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Immunology

The partial amino-acid sequence of an H-2K molecule

(H-2K sequence/radiolabeling/immunoprecipitation)

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ABSTRACT **Eighteen of the NH_2-terminal 27 amino acids of a murine H-2K molecule have been assigned. The approach used was to label murine splenocytes with a single radioactive amino acid, isolate the H-2K molecule by specific immunoprecipitation, electrophorese the dissolved precipitate on sodium dodecyl sulfate polyacrylamide gels, and subject the isolated H-2K peak to amino-acid analysis and automated sequencing.**

The *H-2* complex of the mouse is of interest to immunologists, geneticists, virologists, and membrane biochemists (1). Recent studies have demonstrated that the complex consists of several genetic regions (at least five have been identified thus far, namely, *K*, *I*, *S*, *G*, and *D*), with each region containing one or more loci. The *K* and *D* regions, located on the opposite ends of the *H-2* complex, are known to code for antigens capable of stimulation of alloantibody production (2) and graft rejection (3). The H-2K or H-2D antigens are serologically complex (4) and genetically extremely polymorphic (5). Serological analysis of these antigens indicates that a product of each *H-2K* or *H-2D* allele is characterized by one private antigen, i.e., antigen specific for the particular allele (6), and an array of public antigens, i.e., antigens shared by products of several alleles (7). It should be emphasized that all antigens coded for by a particular allele are apparently carried by the same molecule or molecular complex (8). Biochemical studies of the H-2K and H-2D antigens have revealed that the antigens are glycoproteins with a molecular weight of 44,000 (9, 10) which are attached noncovalently to β2-microglobulin (11–15), a 12,000-molecular-weight protein devoid of carbohydrate (15).

Although there have been numerous speculations concerning the function of the *H-2K* and *H-2D* loci and the genetic relationship of these loci to other immunologically important components of the cell membrane (16–18), no satisfactory interpretation of the biological role of the H-2K and H-2D antigens is available. In the hope that elucidation of the primary structure of H-2 antigens might help in understanding their function and evolution, we have undertaken the task of determining the amino acid sequence of several H-2 molecules. In this report we describe the assignment of 18 of the 27 NH_2-terminal residues of an H-2K molecule.

MATERIALS AND METHODS

Labeling of murine splenocytes

^{3}H- and ^{35}S-Labeled Amino Acids. Each cell culture, consisting of 2.7 to 4.4 $\times$ 10^8 cells, was prepared from spleens of three to six adult A/J mice (The Jackson Laboratory, Bar Harbor, Me.). Mice were killed by cervical dislocation and the spleens excised. Cells were teased into phosphate-buffered saline, pH 7.3, centrifuged, washed once in phosphate-buffered saline, once in medium, and then filtered through a 200 mesh stainless steel screen. The medium used in all experiments was Eagle's minimal essential medium (GIBCO, Grand Island, N.Y.) supplemented with nonessential amino acids, 10% fetal calf serum (GIBCO), and 2% antibiotic-antimycotic mixture. To prepare each medium for labeling with a single essential radioactive amino acid, the one amino acid was deleted from the medium. When a nonessential amino acid was to be used for labeling, all the nonessential amino acids were deleted from the medium. The labeled amino acids used in these experiments are described in Table 1. All media were sterilized by filtration immediately prior to use and were supplemented with 50–150 μCi/ml of the particular isotope used for labeling. Cells were cultured at 10^7/ml.

Radioiodination. Cells (5 to 10 $\times$ 10^7) from C57BL/10Sn [B10(*H-2*^{b})], B10.A(*H-2*^{a}), or (B10 $\times$ A.TL)F_1 (*H-2*^{b}/*H-2*^{t1}) mice were radioiodinated as described (19), using 1–2 mCi of carrier-free, thiosulfate-free Na^{125}I (Amersham-Searle).

Cells labeled with either isotopic amino acids or ^{125}I were washed, lysed in 0.5% Nonidet P40 (Shell) (19), and centrifuged to remove the nuclei. Fetal calf serum was added to each lysate to a final concentration of 5% and the lysates were dialyzed for 16 hr at 4° against phosphate-buffered saline. The addition of fetal calf serum effectively prevented proteolysis of the H-2 molecules present in the lysate.

Antisera

(*1*) Rabbit anti-mouse Ig. This serum, prepared against purified murine myeloma proteins MOPC-104E (μ,λ), TEPC-15 (α,κ), and IgG (γ,κ,λ) contained antibodies against mouse μ, γ, α, κ, and λ chains.

(*2*) Goat anti-rabbit Ig. This serum contained antibodies against rabbit γ and light chains.

(*3*) Goat anti-mouse Ig. This serum contained antibodies against mouse γ and light chains.

(*4*) Anti-H-2.23 serum (see *Results*).

Cytotoxic tests

The microcytotoxic test was performed as described elsewhere (20). The source of complement was a mixture of normal guinea pig and normal rabbit serum (3:1).

Immunoprecipitation of cell lysates

After dialysis, lysates were centrifuged, the total radioactive protein was assayed by precipitating small aliquots of the lysate in 5% trichloroacetic acid (19), and the labeled Ig was removed by immunoprecipitation with rabbit anti-mouse Ig and goat anti-rabbit Ig (13). The supernatant of the anti-Ig precipitate was treated with a saturating amount of anti-H-2.23 (35 μl/10^8 cell equivalents), and the complexes were precipitated with a slight excess of goat anti-mouse Ig. Precipitates were washed, dissolved in 1% sodium dodecyl sulfate ($NaDodSO_4$) containing 8 M urea, pH 8.4 (13, 19), and reduced with 0.1 M 2-mercaptoethanol. Multiple aliquots

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight; PTH, phenylthiohydantoin.

Table 1. Isotopic labeling and extraction of H-2.23 radioactivity[a]

Isotope and source	(*1*) No. cells labeled $\times 10^{-8}$	(*2*) Protein, cpm $\times 10^{-6}$	(*3*) H-2.23 cpm	(*4*) H-2K peak recovered from gel, cpm	% of Total protein-associated radioactivity in (*5*) Immunoprecipitate	% of Total protein-associated radioactivity in (*6*) Gel extract	(*7*) Radioactivity recovered from amino-acid analyzer present in amino acid used for labeling, %[d]
L-[*3*-^{3}H]Alanine, 13.2 Ci/mmol[b]	2.7	7.4	55,470	10,500	0.73	0.14	ND[e]
L-[*3*-^{3}H(N)]Arginine, 30 Ci/mmol[b]	3.0	166.8	1,719,500	299,280	1.03	0.18	90
L-[*2,5*-^{3}H]Histidine, 55 Ci/mmol[c]	3.7	23.1	73,380	24,300	0.30	0.10	100
L-[*4,5*(N)-^{3}H]Isoleucine, 26 Ci/mmol[c]	3.7	78.8	247,710	74,100	0.30	0.09	85
L-[*4,5*-^{3}H]Leucine, 55 Ci/mmol[b]	3.0	59.8	177,470	25,536	0.28	0.04	92
L-[*4,5*-^{3}H(N)], Lysine, 38.9 Ci/mmol[b]	3.0	139.1	607,200	55,000	0.44	0.04	100
L-[^{35}S]Methionine, 361.2 Ci/mmol[b]	4.4	468.7	3,398,560	820,800	0.75	0.18	100
L-[*4*-^{3}H]Phenylalanine, 15 Ci/mmol[c]	3.5	44.9	409.840	89,550	0.92	0.20	ND
L-[*3,4*(N)-^{3}H]Proline, 46 Ci/mmol[c]	3.4	6.6	42,960	9,000	0.67	0.14	95
L-[*6*-^{3}H]Tryptophan, 11 Ci/mmol[c]	3.0	36.7	319,920	27,000	0.83	0.07	ND
L-[*3,5*-^{3}H]Tyrosine, 54 Ci/mmol[c]	3.0	87.3	333,420	69,479	0.38	0.08	ND
L-[*2,3*-^{3}H]Valine, 27 Ci/mmol[c]	3.3	31.2	159,120	45,000	0.51	0.14	86
Average (12 amino acids)	3.3	95.9	628,712	129,129	0.59	0.12	94

[a] A representative experiment of 2–4 experiments that were done for each isotope.
[b] New England Nuclear.
[c] Amersham-Searle.
[d] Based on acid hydrolysis and amino-acid analysis of the precipitate. All interconversions observed were into one other amino acid only. Radioactivity (40–60%) in the hydrolysate was recovered in labeled amino acids.
[e] ND = Not done.

were electrophoresed for 16 hr on 12.5 cm 7.5% $NaDodSO_4$ polyacrylamide gels at 4 mA per gel. Gels were fractionated (19) and collected in tubes. Radioactivity of small aliquots of these fractions was determined in scintillation vials containing Beckman Cocktail D. The H-2 peak was located; the fractions representing the top of the peak and one tube on either side were pooled and the gel matrix was removed by filtration through a 200 mesh stainless steel screen. The radioactivity in the extract was determined in a small aliquot in a Biosolv (Beckman)-containing toluene scintillant. Samples containing less than 5000 cpm were not processed further. Other samples were subjected to gel filtration on a 25 $\times$ 2.5 column of Sephadex G-25 in order to remove free $NaDodSO_4$ and salt. The desalted fractions were lyophilized.

Amino-acid analysis

To assure that extensive interconversion of the single isotopic amino acid had not occurred during the period of cell culture, a portion of the gel extract, or the undissolved H-2 or Ig precipitate, was combined with carrier protein and hydrolyzed under reduced pressure for 18 hr at 110° with 6 M HCl. Hydrolysates were analyzed on a Durrum D500 amino-acid analyzer equipped with a 590 and 440 channel and a fraction collector. A time delay system coupled to the integration circuit of the amino-acid analyzer allowed the collection of each amino acid in a separate test tube. This system is similar to one previously described (21). Radioactivity of aliquots of the fractions corresponding to each carrier amino acid was determined, and the position of the labled amino acid was ascertained.

Sequencing

The lyophilized gel extract was taken up in 0.5 ml of 25% acetic acid and loaded into a Beckman sequencer. The use of the automated Beckman Sequencer in this laboratory utilizing DMAA programs has been previously described in detail (22–24). The following modifications were utilized for these studies: (*i*) Liquid N_2 was used rather than the usual prepurified N_2 gas (25); (*ii*) a 50:50 (vol/vol) mixture of benzene and ethyl acetate was utilized as a washing solvent (D. Klapper and J. D. Capra, unpublished observation); and (*iii*) a carrier protein (myoglobin) was mixed with each sample prior to the sequencer runs. When it was known that the amino-acid histidine was present in position 3, subsequent sequencer runs were done in Quadrol buffer. The thiazolinone derivatives of the amino acids were collected and evaporated to dryness; radioactivity was determined with Cocktail D in order to determine the position of the single amino acid used for labeling. When sufficient radioactivity was present in the initial experiment, the fractions from repeat experiments containing labeled thiazolinone derivatives were thermally converted to phenylthiohydantoin (PTH)

Table 2. Reactivity in the cytotoxic test of antiserum (C3H.OH × 129/J)F_1 anti-C3H/HeJ with strains carrying *H-2* haplotypes of independent origin

Strain	*H-2* haplotype	Reciprocal of titer
C57BL/10Sn	*b*	0
B10.D2	*d*	0
B10.M	*f*	0
B10.WB	*j*	0
B10.BR	*k*	4096
C3H.NB	*p*	0
B10.Q	*q*	128
B10.RIII(71NS)	*r*	1024
B10.S	*s*	0

amino acids (26). These derivatized amino acids were mixed with PTH carrier amino acids and subjected to high pressure liquid chromatography (Waters Associates, Watertown, Mass.); each PTH derivative was collected in a single scintillation vial. The radioactivity of these samples was then determined to confirm the exact amino acid labeled. This technique was especially useful when limited interconversions of the labeled amino acids into another amino acid had occurred during the labeling period. The myoglobin mixed with each sample was utilized as follows: 2% of the thiazolinone derivative from steps 1, 2, 9, 10, 11, 17, 21, 28, 29, and 30 (the leucine, isoleucine, and valines) was converted to the PTH derivative by aqueous HCl and the total sample was injected onto a Beckman GC-65 gas chromatograph. This allowed the calculation of the repetitive yield for each run (average 95.7%). This procedure served as an internal control to monitor the mechanical action of the sequencer.

RESULTS

Analysis of the anti-H-2.23 serum

Because the specificity of the anti-H-2 serum used in these studies is critical to the interpretation of the results, the assays regarding specificity will be described in detail.

1. Cytotoxic Assay. The anti-H-2 serum was produced by hyperimmunization of (C3H.OH × 129/J)F_1 hybrids with cells from the thymus, spleen, and lymph nodes of C3H/HeJ mice. The strain combination (27) is such that only antibodies against antigens controlled by the *K* end of $H\text{-}2^k$ can be produced (the donor and the recipient share the *D* end). The antiserum reacts not only with cells of the immunizing haplotype ($H\text{-}2^k$), but also with those of $H\text{-}2^q$ and $H\text{-}2^r$ (Table 2). This reactivity pattern, as well as absorption experiments performed with this antiserum (data not shown), indicate that the antiserum contains antibodies against antigens H-2K.23 (private antigen of $H\text{-}2^k$), H-2K.25 (an antigen shared by $H\text{-}2^k$ and $H\text{-}2^r$), and H-2K.11 (an antigen shared by $H\text{-}2^k$, $H\text{-}2^r$, and $H\text{-}2^q$). All three antigens are known to be coded for by the same region (*K*) and to be present on the same molecule in the $H\text{-}2^k$ or $H\text{-}2^a$ strains (28). Typing *H-2* recombinant strains (Table 3) indicates that the antiserum also contains antibodies against antigens controlled by the *I* region (Ia antigens), specifically by the IA^k subregion. For example, the antiserum reacts with A.TL lymphocytes, which share the *I* region with $H\text{-}2^k$, but does not react with A.TH cells, which are isogenic with A.TL except for the central *H-2* regions.

2. Immunoprecipitation Assay. As seen in Fig. 1, analysis of anti-H-2.23 precipitates prepared from lysates of

Table 3. Reactivity in the cytotoxic test of antisera (C3H.OH × 129/J)F_1 anti-C3H/HeJ with strains carrying *H-2* recombinant haplotypes

Strain	*H-2* haplotype	Origin of *H-2* regions*					Reciprocal of titer
		K	*I*	*S*	*G*	*D*	
B10.A	*a*	(k)	(k)/*d*	*d*	*d*	*d*	2048
A.AL	*a1*	(k)	(k)	*k*	*k*	*d*	4096
A.TFR1	*an1*	*s*	(k)	*k*	*f*	*f*	64
B10.D2(R101)	*g1*	*d*	*d*	*d*	.	*b*	0
B10.A(2R)	*h2*	(k)	(k)/*d*	*d*	.	*b*	2048
B10.A(4R)	*h4*	(k)	(k)/*b*	*b*	*b*	*b*	2048
B10.A(5R)	*i5*	*b*	*b/d*	*d*	*d*	*d*	0
B10.AKM	*m*	(k)	(k)	*k*	*k*	*q*	2048
C3H.OL	*o1*	*d*	*d*	*k*	*k*	*k*	0
C3H.OH	*o2*	*d*	*d*	*d*	*d*	*k*	0
A.TL	*t1*	*s*	(k)	*k*	*k*	*d*	64
A.TH	*t2*	*s*	*s*	*s*	*s*	*d*	0
B10.HTT	*t3*	*s*	*s/k*	*k*	*k*	*d*	0
B10.S(9R)	*t4*	*s*	*s/d*	*d*	*d*	*d*	0
B10.AQR	*y1*	(q)	(k)/*d*	*d*	*d*	*d*	128
B10.T(6R)	*y2*	(q)	(q)	*q*	*q*	*d*	32

* Regions coding for antigens with which the antiserum is reacting are circled.

B10.A($H\text{-}2^a$) splenocytes revealed three peaks after Na-DodSO$_4$-polyacrylamide gel electrophoresis. These peaks corresponded to molecules of molecular weight (M_r) 44,000, 31,000, and 12,000. Occasionally, molecules of M_r 22,000 were observed (not shown in the figure). None of these peaks was observed when equal aliquots of B10($H\text{-}2^b$) lysates from cells labeled to the same specific activity (cpm/10^7 cells) were used. However, when (C57BL/10 × A.TL)F_1 ($H\text{-}2^b/H\text{-}2^{t1}$) lysates were used, only a portion of the 31,000 M_r peak was precipitated. Since the antiserum would not be expected to react with the H-2 antigen of these animals but would recognize at least one parental Ia, it is likely that the 31,000 M_r peak is Ia (29, 30) and the 44,000 M_r peak is H-2K (9, 10). The 12,000 M_r peak presumably represents β2-microglobulin (11–15). The nature of the 22,000 M_r peak noted in previous studies (31) is unknown. As seen in Fig. 1, the H-2K and Ia peaks are well separated. Nevertheless, only the three peak tubes of H-2K were pooled to prevent con-

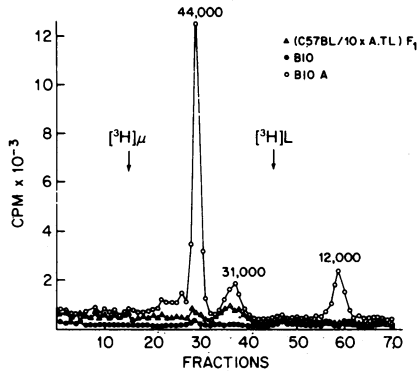

FIG. 1. H-2.23 antigens precipitated from lysates of radioiodinated splenocytes. Precipitates were dissolved, reduced, mixed with [^{3}H]μ and [^{3}H]L chain markers, and subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis. In plotting the three different gel patterns, the marker proteins were aligned. The calculated molecular weights shown are based on the apparent molecular weights of μ and L chains on 7.5% NaDodSO$_4$-gels (U. Melcher and J. W. Uhr, unpublished observations) (80,000 and 23,000).

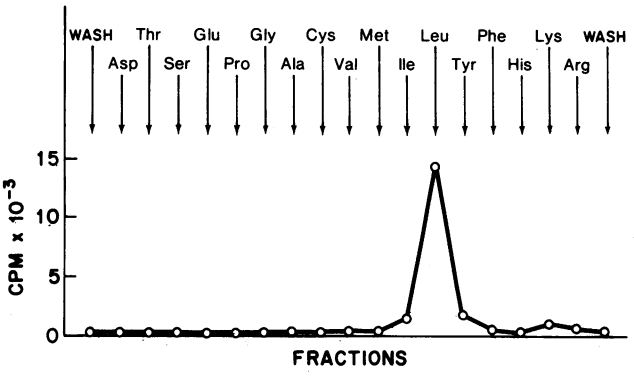

FIG. 2. Amino-acid analysis of an H-2.23 immunoprecipitate prepared from [^{3}H]leucine-labeled A/J splenocytes. The precipitate was hydrolyzed and analyzed on a Durrum D500 amino-acid analyzer equipped with a fraction collector designed to collect each amino acid in a separate tube.

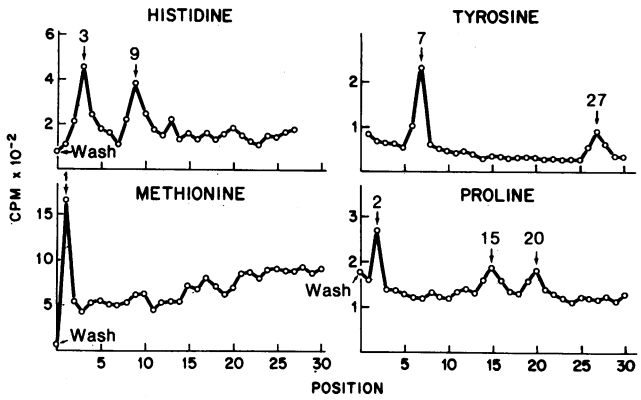

FIG. 3. Sequence analysis of H-2.23 antigen that has been labeled with four different amino acids.

tamination with Ia molecules. When portions of this extract were reelectrophoresed, only the 44,000 M_r peak was observed (not shown).

Cell Labeling and Recovery of H-2K. As seen in Table 1, when different amino acids were used for labeling, an average of 95.9×10^6 acid-precipitable cpm were obtained for 3.3×10^8 cells (columns 1 and 2). Of this radioactivity, 0.6% could be precipitated with anti-H-2.23 serum (column 5), consistent with previous reports (32, 33). Of the immunoprecipitable radioactivity, 40–60% appeared on the gel in the H-2K peak (not shown) and 60–70% of this peak could be recovered in the extract (not shown). Thus, an average of 21% of the H-2 associated radioactivity (column 4/column 3 × 100) that was electrophoresed was recovered in the extract. This represented approximately 0.12% of the initial acid-precipitable radioactivity (column 6).

After the sample was desalted on Sephadex G-25, 30–50% of the radioactivity was recovered. In addition, the approximated recoveries of radioactivity from the sequencer were 20% of radioactivity placed into the instrument. This latter low recovery was probably due either to the artifactual "blocking" of the majority of the H-2 molecules by urea or by difficulties in sequencing caused by the presence of protein-bound $NaDodSO_4$. This blocking did not, however, result in an incorrect sequence, as shown by the several experiments using the undissolved immunoprecipitate. In these experiments, a portion of the immunoprecipitate was sequenced directly without the purification by $NaDodSO_4$-polyacrylamide gel electrophoresis of the 44,000 M_r peak. In these experiments, the majority of the radioactivity was recovered in the same peaks obtained with the gel extract. There were also several additional peaks, reflecting presumably the Ia and/or β2-microglobulin molecules. The recovered radioactivity from the sequence of the H-2K immunoprecipitate obtained in these experiments was five to ten times higher than seen with the gel extract. The labeled residues were in the same positions however. Because of the increased accuracy of sequencing H-2 in the absence of Ia and β2-microglobulin, the gel extract was routinely used as the source of material.

Amino-Acid Analysis. When a culture period of 4–5 hr was used, virtually all of the radioactivity was recovered in the particular precursor amino acid used in those samples tested (Table 1, column 7). A representative experiment performed with [^{3}H]leucine-labeled H-2 antigen is shown in Fig. 2. Since some ^{3}H exchange occurs during hydrolysis, a portion of the radioactivity (approximately 40%) was lost during this procedure. Nonetheless, the experiment documents that virtually all radioactivity remained within the labeled amino acid used in the culture.

Sequencer Analysis. As seen in Fig. 3, by determining the radioactivity of the samples from the sequencer in representative experiments using four amino acids, it was possible to assign eight positions. The peaks corresponding to labeled tyrosine were further analyzed by high pressure liquid chromatography, which confirmed that no interconversions had occurred. The assignment of 18 of the NH_2-terminal residues of the H-2.23 molecule is shown in Fig. 4. The nine positions in which assignments have yet to be made presumably represent those amino acids not tested to date. Tryptophan is excluded in this region of the molecule since, in two separate experiments, it was not detected in the NH_2-terminal 40 positions. In deliberate mixing experiments, a secondary amino acid at a particular position would have been detected if it were present in greater than 15% of the molecules.

DISCUSSION

This paper describes a procedure for sequencing trace amounts of radioactively labeled cell surface molecules. Utilizing this technique, 18 of the NH_2-terminal 27 amino acids have been assigned in an H-2K molecule. The procedure has two significant additional features in comparison to the approach previously described by Silver and Hood (34). (*i*) Prior to sequencing, a small aliquot of the dissolved H-2 or Ig precipitates or the H-2 gel peak was hydrolyzed and analyzed on the amino-acid analyzer. This step was essential in determining whether radioactivity remained associated only with the amino acid used for labeling or whether interconversion had taken place during the period of culture. If the former were the case, the radioactivity of samples from the sequencer could be determined directly. (*ii*) If interconversion into other amino acids had occurred, a portion of the samples from the sequencer was analyzed by high pressure liquid chromatography. This latter procedure (which separates 18 of 20 PTH amino acids in a single run) allowed the determination of the interconverted amino acids. Using

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                5                  10
Met Pro His — Leu Arg Tyr Phe His —
               15                  20
Ala Val — Ile Pro — Leu — Lys Pro
               25
Phe Ala — — —  — Tyr
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FIG. 4. The partial NH_2-terminal amino-acid sequence of H-2.23.

these approaches, an unambiguous assignment of amino acids to particular positions could be made.

The major advantage of this approach is that only three to six mouse spleens are necessary to prepare the H-2 antigen labeled with a single amino acid, and that by using immunological techniques, virtually all of the conventional protein isolation techniques can be avoided. The net result is a rapid and efficient approach for sequencing a surface molecule using only trace amounts of protein. A significant disadvantage is the relatively small yield of material from the sequencer due presumably to binding of $NaDodSO_4$ by protein. However, as demonstrated in these experiments, using isotopic amino acids of high specific activity, it is possible to recover significant and reproducible amounts of radioactivity. The electrophoresis of the immunoprecipitates on $NaDodSO_4$-gels was necessary because of the presence of Ia and β2-microglobulin in the precipitate. [All attempts to completely remove protein-bound $NaDodSO_4$ by published techniques (35, 36) have resulted in simultaneous loss of H-2 antigen.]

Silver and Hood (34) have presented evidence that an H-2K antigen, sequenced by similar techniques, contains a leucine at position 5 and a tyrosine at position 7. Our results confirm that finding, and extend them to include 10 additional amino acids, and assign leucine to position 17 and tyrosine to position 27 as well.

In the present studies, only a single amino acid (of the 12 studied) could be assigned to each position. Thus, this preliminary analysis suggests that at least at the NH_2-terminus, the H-2K molecule is homogeneous within the experimental error of this system.

The incomplete sequence presented in Fig. 4 has been compared to several immunoglobulins from a variety of species and a modest homology to human Cμ3(341-368) and human Cδ2 is apparent. By introducing two deletions in the 27 residues of the H-2K molecule (positions 11 and 18), there are four identities to each of these immunoglobulin constant region domains. This degree of homology (4 of 18, or 22%) is approximately that seen between β2-microglobulin and immunoglobulin. It should be emphasized that if histocompatibility and immunoglobulin genes arose from a common ancestral gene during invertebrate life, only a limited homology would be expected. Obviously, additional amino-acid sequence information will be necessary before a definitive comparison can be made. The presence or absence of the cysteine in position 25 or 26 will be crucial to this argument.

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