to a class of cell surface molecules (ie, H-2) that play critical roles in immune recognition processes.

METHODS

Mice

Adult mice 6–10 weeks of age were used in these experiments. C57BL/6 mice (B6) were purchased from the Jackson Labs, Bar Harbor, Maine. The B10.A and B6•K1 strains were inbred strains obtained from the colony maintained at the University of Texas Health Science Center at Dallas.

Antisera

Antisera reactive against Qa-2 were prepared by immunizing B6•K1 mice with B6 spleen and lymph node cells as described previously [19]. The anti-D^b serum ([B10.A (5R) $\times$ R III] F_1 α B10) was obtained from the Research Resources Branch of the National Institutes of Health. All other anti-H-2 alloantisera were

prepared by immunizing the appropriate recipients with donor spleen and lymph node cells as follows: 1) anti-K^b; [B10.D2 (K^d D^d) × A/J (K^k D^d)] α B10.A(5R) (K^b D^d); 2) anti-H-2^d, B10.BR (K^k D^k) α B10.D2 (K^dD^d); 3) anti-H-2^k, [BALB/c Kh (K^dD^d × B10.D2 (K^dD^d) α B10.BR (K^kD^k). The specificity of the alloantisera was determined by immunoprecipitation analysis of lysates from spleen cells of appropriate recombinant mice. Rabbit antimouse Ig (RAMIg) and goat antimouse Ig (GAMIg) were prepared as described previously [18, 20]. Normal mouse serum (NMS, Pel-Freeze) was obtained from outbred mice.

Preparation and Labeling of Mitogen-Activated Spleen Cells

Mouse spleens were teased into Hank's balanced salt solutions (HBSS), and single cell suspensions were prepared as described previously [21]. Cell viability was assessed using trypan blue exclusion. Concanavalin A (Con A)-activated spleen cells were routinely prepared by resuspending 7.2×10^8 viable cells in 200 ml RPMI-1640 containing 5% FCS, 5 μg/ml Con A (ICN Pharmaceuticals), 5×10^{-5} M 2-mercaptoethanol (2-ME), glutamine, and antibiotics. To obtain lipopolysaccharide (LPS)-activated cells 3×10^8 viable spleen cells were resuspended in 150 ml RPMI-1640 containing 20% FCS, 5×10^{-5} M 2-ME, 2 × nonessential amino acids and glutamine, 1 mM sodium pyruvate, 50 μg/ml LPS (Difco, Salmonella typhosa 0901), and antibiotics. The cultures were then gassed with 10% CO_2 and incubated for 48 hours at 37°C.

The mitogen-activated cells were harvested and labeled at 1×10^7 cells/ml for 8 hours with ^3H (150 μCi/ml) or ^{14}C (15 μCi/ml) amino acids (New England Nuclear or Amersham-Searle) as detailed elsewhere [22]. Labeled cells were harvested by centrifugation, washed twice in Tris-buffered saline (TBS), pH 7.4, and lysed for 15 min at 4°C in 5–6 ml TBS containing 0.5% Nonidet P-40 (NP-40, Gallard, Schlesinger, New York). Nuclei and debris were subsequently removed by centrifugation for 15 min at 2,000g.

Immunoprecipitation and Isolation of H-2K, H-2D, and Qa-2 Molecules

Glycoproteins were isolated from NP-40 lysates of labeled cells by fractionation on Lentil-lectin Sepharose [23]. Lentil-lectin was purified from Lens culinaris and coupled to Sepharose 4B as described [23]. Lysates were applied to the lectin column in TBS containing 0.25% NP-40, and the nonadherent proteins were eluted in the same buffer. The adherent material was eluted with 0.3 M α-methylmannoside in TBS with 0.25% NP-40 and concentrated to approximately 5 ml by negative pressure dialysis.

The concentrated glycoprotein pools were centrifuged at 10,000g for 15 min prior to immunoprecipitation. Immunoglobulin was immunoprecipitated from the concentrated glycoprotein pool by incubation with RAMIg (300 μl/3×10^8 cells), for 15 min at 37°C and 30 min at 4°C. Immune complexes were removed by the addition of protein A-bearing Cowan I strain of Staphyloccoccus aureus followed by incubation for 15 min at 37°C and 30 min at 4°C [24]. Immunoglobulin-depleted glycoprotein pools were then depleted of "nonspecific" material by adding NMS (250 μl/3×10^8 cells), for 15 min at 37°C and 30 min at 4°C followed by two treatments with Cowan strain I of S aureus. "Precleared" glycoprotein pools were then reacted with the appropriate alloantisera at 37°C for 15 min and 4°C for 2–12 hours. All alloantisera were titered so as to remove all reactive molecules.

Immune complexes were removed by either immunoprecipitating them with GAMIg or by absorbing them to S aureus. Immunoprecipitates or S aureus-bound complexes were washed three times with TBS containing 0.1% SDS, 0.25% NP-40, and 0.2% deoxycholate followed by one wash with TBS. Immune complexes were eluted from the S aureus or immune precipitates were dissolved by boiling for 2 minutes in electrophoresis sample buffer with 5% 2-ME. All insoluble material was removed by centrifugation at 10,000g for 5 min. Samples were electrophoresed for 4–5 hours on 12.5% polyacrylamide gels using the Laemmli discontinuous system as described previously [25]. All gels were fractionated using a Savant gel crusher. In some experiments, gels were fractionated directly into scintillation vials and then counted in Beckman EP cocktail. Alternatively, the fractionated gels were incubated overnight in 0.01% SDS to allow the radiolabeled protein to elute from the gel matrix. Aliquots of each fraction were then removed and counted in scintillation cocktail. The appropriate fractions were pooled, gel pieces were removed by filtration through a 0.45-μ Millipore filter, and the samples were then lyophilized.

Trypsin Digestion and Comparative Peptide Mapping

The techniques described below have been detailed elsewhere [26, 27]. Lyophilized H-2D, H-2K, or Qa-2 molecules were redissolved in 2–3 ml dH_20 containing 0.5–1.0 mg human gamma globulin (HGG) as carrier and dialyzed against several changes of 0.1 M NH_4HCO_3, pH 8.0. The molecules to be compared (eg, ^{3}H-Qa-2 and ^{14}C-H-2K^k) were then mixed at a ^{3}H/^{14}C ratio of 2–3:1 and relyophilized. The lyophilized samples were dissolved in 1.0 ml 0.1 M NH_4HCO_3, pH 8.0, and 200 μg TPCK-trypsin was added. After a 1-hour incubation at 37°C, additional trypsin (100–200 μg) was added, and the incubation continued for 12–16 hours at 37°C. The pH was then lowered by the addition of 2–3 drops of glacial acetic acid, and the sample was lyophilized. Prior to chromatography, the peptides were solubilized in 2.0 ml 0.05 M pyridine acetate (PA), pH 3.13. Insoluble material was removed by centrifugation at 10,000g for 5 min. Routinely, 75–95% of the input cpm was recovered in the acid-soluble fraction. Tryptic peptides were analyzed by cation exchange chromatography on Technicon chromobeads type P using a 3 mm × 150 mm microbore column maintained at 54°C by a water jacket. The solubilized peptides were applied to the column in 0.05 M PA, pH 3.13. After washing the column with 5–6 ml of starting buffer, a pH-ionic strength gradient was applied using a Varigard gradient maker. Thirty ml of each of the following PA buffers was used: 0.05 M PA, pH 3.13; 0.1 M, pH 3.54; 0.2 M, pH 4.02; 0.5 M, pH 4.50; 2.0 M, pH 5.0. Following completion of the gradient, 2–3 ml of 2N NaOH was applied to remove all bound material. Twelve drop fractions were collected into minivials and allowed to evaporate. Water (0.35 ml) and scintillation fluid were added and the samples counted. The ^{3}H and ^{14}C cpm were normalized and corrected for channel spillover.

RESULTS

Isolation of Qa-2 and H-2 Alloantigens

Unlike H-2 alloantigens, the Qa-2 antigen is not expressed on all nucleated cells, but rather is found only on subpopulations of hematopoietic cells. In addi-

tion, the surface density of Qa-2, as determined by immunoprecipitation, is several-fold less than H-2K and H-2D alloantigens [16]. It was therefore to our advantage to obtain a populations of lymphocytes that was enriched for Qa-2 expression. Studies by Flaherty et al [12] have demonstrated that 75–90% of the cells in populations of mitogen (Con-A, PHA, LPS)-activated lymphocytes express serologically detectable Qa-2. Therefore, Con A-stimulated spleen cells were used for preparation of Qa-2 molecules. An SDS gel profile of a typical immunoprecipitate using extracts prepared from B6 Con A blasts is shown in Figure 1A. As demonstrated earlier [16] B6•K1αB6 (αQa-2) serum recognizes a polypeptide of approximately 44,000 daltons. β-2 Microglobulin, which is known to be associated with Qa-2 molecules, was allowed to migrate off the gel under the electrophoretic conditions employed. The specificity of our antisera is demonstrated by the observation that no 44,000-dalton polypeptide is precipitated when glycoprotein pools from B6•K1 (Qa-2$^-$) Con A blasts were reacted with αQa-2 (Fig. 1B). Note that when glycoprotein pools were reacted with NMS a significant amount of material with a molecular weight of 44,000 daltons could be detected. Additional experiments demonstrated that this "nonspecific" material could be significantly reduced by preincubating glycoprotein pools with NMS and S aureus.

The immunoprecipitation procedure used for isolation of Qa-2 alloantigens was equally applicable for the isolation of H-2 alloantigens from mitogen-stimulated spleen cells. It should be noted that the anti-H-2 sera employed are complex and contain reactivities toward both H-2K, D, and I region determinants. With the availability of appropriate recombinant congenic strains, specific K or D end precipitation can be obtained. For example, B10•D2αB10•BR will contain reactivity toward determinants carried by K^k, I-A^k, I-E/C^k and D^k. By using B10.A (K^k D^d) cells, reactivity is confined to the *K* end. Any *I* region controlled molecules that are coprecipitated can be readily separated from H-2 by SDS-PAGE.

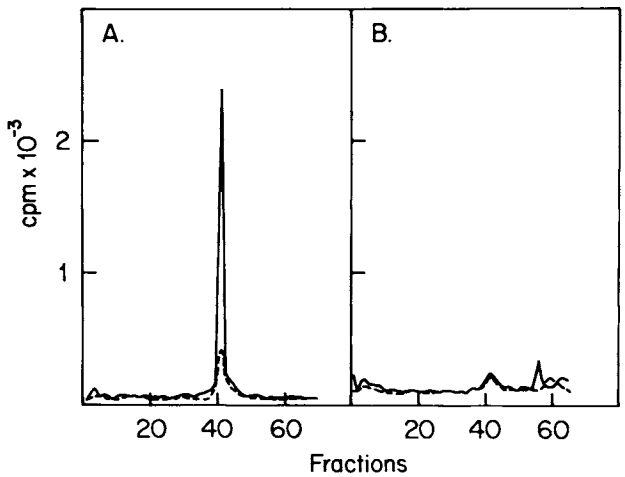

Fig. 1. SDS-PAGE analysis of an αQa-2 immunoprecipitate. Glycoprotein pools obtained from ^{3}H-arginine-labeled B6(A) or B6•K1(B) Con A-activated spleen cells were precleared with RAMIg and NMS as described in Methods. Aliquots (approximately 2 × 10^7 cell equivalents) of the precleared glycoprotein pool were removed and incubated with 30 μl αQa-2 (———) or NMS (--------). Immunoprecipitates were formed by the addition of GAMIg and subsequently were washed, solubilized, reduced, and analyzed on 12.5% polyacrylamide gels.

Tryptic Peptides of Isolated ^{3}H-Arginine-Labeled Qa-2 Molecules

Figure 2 (top) shows a tryptic peptide map in which ^{3}H-arginine and ^{14}C-arginine-labeled Qa-2 were coanalyzed. Routinely, 85–95% of the input radioactivity is soluble in the acidic (pH 3.13) starting buffer, and 85–95% of this acid soluble radioactivity is recovered from the column. Therefore, the vast majority of arginine-labeled peptides are being detected by this analysis. The maps generated from ^{3}H-arginine or ^{14}C-arginine labeled Qa-2 are completely super-imposable, thus demonstrating the reproducibility of this "double-label" mapping technique. In addition, the amount of radioactivity under the majority of the peaks is approximately equal throughout the map (Fig. 2, top). The simplest interpretation of this observation is that these peptides are derived from a single major molecular species. However, we observe several exceptions, the most notable being the large peak eluting at fractions 135–140. Subsequent studies revealed that this peak cochromatographs with free arginine (data not shown). Several nonreproducible minor peaks are also observed. The antisera used for immunoprecipitation of

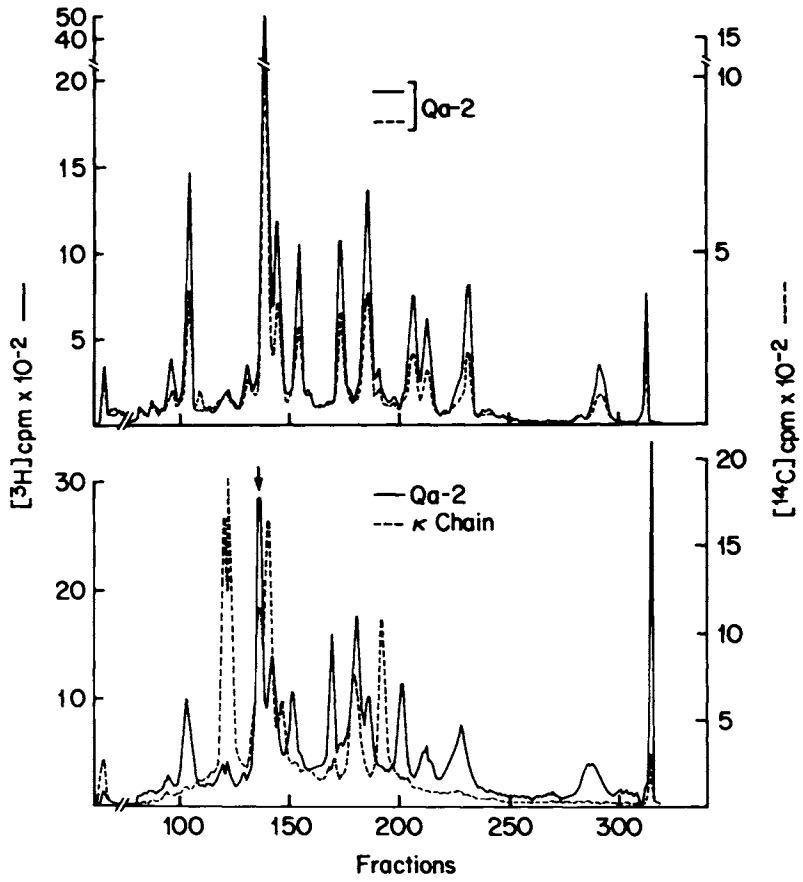

Fig. 2. Ion exchange chromatography of tryptic digests of Qa-2 alloantigen. Qa-2 alloantigen labeled with ^{3}H-arginine was compared with ^{14}C-arginine-labeled Qa-2 (top) or with a monoclonal κ chain labeled with ^{14}C-arginine (bottom). The bold arrow in the bottom panel indicates where free arginine and the major Qa-2 peak coelute.

Qa-2 is potentially polyvalent with reactivities toward Qa-2, 3, 4, and 5 [10]. Reactivity toward Qa-4 and Qa-5 has only been observed using monoclonal antibodies [10]. Perhaps the minor peaks observed are derived from a 44,000-dalton polypeptide bearing the Qa-3 alloantigen. Alternatively these minor peaks could represent 1) peptide generated from nonspecific 44,000-dalton material (see above), 2) incomplete digestion products, or 3) result from occasional trypsin-mediated cleavage at tyrosine residues [28]. However, these minor species will not detract from our analysis, since only major peaks are scored in the comparisons.

The sensitivity of the cation exchange chromatography technique in detecting single amino acid substitution in a given peptide is well documented [28–31]. Hence, structurally distinct polypeptides should show little peptide homology. Figure 2 (bottom) shows a peptide map comparison of Qa-2 with a mouse immunoglobulin kappa chain isolated from the IgG $2A_x$ secreted by P3 myeloma cells [32]. None of the 12 Qa-2 peptides coeluted with peptides derived from this kappa chain. The free arginine peptide is indicated by the arrow. This result confirms that structurally unrelated molecules show little or no homology at the level of comparative peptide mapping.

Peptide Map Comparison of Qa-2 and H-2 Alloantigens

A comparative peptide map analysis of Qa-2, H-2D^b, and H-2K^b molecules isolated from B6 Con A blasts is shown in Figure 3. Numerous peptide differences are observed implying that Qa-2, H-2K^b, and H-2D^b are distinct molecular species. In addition, several Qa-2 peptides coelute with peptides derived from H-2K^b (5/12, 42%) or H-2D^b (2/12, 17%). Interestingly, this level of peptide homology is similar to that reported for various H-2K, H-2D, and H-2L alloantigens when their tryptic peptides are compared [27, 33, 34].

Figure 4 shows comparative peptide maps of Qa-2 isolated from B6 cells and H-2K^k or H-2D^d isolated from B10.A cells. Again considerable structural homology is observed with 4/12 (33%) and 5/12 (42%) Qa-2 peptides coeluting with H-2K^k and H-2D^d peptides, respectively. A summary of the peptide map comparisons of Qa-2 versus H-2 alloantigens is shown in Table I.

The tryptic peptide comparisons indicate that Qa-2 is structurally related to H-2K and H-2D alloantigens, and that the degree of relatedness is equivalent to that observed among H-2 alloantigens [27, 33, 34].

DISCUSSION

The tryptic peptide map comparison of Qa-2 with several H-2 alloantigens has demonstrated that Qa-2 is distinct from H-2 alloantigen at the primary structural level. These observations together with previous genetic and serological studies [10, 11] argue strongly that the Qa-2 alloantigen is not a posttranslationally modified H-2 antigen, but instead is the gene product of a locus distinct from that encoding H-2K and H-2D.

The comparative peptide mapping of Qa-2 antigen with several H-2 alloantigens has shown that 21–43% of their arginine-labeled tryptic peptides are homologous (Table I). The degree of structural homology is also observed when various H-2K, H-2D, and H-2L alloantigens are compared [27, 33, 34]. Molecules showing such levels of peptide homology have been repeatedly demonstrated to exhibit

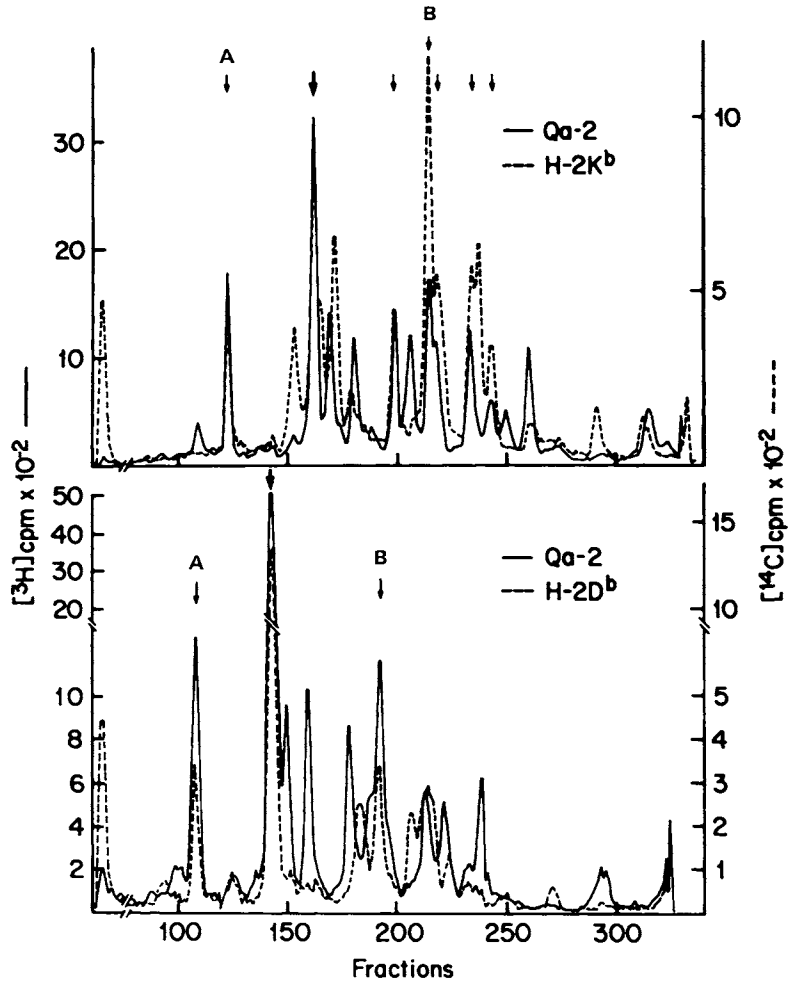

Fig. 3. Ion exchange chromatography of the arginine-labeled tryptic peptides from Qa-2, and H-2^b alloantigens. Qa-2 labeled with ^{3}H-arginine was compared to ^{14}C-arginine-labeled H-2K^b (top) or H-2D^b (bottom) isolated from B6-Con A blasts. The arrows indicate peptides that coelute. The bold arrow marks the position of the major Qa-2 peak, which was identified as free arginine. Peaks labeled A and B denote Qa-2 peptides, which always have an H-2-derived homologue.

75–90% amino acid sequence homology [35–39]. It is therefore possible that Qa-2 and H-2 alloantigens will show similar degrees of amino acid sequence homology. This is the first observation that there exist genes in the T1a region that show primary structural homology to H-2K and H-2D gene products. Indeed, these results further suggest that Qa-2 and H-2 alloantigens arose via duplication of a common primordial gene.

The Qa-2 and H-2 alloantigens share several biochemical properties. For example, all bind β-2 microglobulin, are glycosylated, and are membrane proteins [3, 18]. In this regard, our comparisons have detected two Qa-2 peptides that appear to have an H-2-derived homologue in all alleles and loci tested (see Figs. 3 and 4, peaks labeled A and B). Hence, some of the homologies observed may re-

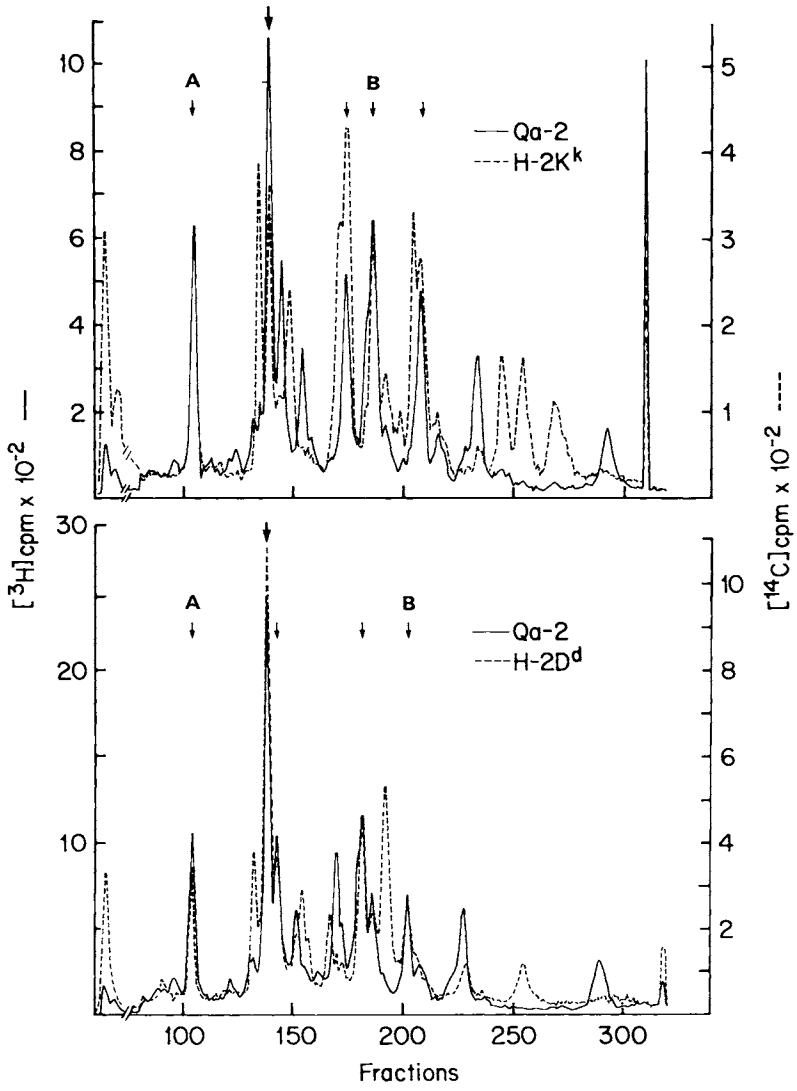

Fig. 4. Ion exchange chromatography of the arginine-labeled tryptic peptides from Qa-2 and H-2^a alloantigens. Qa-2 labeled with ^{3}H-arginine was compared to ^{14}C-arginine-labeled H-2K^k (top) or H-2D^d (bottom) isolated from B10.A LPS blasts. The arrows indicate peptides that coelute. The bold arrow marks the position of the major Qa-2 peak, which was identified as free arginine. Peaks labeled A and B denote Qa-2 peptides, which always have a H-2-derived homologue.

TABLE 1. Average Peptide Homology Between Qa-2 and H-2 Alloantigens

	H-2K^b	H-2D^b	H-2K^k	H-2D^d
Qa-2	$\frac{8}{24}$ (33)[a]	$\frac{4}{19}$ (21)	$\frac{8}{27}$ (30)	$\frac{10}{23}$ (43)

[a]Percentage homology = $\frac{2 \times \text{number of peptides in common}}{\text{number Qa-2 peptides} + \text{number H-2 peptides}}$

present regions of the molecule involved in glycosylation, membrane attachment, or interactions with β-2 microglobulin.

Two additional T1a region antigens, namely Qa-1 and TL, share biochemical properties with Qa-2. Our findings regarding Qa-2 alloantigens suggest that there exists in the T1a region a cluster of genes encoding glycoproteins that structurally resemble a class of self-recognition units (ie, H-2K, D, and L) involved in immune processes.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Richard Cook for advice on the peptide mapping procedure and Dr. Dorothy Yuan for her gift of the P3 myeloma cells. We also thank Dr. Shen, Sloan Kettering, for providing some of the antiserum used in the early phases of this study. Special thanks also go to Ms. Shirley Shanahan, Ms. Linda Trahan, and Ms. Kay Snave for expert technical assistance. We also thank Ms. Daisi Marcoulides for patiently and expertly typing this manuscript.

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