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Immunology

Detergent-soluble HLA antigens contain a hydrophilic region at the COOH-terminus and a penultimate hydrophobic region

(membrane integration/histocompatibility)

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ABSTRACT **Purified, detergent-soluble HLA antigens (p44,12) are composed of a glycoprotein of molecular weight 44,000 (p44) and a peptide of molecular weight 12,000 (p12), β_2-microglobulin. Upon digestion with papain, p44,12 is converted to p39,12, then to p34,12, which retains antigenic activity. The NH_2-terminal amino acid sequences of p34 and p44 are identical. p44, p39, and p34 were purified, and comparison of their amino acid compositions showed that the COOH-terminal peptide removed by the first papain cleavage is hydrophilic and contains cysteine that can be alkylated after mild reduction. The penultimate COOH-terminal peptide removed by the second papain cleavage is hydrophobic, and presumably anchors HLA antigens to the membrane. This correlates with the observation that p44,12 and p39,12 bind detergent, while p34,12 does not. The orientation and integration of HLA antigens in the lymphocyte membrane were thus defined, and the structure suggests that HLA antigens span the plasma membrane.**

The major histocompatibility complex (MHC) of higher vertebrates contains a series of closely linked genetic loci, some of which code for cell surface structures carrying the immunologic determinants involved in graft rejection and virus recognition (1–7) and some of which govern immune response, disease susceptibility, immune cell interactions, and serum complement components (1, 2). The MHC of humans (*HLA*), on chromosome six (8), contains two loci, *HLA-A* and *HLA-B*, which have a large number of alleles coding for biochemically similar, serologically detectable, alloantigens. Recent studies show that the detergent-solubilized HLA-A and HLA-B antigens (for simplicity here called the HLA antigens) are composed of two noncovalently associated subunits, a glycoprotein of molecular weight (M_r) 44,000 (p44) carrying the antigenic specificity, and a polypeptide of M_r 12,000, β_2-microglobulin (p12) (9–14), which is common to all antigenic specificities, and the gene for which is on chromosome fifteen (15). HLA antigens, when solubilized with papain, contain β_2-microglobulin and a subunit of M_r 34,000 (p34) that contains antigenic activity (16, 17). NH_2-terminal sequencing and amino acid analysis of p34 have shown that the antigens of the *A* and *B* loci have highly homologous but distinct amino acid sequences (18). Detergent-soluble HLA antigens have recently been purified to homogeneity* (9, 19, 20). Their structures are presumably very close to that of the native membrane molecules, differing only in that detergent is substituted for a lipid environment (9). Since p44 contains one or two sulfhydryl groups not found on p34, S–S linked dimers and multimers of p44 (and their noncovalently associated p12) may be formed (10, 21–23). The present paper describes amino acid analysis, NH_2-terminal sequencing, and cysteine content of p44,p39 (an intermediate in the papain digestion) and p34, all of which define the orientation and integration of HLA antigens in the membrane, and suggest that these molecules span the membrane.

Abbreviations: Brij 99:97, Brij 99:Brij 97 (2:1); M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate.

* T. A. Springer, D. L. Mann, A. L. DeFranco, and J. L. Strominger, submitted for publication.

MATERIALS AND METHODS

Reagents were obtained from the indicated sources: papain, twice crystallized (Worthington); guanidine·HCl (Heico); Sepharose CL 6B (Pharmacia); Bio-Gel A-1.5m, 200–400 mesh (Bio-Rad); sodium iodoacetate and dithiothreitol (Sigma); iodo[^{14}C]- and [^{3}H]acetic acid (New England Nuclear). HLA antisera were as described previously* (24). Detergent-soluble HLA antigens (a mixture of the specificities A2; B7,12) were purified by a lectin affinity chromatography step and two Bio-Gel A-5m filtration steps to homogeneity by the criterion of sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis and NH_2-terminal analysis (9).*

Mild reduction and alkylation was performed by incubating HLA antigens with 1 mM dithiothreitol in 20 mM Tris·HCl, pH 8.2, under N_2 for 2 hr at 37°. Iodo[^{3}H]- or [^{14}C]acetate and Tris·HCl, pH 8.4, were added to 2.4 and 50 mM, respectively, allowed to stand 1 hr at room temperature, and samples were dialyzed versus 20 mM Tris·HCl, pH 8.0, 0.1 mM EDTA. Non-protein-bound label and excess detergent were removed by adsorbing HLA antigen to a DEAE-cellulose DE-52 (Reeve-Angel) column (5 mg of HLA per ml of DE-52) in 20 mM Tris·HCl, pH 8.0, and elution with 0.5 M NaCl, 20 mM Tris·HCl, pH 8.0, 0.05% Brij 99:Brij 97, 2:1 (abbreviated Brij 99:97). The samples were then filtered on Bio-Gel A-1.5m and concentrated in dialysis sacks versus Sephadex G-200 (Pharmacia).

Papain digestion was performed by adding to 1 volume of HLA antigen in 20 mM Tris·HCl, pH 8.0, and Brij 99:97 in a 6 × 50 mm tube, 0.25 volume of papain, freshly dissolved in 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 60 mM 2-mercaptoethanol. The tubes were incubated in a desiccator partially filled with water, and thoroughly flushed with N_2, in a 37° waterbath for 2 hr. An aliquot (0.25 volume) of 80 mM sodium iodoacetate, 300 mM Tris·HCl, pH 8.4, was added and the tubes were allowed to stand at 21° for 20 min. HLA antigens were assayed as described (9).

Amino acids were analyzed on a Beckman 121 M analyzer equipped with a microbore single column system. Samples were lyophilized and hydrolyzed in 5.7 M HCl, 0.05% mercaptoethanol, for 24, 48, and 72 hr, and were analyzed at two different concentrations.

RESULTS

Digestion of HLA Antigens with Papain. Earlier studies showed that detergent-solubilized and papain-solubilized HLA antigen heavy chains (p44 and p34, respectively) both contain four half-cystines in intrachain disulfides that are relatively difficult to reduce, but detergent-solubilized HLA antigens

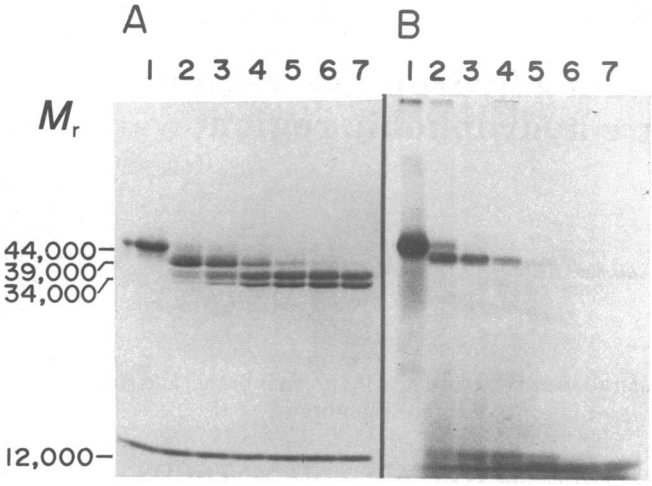

FIG. 1. $NaDodSO_4$ slab gel electrophoresis of iodo[^{14}C]acetate-labeled HLA antigen. Antigen was digested with the indicated ratios of papain to protein: 1, none; 2, 1:3840; 3, 1:1920; 4, 1:960; 5, 1:480; 6, 1:240; 7, 1:120. Each aliquot was divided into halves and electrophoresed on two sides of an 11% acrylamide $NaDodSO_4$ slab gel (32). One half of the slab gel was stained (A); the other half was autoradiographed (B).

contain, in addition, sulfhydryls that can be reduced under mild conditions (18, 21). HLA antigens were labeled with iodo[^{14}C]acetate after mild reduction, digested with graded amounts of papain, electrophoresed in $NaDodSO_4$ gels, and either stained with Coomassie brilliant blue (Fig. 1A) or autoradiographed (Fig. 1B). The digestion proceeded in two steps; p44 was converted rapidly to p39, and then more slowly to p34. When electrophoresed in high resolution $NaDodSO_4$ slab gels, such as Laemmli (Fig. 1) and Laemmli acrylamide gradient gels (Fig. 2), p44, p39, and p34 were each resolved into triplet bands, which probably correspond to different specificities (9, 24). The autoradiogram (Fig. 1B) showed that [^{14}C]carboxymethyl cysteine(s) are present in p44, and to a considerably lesser extent in the weakly staining upper two bands of the p39 triplet, but not in the lower band of the p39 triplet. This was confirmed by cutting out, and counting in scintillation fluid, each of the stained p39 triplet bands in Fig. 2. Concomitant with conversion of p44 to p39, radioactivity appeared in small peptides electrophoresing near the buffer front in the Laemmli system (Fig. 1B, lane 2). At higher papain concentrations, some degradation of the small peptides occurred (Fig. 1B, lanes 5, 6, and 7).

In a similar experiment, bands were identified in unstained gels by autoradiography, cut out, and counted in scintillation fluid. At a low concentration of papain, 46% of the radioactivity was removed from p44 and 18% of the radioactivity was found in the p39 upper doublet. Therefore, about 18/46 = 39% of the [^{14}C]carboxymethylcysteine(s) present in the p44 were also present in p39.

Preparation of p44, p39, and p34 for Amino Acid Analysis. Since p44 and p39 both contain detergent-binding sites and chromatograph at high molecular weights due to bound detergent, while p34 does not (9), it was hypothesized that p44 and p39 both contain a region of highly hydrophobic amino acids, which is removed by papain to form p34. It was therefore decided to purify p44, p39, and p34, and, by difference of their amino acid compositions, to obtain the compositions of the two peptides that are removed by papain. The small peptides were subject to further papain digestion after release from the large peptide, and have not yet been obtained in a form suitable for

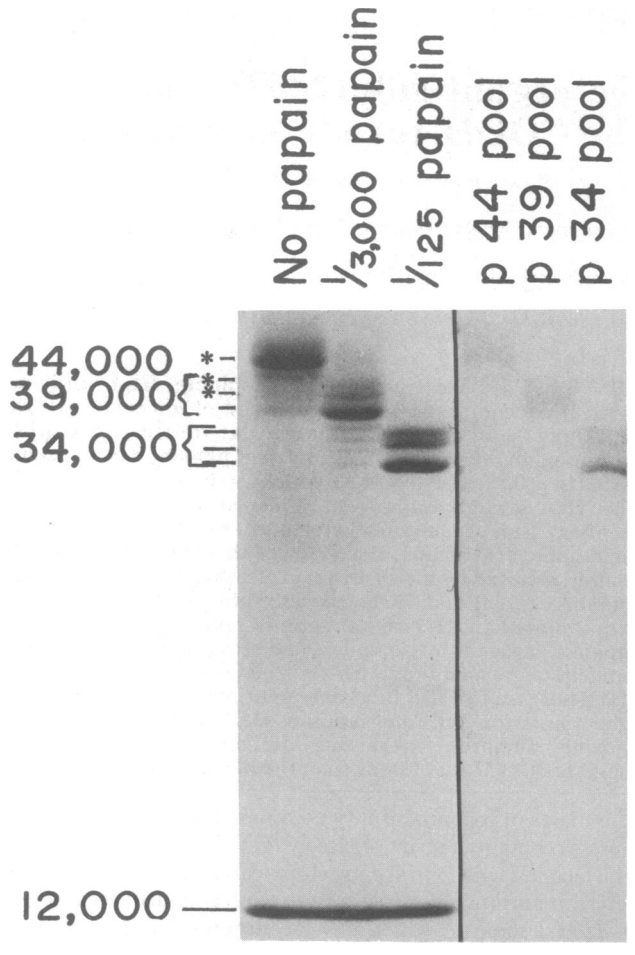

FIG. 2. $NaDodSO_4$ gel electrophoresis of materials at steps in the purification of p44, p39, and p34 for amino acid analysis. Aliquots removed after papain digestion (see legend to Fig. 3) and from the M_r 44,000, 39,000, and 34,000 polypeptide pools from the 6 M guanidine·HCl column after dialysis (see Fig. 4) were lyophilized and electrophoresed on 7–15% acrylamide gradient gels (32). The asterisks indicate bands containing ^{3}H-labeled, easily reduced sulfhydryls, which were determined by cutting out and counting them in scintillation fluid.

analysis. Purified HLA antigens, with the easily reduced sulfhydryls labeled with iodo[^{3}H]acetate, were treated with no papain, yielding p44,12; $1/3000$ papain, yielding p39,12 contaminated with a small amount of p34,12; and $1/125$ papain yielding p34,12 (Fig. 2). These samples were chromatographed on a Bio-Gel A-1.5m column (Fig. 3). After digestion with $1/3000$ papain (Fig. 3B), most of the HLA antigen (p39,12, bound to a detergent micelle) eluted at the same molecular weight as undigested HLA antigen (p44,12, also bound to a detergent micelle) (Fig. 3A), and was separated from a smaller amount of HLA antigen at M_r 45,000 (p34,12). Thus, p39,12 was obtained in pure form, by adjusting the papain concentration to yield p39,12 contaminated with p34,12, rather than with p44,12, and separation of p39,12 from p34,12 by gel filtration (9). Compared to the iodo[^{3}H]acetate labeling pattern without papain digestion (Fig. 3A), the material digested with $1/3000$ papain (Fig. 3B) had a reduced amount of ^{3}H associated with HLA activity bound to a detergent micelle at M_r 410,000 (p39,12). No ^{3}H was associated with papain-solubilized HLA antigen at M_r 45,000 (p34,12). A peak of ^{3}H not associated with HLA activity eluted in a low-molecular-weight region corresponding to small peptides. After digestion with $1/125$ papain,

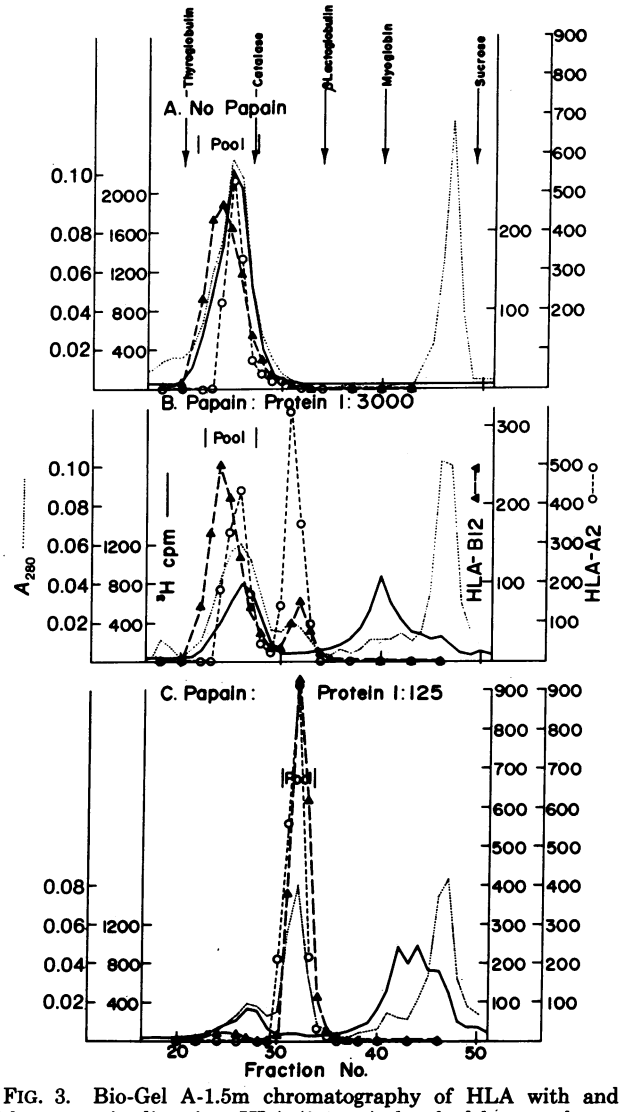

FIG. 3. Bio-Gel A-1.5m chromatography of HLA with and without papain digestion. HLA (0.4 mg) that had been carboxymethylated with iodo[^{3}H]acetate under mild conditions was incubated with no papain (A), papain:protein 1:3000 (B), or papain:protein 1:125 (C). Aliquots were reserved for NaDodSO$_4$ gel electrophoresis (Fig. 2) and the remainder was chromatographed on a 0.9 × 90 cm Bio-Gel A-1.5m column equilibrated with 0.05% Brij 99:97, 0.05 M Tris·HCl at pH 8.0, 0.02% NaN_3. Aliquots (75 μl) were mixed with 5 ml Aquasol for scintillation counting. The large A_{280} peak near the total column volume at the right is due to dithiothreitol.

essentially all of the HLA antigen chromatographed at M_r 45,000 (p34,12). A large peak of ^{3}H, corresponding to small peptides, was at a molecular weight still lower than after 1/3000 papain, indicating further degradation of the released peptide had occurred (compare Fig. 1B).

To separate HLA heavy chains from β_2-microglobulin, the material from agarose chromatography was placed in appropriate pools, lyophilized, dissolved in 6 M guanidine·HCl, completely reduced, alkylated with iodo[^{14}C]acetate, and chromatographed in 6 M guanidine·HCl on Sepharose CL 6B (Fig. 4). ^{3}H label was associated with p44 and p39, but not with p34 or β_2-microglobulin, while ^{14}C was incorporated into the intrachain half cystines of all the polypeptides. By comparing the ratios ^{3}H:^{14}C of p44 and p39, or the ratio of ^{3}H in heavy chain to ^{14}C in β_2-microglobulin in each preparation, the ratio of easily reduced sulfhydryls in p44:p39 was measured as 2.1 and 2.6, respectively.

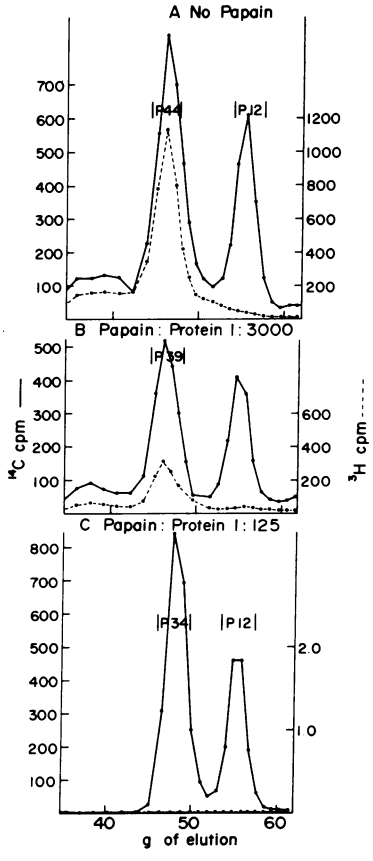

FIG. 4. Purification of the M_r 44,000, 39,000, and 34,000 polypeptides by gel filtration in 6 M guanidine·HCl on Sepharose CL 6B. The pools from Bio-Gel A-1.5m filtration (Fig. 3) were lyophilized and dissolved in 0.25 ml of 6 M guanidine·HCl, the pH was adjusted to 8.6 with 1 M NaOH, dithiothreitol was added to a final concentration of 5 mM, and reduction was carried out at 37° under a N_2 atmosphere for 4 hr. Then iodo[^{14}C]acetate was added to a final concentration of 12 mM, and alkylation was carried out at 37° for 1 hr. The samples were chromatographed on a 0.9 × 90 cm Sepharose CL 6B column equilibrated with 6 M guanidine·HCl, pH 5.5, 0.1 mM NH_4Cl. Radioactivity was determined by counting 25 μl aliquots in 5 ml of Aquasol containing NaDodSO$_4$ (16).

Amino Acid Analysis. p44, p39, p34, and β_2-microglobulin peaks were pooled and dialyzed exhaustively versus 0.1 M acetic acid before amino acid analysis. Aliquots of the peptides were also analyzed by NaDodSO$_4$ slab gel electrophoresis, and were homogeneous and free of β_2-microglobulin (Fig. 2). A molecular weight of 29,000 was assumed for the peptide moiety of p34 (18), and since NaDodSO$_4$ gel molecular weight data indicated that peptides totalling M_r 10,000 are released by papain digestion, molecular weights of 33,500 and 39,200 were assumed for the peptide moieties of p39 and p44, respectively, which were compatible with both molecular weight and amino acid analysis data, leucines being fixed at 17, 24, and 24 for p34, p39, and p44, respectively.

The compositions of p44, p39, p34, and p12 [β_2-microglobulin (β_2-M) from both p44,12 and p34,12] are shown in Table 1. The composition found for papain-digested detergent-soluble antigens is in excellent agreement with that found earlier (18) for the average of HLA-A2 and HLA-B7,12. p39 had a more hydrophobic composition than p34 or p44, and the subtractive comparison of the compositions of p44, p39, and p34 shows that the first peptide released by papain had a highly hydrophilic composition, while the second peptide had a highly hydrophobic composition. The hydrophilic and hydrophobic peptides

Table 1. Amino acid composition, residues/molecule

Amino acid	p44	p39	p34	Δ p44-p39	Δ p39-p34	β_2-M from p44,12	β_2-M from p34,12
CM-Cys	4.5	4.2	3.6	0.3	0.6	2.1	1.8
Asp	30	22	21	8	1	12	12
Thr*	24	19	19	5	0	5	5
Ser*	23	18	13	5	5	8	9
Glu	44	36	36	8	0	11	11
Pro	18	14	12	4	2	5	5
Gly	27	22	12	4	4	4	4
Ala	29	23	22	6	1	3	3
Val‡	21	21	15	0	6	7	7
Met†	5	5	4	0	1	1	1
Ile‡	12	12	6	0	6	5	5
Leu	24	24	17	0	7	7	7
Tyr†	15	13	13	2	0	6	6
Phe	11	10	7	2	3	5	5
His	10	10	10	0	0	4	4
Lys	13	11	9	2	2	8	8
Arg	25	21	21	4	0	5	5
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Polarity§	49.2	46.7	50.7	63.7	20.7		

Determinations are rounded to the nearest integer, except for CM-Cys. n.d. indicates not determined.
* Extrapolated to zero time values.
† 24 hr values.
‡ 72 hr values.
§ Calculated according to ref. 33.

contained 0.6 mol and 0.3 mol of CM-cysteine, respectively. The relative amounts of CM-cysteine were in agreement with radioactive labeling experiments described in this paper. However, the total amount of CM-cysteine removed by papain, 0.9 mol, was at variance with other measurements in which 2 mol were found (21).

The compositions of β_2-microglobulin isolated from p44,12 and p34,12 were highly similar to one another, and to the composition of β_2-microglobulin isolated from urine (25), indicating that β_2-microglobulin is not degraded after papain treatment or before excretion in urine.

NH_2-Terminal Sequence of p44. The NH_2-terminal amino acids of p44 and p12 have been identified as glycine and isoleucine, respectively*, which are identical to the NH_2-terminal amino acids of p34,12 (18, 26). NH_2-terminal sequencing of the first five residues of p44 by the $NaDodSO_4$-dansyl-Edman technique (27) (Table 2) established that the NH_2-terminal sequence of p44 and p34 are the same, and therefore, the peptides released by papain digestion are derived from the COOH-terminal portion of p44.

Table 2. Amino terminal sequence

	Residue				
	1	2	3	4	5
p44	Gly	Ser	—	Ser	Met
p34*	Gly	Ser	—	Ser	Met

Purified HLA antigens (250 μg) were acetone precipitated, and p44 was prepared by electrophoresis on 0.8 × 12 cm, 10% polyacrylamide $NaDodSO_4$ gels, and sequenced by the $NaDodSO_4$-Edman-dansyl technique (27).
* Sequence from ref. 18.

DISCUSSION

NH_2-terminal analysis of HLA antigens showed that during papain digestion of p44,12 to p34,12 the small peptides are released from the COOH-terminal end of p44. Since the p34,12 portion of p44,12 contains HLA alloantigenic activity and is known to be exposed on the cell surface to antibodies and papain, HLA antigens must be oriented with the NH_2-terminal end extending outside the cell membrane. It has recently been shown that the H-2 antigens of mice have the same orientation (28, 29). Digestion of p44 to p39 released a COOH-terminal peptide containing a high proportion of hydrophilic amino acids (polarity = 63.6%) and an easily reducible cysteine. Digestion of p39 to p34 released the penultimate COOH-terminal peptide(s) which contains a high proportion of hydrophobic amino acids (polarity = 20.7%) and which, in the case of some molecules, contains an easily reducible cysteine, probably depending on the antigenic specificity or the exact extent of papain digestion. The free sulfhydryls found in p44 are responsible for the formation of heavy chain disulfide-linked dimers (10, 21, 22, 28), although recent evidence from our laboratory indicates that these dimers result from oxidation during isolation, and cross-linking experiments have shown that, in the absence of disulfide linkage, heavy chains are not noncovalently associated† (21). The finding that p44 and p39 have a hydrophobic region, while p34 does not, is in agreement with the fact that p44,12 and p39,12 bind to detergent micelles and chromatograph at a high molecular weight in gel filtration, while upon conversion of p39,12 to p34,12, the detergent binding site is lost (9). The hydrophobic nature of this peptide and its role in detergent binding indicate it anchors HLA antigens to the cell membrane through hydrophobic bonds. Since the COOH-terminal peptide is highly hydrophilic, it is presumably present in the aqueous phase. The hydrophilic peptide was found to bear no alloantigenic activity, which is consistent with the hypothesis that it lies inside the cell. Glycophorin, a major erythrocyte membrane protein, has an NH_2-terminal glycosylated region lying outside the cell, a highly hydrophobic region, and a highly hydrophilic region at the COOH-terminal, which lies inside the cell (30). The structural resemblance of HLA antigens to glycophorin suggests that HLA antigens also span the membrane, although a more complicated U-shaped arrangement, in which the hydrophilic COOH-terminus is at the outside, cannot be excluded. The structural data concerning the orientation of HLA in the membrane, the position of sulfhydryls, hydrophilic and hydrophobic regions, and papain cleavage sites are summarized in Fig. 5, which also incorporates the hypothesis that HLA antigens span the membrane.

If HLA has a role in the immune system, which has been suggested by certain structural resemblances to immunoglobulins (10, 21, 22) and by the role H-2 antigens may have in virus recognition (4–6), it might be important for perturbations of HLA on the exterior to be communicated to the cell interior. The proposed structure for HLA antigens allows such communication, either by conformational changes through the cell membrane or by lateral diffusion in the membrane. Capping of histocompatibility antigens has been shown to be dependent on metabolic energy and the integrity of the cytoskeleton (31), which suggests that a link exists between histocompatibility antigens and the cytoskeleton. The COOH-terminal hydro-

† T. Springer, R. Robb, C. Terhorst, and J. L. Strominger, submitted for publication.

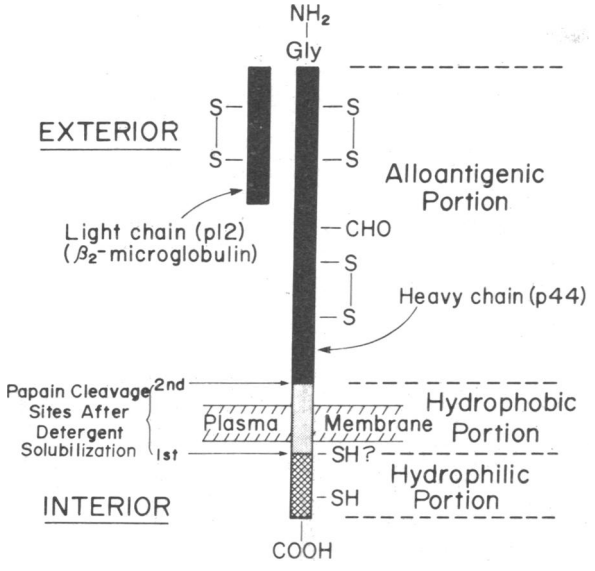

FIG. 5. Arrangement of HLA antigen in the membrane, with the hydrophilic peptide hypothetically shown inside the cell membrane. The position of β_2-microglobulin relative to the alloantigenic portion of the heavy chain is not known. The possible presence and position of a second sulfhydryl, shown near the first papain cleavage site, is unclear. It may be present on only some HLA specificities, or the exact position of papain cleavage relative to it may vary. CHO indicates carbohydrate. The position of the disulfide bonds within the alloantigenic fragment is not known.

philic peptide could provide such a link, if, as suggested, it lies inside the cell.

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