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## A molecular basis for the Ia.2 and Ia.19 antigenic determinants

(major histocompatibility complex/Ia antigens/monoclonal antibodies/T-cell restriction/DNA sequencing)

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**ABSTRACT** The murine Ia antigens, heterodimeric glycoproteins on the surface of immunocompetent cells, restrict immune recognition by their influence on cell–cell interactions. Many serological specificities have been mapped to these molecules, and monoclonal antibodies directed against some of these determinants block antigen presentation to T lymphocytes. As a step toward a better understanding of Ia function, we sought to define the molecular basis of Ia.2 and Ia.19, specificities found on the  $A_\alpha$  chain of only the *k* or of both *k* and *r* haplotypes, respectively. We report nucleotide sequences for the  $A_\alpha$  chain cDNAs of the *k*, *r*, and *s* haplotypes, which, when compared to previously published  $A_\alpha$  sequences, demonstrate the existence of one *k*-specific amino acid residue and of another present only in the *k* and *r* haplotypes. These residues must thus play an important role in the generation of Ia.2 and Ia.19 specificities.

Painstaking serological studies have played an important role in revealing the fascinating diversity of the major histocompatibility complex (MHC) (for reviews, see refs. 1–3). A battery of antisera specific for mouse MHC antigens has been produced by immunizing mice of various recipient strains with lymphocytes from donor strains differing only within the *H-2* region of chromosome 17. These antisera have been used to define more than 150 determinants encoded within *H-2* and have allowed the designation of multiple alleles for most of the murine MHC loci. More recently, monoclonal antibodies (mAbs) have replaced conventional antisera as typing reagents. Some new determinants have thus been revealed, but many of the mAb specificities mimic those originally identified by using antisera.

More than 50 serological specificities are controlled by the *H-2 I* region, which codes for the Ia antigens:  $A_\alpha$ ,  $A_\beta$ ,  $E_\alpha$ , and  $E_\beta$ . These polymorphic polypeptides assemble as dimeric complexes ( $A_\alpha:A_\beta$  and  $E_\alpha:E_\beta$ ) on the surface of antigen-presenting cells (APCs), B cells, and perhaps activated T cells (for reviews, see refs. 4–6). They have been implicated as restricting elements for regulatory T cells; in particular, they seem to play a crucial role in the presentation of antigen by APCs to T cells and, subsequently, in T-cell facilitation of B-cell antibody production. Although much is known about the cellular manifestations of Ia restriction of T cells, details of the molecular mechanisms remain elusive.

Serological reagents have become important tools for those attempting to study such question. For example, anti-Ia antibodies have been used as probes to localize increasingly more precisely what a T cell “sees” on the Ia complex. Since immunoprecipitation experiments have shown that an antibody (Ab) usually recognizes only one of the dimeric Ia complexes, one can assess by Ab-blocking studies whether the  $A_\alpha:A_\beta$  or  $E_\alpha:E_\beta$  complex is involved in antigen presentation to T cells. Results indicate that some antigens are

presented in the context of the E complex, others in the context of A (6). Recently, it has been possible to assign certain of the Ia determinants to specific chains on the basis of a mAb's ability to precipitate isolated or hybrid chains (7–10). Blocking experiments using these reagents have revealed that both the  $\alpha$  and  $\beta$  chains can restrict T cells (8, 9). Finally, competitive binding assays with pairs of anti-Ia mAbs have revealed that the A and E complexes contain several partially or non-overlapping epitopes (11, 12). Parallel Ab-blocking studies with these mAbs have shown that T cells, too, are capable of distinguishing several epitopes on an Ia molecule (8, 9, 13). In summary, it seems that the understanding of what a T cell “sees” on an Ia antigen has increased in parallel with our ability to precisely assign Ia determinants.

Amino acid sequence information may provide the next level of resolution in delimiting Ia determinants. Multiple alleles have now been sequenced for all four Ia chains (ref. 14 and our unpublished data). By correlating the presence or absence of polymorphic amino acids with the ability or inability of a chain to be recognized by a particular anti-Ia mAb, it may be possible to pinpoint the molecular basis of certain Ia determinants. We have attempted to define the Ia.2 and Ia.19 determinants in such a manner for the following reasons: (i) They are relatively rare specificities. Ia.2 is found on Ia molecules from only *k* haplotype mice, and Ia.19 is limited to the *k* and *r* haplotypes of common inbred strains. (ii) Although these specificities were originally defined by using conventional antisera, it has been possible to isolate mAbs recognizing similar or identical determinants. Notably, Pierres *et al.* (11) have described six such mAbs derived from A.TH (*H-2<sup>k</sup>*, *Ia<sup>k</sup>*) anti-A.TL (*H-2<sup>l</sup>*, *Ia<sup>k</sup>*) immunizations. (iii) Both Ia.2 and Ia.19 have been assigned (at least in part) to the  $A_\alpha$  chain (8, 9). We have previously sequenced six  $A_\alpha$  alleles—*k*, *b*, *d*, *f*, *q*, and *u*—thus providing a strong data base for correlative studies (15). (iv) Antibodies recognizing Ia.2 and Ia.19 determinants have been shown to block the ability of certain T-cell clones to react to antigen presentation (8, 9, 13).

Hence, we have sequenced  $A_\alpha$  cDNA clones derived from A.TH, A.TL, and B10.RIII (*H-2<sup>r</sup>*, *Ia<sup>r</sup>*) mice by using a newly developed, very rapid sequencing strategy. We have identified one *k*-specific and one *k*- and *r*-specific amino acid and suggest that these residues are crucial to the existence of Ia.2 and Ia.19. The possibility that these residues are involved in T-cell recognition is discussed.

### MATERIALS AND METHODS

**Mice.** Strains A.TL (*H-2<sup>l</sup>*, *Ia<sup>k</sup>*), A.TH (*H-2<sup>k</sup>*, *Ia<sup>s</sup>*), and B10.RIII (*H-2<sup>r</sup>*, *Ia<sup>r</sup>*) were furnished by the Centre de

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Abbreviations: MHC, major histocompatibility complex; H-2, mouse MHC; APC, antigen-presenting cell; Ab, antibody; mAb, monoclonal Ab.

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**RNA Isolation and cDNA Cloning.** Poly(A)<sup>+</sup> RNAs were isolated from splenocytes and analyzed as described (16). cDNA libraries, carried in the phage  $\lambda$ gt10, were constructed and screened with an A $\alpha$ <sup>k</sup> cDNA probe (pAAC6) by standard methods (17). The longest A $\alpha$  cDNA inserts were purified on sucrose gradients and inserted into the *Eco*RI site of M13tg130 or M13tg131 for sequencing (18).

**DNA Sequencing.** Oligonucleotide sequencing primers, chosen as described in the text, were synthesized by methods detailed elsewhere (19). Sequences were determined by using the dideoxy sequencing method of Biggin *et al.* (20).

## RESULTS

**Multiple-Oligonucleotide-Primer Sequencing Strategy.** To facilitate the rapid sequencing of cDNA clones coding for allelic proteins, we have developed a sequencing strategy based on the use of multiple oligonucleotide primers. Most full-length A $\alpha$  cDNA clones are approximately 1 kilobase pair long, but the limit of high-confidence reading for any one sequencing gel run is usually 250–300 bases. Thus, only 250–300 bases of one strand on each end of a cDNA clone are normally available for sequence determination using the universal M13 primer. To overcome this limitation, we synthesized a series of primers that had sequences within the A $\alpha$  cDNA insert. Three pentadecamer pairs (see Fig. 2) were chosen according to the following criteria: (i) they were spaced approximately 200 bases apart along the DNA sequence; (ii) they occurred in allelically invariant regions of A $\alpha$  (15); and (iii) they coded for some relatively rare amino acids of complex structure (21), again favoring their conservation within any allelic cDNA. Both strands of each 15-base sequence were used as primers, and the cDNA insert was cloned in both orientations in the M13 vector so that, as illustrated in Fig. 1, the complete sequence on both strands could be determined. By using this strategy, it is possible to sequence in 1 day an entire A $\alpha$  cDNA insert cloned in M13 and to display the sequence on two gels.

**Nucleotide Sequences.** The nucleotide sequences of the A.TH (*H-2<sup>k</sup>*), A.TL (*H-2<sup>l</sup>*), and B10.RIII (*H-2<sup>r</sup>*) A $\alpha$  cDNA clones are presented in Fig. 2. Consistent with previously published results (15), most diversity occurs in the exon coding for the first external protein domain, D1. To confirm our assignments for certain of the variable nucleotides, we did Southern blotting of genomic liver DNA from the three strains. Results from both *Hind*III and *Msp* I digests agreed with our sequence assignments (data not shown).

We sequenced the A $\alpha$ <sup>k</sup> allele for A.TL mice (*H-2<sup>l</sup>*) even though the A $\alpha$ <sup>k</sup> sequence from A/J mice (*H-2<sup>a</sup>*) was already known, because it had been claimed on the basis of tryptic peptide mapping that the A $\alpha$  chain in A.TL mice is a hybrid of A $\alpha$ <sup>k</sup> and A $\alpha$ <sup>s</sup> (22). We found that the nucleotide sequences of A $\alpha$ <sup>k</sup> cDNAs from A/J and A.TL mice are identical.

**Amino Acid Sequences.** Fig. 3 shows the D1 amino acid sequences for all murine A $\alpha$  chains thus far studied: i.e., the A $\alpha$  chains of the *a*, *d*, *b*, *f*, *q*, *u*, *t2*, *r*, and *t1* *H-2* haplotypes. As discussed extensively in a previous paper (15), regions of allelic hypervariability are evident at positions 11–15 and 56–77; the newly determined polymorphic residues in the A $\alpha$ <sup>s</sup> and A $\alpha$ <sup>r</sup> sequences also fall within these stretches.

There is one *k*- and *r*-specific residue, Glu-75. One residue, Arg-57, occurs only in the *k*-haplotype A $\alpha$  alleles. Both of these differences result in the substitution in *k* or in *r* and *k* of a charged residue for a non-charged amino acid present for all of the other haplotypes. The only two *s*-specific residues, Tyr-72 and Thr-73, should also result in structurally significant amino acid changes (21). There are two *r*-specific residues, Phe-26 and Ala-87.

In the second external protein domain, D2, the *s* and *r* alleles (like *d*, *f*, and *q*) differ from the *k* allele by encoding a leucine (instead of a phenylalanine) at position 142 and a histidine (instead of a tyrosine) at position 147.

**Hydrophilicity Plots.** We have used the method of Hopp and Woods (23) to plot local hydrophilicity in an attempt to visualize possible A $\alpha$  antigenic determinants in A.TH anti-A.TL immunizations. The plots of A $\alpha$ <sup>s</sup> and A $\alpha$ <sup>k</sup> hydrophilicity are superimposed in Fig. 4. What appears striking is that the *k*- and *r*-specific residue at position 75 and the *k*-specific residue at position 57 occur in regions that are much more hydrophilic in *k* than in *s* and thus more likely to be in an exposed region of the protein. This difference is not just the result of altering these particular amino acids but also of the variation in surrounding residues, such as the -Tyr-Thr-→-His-Asn- alteration at positions 72 and 73.

For the sake of simplicity, we have not shown the hydrophilicity plot for A $\alpha$ <sup>r</sup>. It closely resembles that of A $\alpha$ <sup>s</sup>, as a quick look at the two sequences would confirm, but it does have a slightly higher peak around Glu-75.

## DISCUSSION

Ia.2 and Ia.19 are antigenic specificities that reside on the murine I-A complex but have a very limited strain distribution: Ia.2 is found on the lymphocytes of only *k*-haplotype mice; Ia.19 is restricted to the *k* and *r* haplotypes. Although these specificities were originally defined using polyclonal sera, several mAbs with similar or identical reactivities have

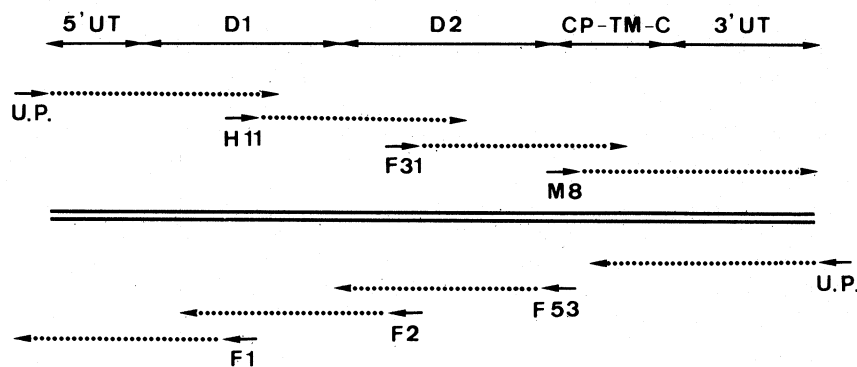


FIG. 1. Sequencing strategy. The double lines symbolize the two strands of A $\alpha$  cDNA inserts cloned in M13. Solid arrows represent the 5' exons of A $\alpha$ : 5' UT, the 5' untranslated region plus the leader peptide; D1, the first external protein domain; D2, the second external protein domain; CP-TM-C, the connecting peptide, the transmembrane region and the cytoplasmic tail; 3' UT, the 3' untranslated region. Dotted arrows depict the DNA stretches sequenced by using the various oligonucleotides as explained in the text. U.P., M13 universal primer.

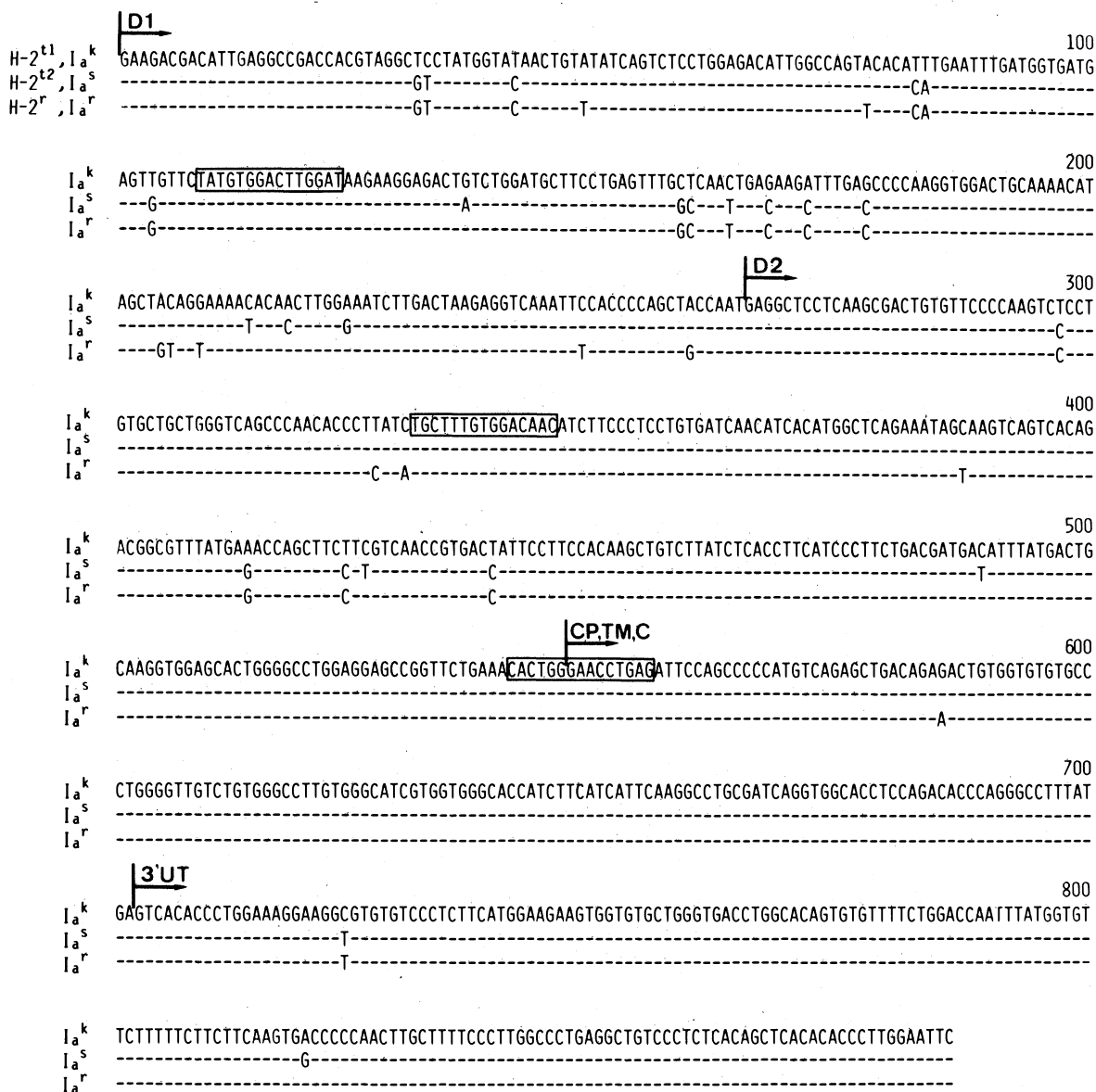


FIG. 2. Nucleotide sequences of allelic  $A_a$  cDNAs. The messenger strand sequence, written in full for the  $k$  haplotype (from A.TL mice), begins with the first residue of D1. The sequences for the  $s$  and  $r$  haplotypes (from A.TH and B10.RIII mice) appear as dashes except at positions where they differ from that of the  $k$  haplotype. Boxes indicate the positions of the sequences corresponding to the oligonucleotides used for sequencing. The regions of the protein are as defined in the legend to Fig. 1.

now been described. In particular, Pierres *et al.* (11) have extensively characterized a series of A.TH anti-A.TL (i.e., haplotype  $s$  anti-haplotype  $k$ ) mAbs which includes five with an Ia.2-like and one with an Ia.19-like pattern of reactivity. Apparently similar mAbs have also been isolated using haplotype  $d$  anti-haplotype  $k$  strain combinations (12).

**The Ia.2 and Ia.19 Determinants May Coincide with Restriction Sites for Some T Cells.** mAbs recognizing Ia.2 or Ia.19 have recently been used in blocking studies aimed at dissecting the structure of T-cell restriction sites on Ia molecules. Dubreuil *et al.* (24) have reported that such Abs are not effective inhibitors of the spleen T-cell response to the antigens poly(Glu<sup>60</sup>Ala<sup>30</sup>Tyr<sup>10</sup>), ovalbumin, and keyhole limpet hemocyanin, and thus conclude that these determinants are not intimately associated with restriction sites for the majority of T cells specific for these three antigens. More recently, however, the response to poly(Glu<sup>60</sup>Ala<sup>30</sup>Tyr<sup>10</sup>) has been studied for isolated T-cell clones and the results appear somewhat different (13). Although none of the clones could be blocked by anti-Ia.2 mAbs, the proliferation of all was

inhibited moderately well by an anti-Ia.19 mAb. A more complex pattern of T-cell blocking has been described by Fathman's group (8, 9). Some but not all clones specific for keyhole limpet hemocyanin, myoglobin, or poly(LTyr, LGlu)-poly(DLAla)-poly(LLys) could be blocked by anti-Ia.19 mAbs. When the same clones were tested with various Ia.2 mAbs, some were blocked severely by a given antibody, some only moderately, and some not at all.

The sum of all these data suggests but does not prove that the Ia.2 and Ia.19 antigenic determinants are part of or are closely associated with the region on an Ia molecule that is recognized by some T cells specific for certain antigens. Arguments may be advanced that the binding of mAbs can cause steric hindrance or result in an allosteric conformational change in Ia structure, but the fact that, for a given antigen, only some T-cell clones are blocked renders this point a less valid objection. With the suggestion in mind that Ia.2 and Ia.19 may coincide with some T-cell restriction sites on Ia, we felt it important to further localize these antigenic determinants.

	1	10	20	30
H-2 <sup>t1</sup> ,Ia <sup>k</sup>	Glu Asp Asp Ile Glu Ala Asp His Val Gly Ser Tyr Gly Ile Thr Val Tyr Gln Ser Pro Gly Asp Ile Gly Gln Tyr Thr Phe Glu Phe			
H-2 <sup>t2</sup> ,Ia <sup>s</sup>	-----	-----	Val ----- Thr -----	----- His -----
H-2 <sup>r</sup> ,Ia <sup>r</sup>	-----	-----	Val ----- Thr -----	----- Phe ----- His -----
H-2 <sup>d</sup> ,Ia <sup>d</sup>	-----	-----	Phe ----- Thr -----	----- His -----
H-2 <sup>b</sup> ,Ia <sup>b</sup>	-----	-----	Thr ----- Ser -----	-----
H-2 <sup>f</sup> ,Ia <sup>f</sup>	-----	-----	Phe ----- Ser -----	-----
H-2 <sup>u</sup> ,Ia <sup>u</sup>	-----	-----	-----	Val -----
H-2 <sup>q</sup> ,Ia <sup>q</sup>	-----	-----	-----	-----
H-2 <sup>a</sup> ,Ia <sup>k</sup>	-----	-----	-----	Val ----- His -----
H-2 <sup>k</sup> ,Ia <sup>k</sup>	-----	-----	-----	-----

	40	50	60	
Ia <sup>k</sup>	Asp Gly Asp Glu Leu Phe Tyr Val Asp Leu Asp Lys Lys Glu Thr Val Trp Met Leu Pro Glu Phe Ala Gln Leu Arg Arg Phe Glu Pro			
Ia <sup>s</sup>	-----	-----	Trp ----- Ile ----- Gly ----- Thr Ser ----- Asp -----	
Ia <sup>r</sup>	-----	-----	Trp ----- Gly ----- Thr Ser ----- Asp -----	
Ia <sup>d</sup>	-----	-----	----- Lys ----- Arg ----- Gly ----- Ile Leu -----	
Ia <sup>b</sup>	-----	-----	-----	Gly ----- Ala Ser ----- Asp -----
Ia <sup>f</sup>	-----	-----	Trp ----- Arg ----- Gly ----- Thr Ser ----- Asp -----	
Ia <sup>u</sup>	-----	-----	-----	Ile ----- Ser ----- Asp -----
Ia <sup>q</sup>	-----	-----	Trp ----- Gly ----- Thr Ser ----- Asp -----	
Ia <sup>k</sup>	-----	-----	-----	-----

	70	80		
Ia <sup>k</sup>	Gln Gly Gly Leu Gln Asn Ile Ala Thr Gly Lys His Asn Leu Glu Ile Leu Thr Lys Arg Ser Asn Ser Thr Pro Ala Thr Asn			
Ia <sup>s</sup>	-----	-----	Tyr Thr ----- Gly -----	
Ia <sup>r</sup>	-----	-----	Val Val ----- Phe ----- Ala -----	
Ia <sup>d</sup>	-----	-----	Ala Glu ----- Gly ----- Phe -----	
Ia <sup>b</sup>	-----	-----	Val Val ----- Gly Val -----	
Ia <sup>f</sup>	-----	-----	Glu ----- Gly Trp ----- Phe -----	
Ia <sup>u</sup>	-----	-----	-----	Gly Val -----
Ia <sup>q</sup>	-----	-----	-----	Gly Gly ----- Phe -----
Ia <sup>k</sup>	-----	-----	-----	-----

FIG. 3. Allelic amino acid sequences of the first external domain (D1). A compilation of all A<sub>α</sub> domain sequences studied to date is shown. The amino acid sequences for the first three haplotypes are deduced from the data of Fig. 2, whereas the remaining (also deduced) sequences are from ref. 15. The mouse strains from which the corresponding cDNA clones were isolated are listed in *Materials and Methods* or in ref. 15.

**Arg-57 on the A<sub>α</sub> Chain Is a Critical Component of the Ia.2 Determinant; Glu-73 Is a Crucial Element of Ia.19.** That Ia.2 and Ia.19 reside, at least in part, on the A<sub>α</sub> chain has recently been shown by immunoprecipitation studies of hybrid Ia molecules as well as by the analysis of blocking behavior on a panel of T-cell clones (8, 9). An earlier report had placed a component of Ia.2 on A<sub>β</sub> because of its ability to precipitate the isolated chain (7), but the significance of this contradiction is unclear since none of the mAbs could precipitate the isolated A<sub>α</sub> chain and since the specificity assignment of the anti-Ia.2 used in these experiments is now doubtful (25). Results from competitive-binding assays suggest that the Ia.2 and Ia.19 determinants are sterically close, but that they are distant from determinants such as Ia.17 and Ia.1 that have recently been localized to the A<sub>β</sub> chain (9, 11, 12).

Thus, we felt that by analyzing the amino acid sequences of several A<sub>α</sub> chains we might be able to pinpoint Ia.2 or Ia.19. A<sub>α</sub> sequences from the H-2 haplotypes *a*, *d*, *b*, *f*, *q*, and *u* were already known (15). Here we have reported the A<sub>α</sub> amino acid sequence from two strains commonly used to

make anti-Ia.2 or anti-Ia.19 antibodies: A.TH (H-2<sup>t2</sup>, Ia<sup>s</sup>) and A.TL (H-2<sup>t1</sup>, Ia<sup>k</sup>). We have also sequenced A<sub>α</sub> from B10.RIII mice, since Ia.19 is found on A<sub>α</sub><sup>r</sup> as well as A<sub>α</sub><sup>k</sup>.

Analysis of the nine A<sub>α</sub> sequences thus far determined reveals that there is only one amino acid, Glu-75, that is restricted to the *k* and *r* haplotypes. Likewise, only Arg-57 is *k*-specific. Surrounding this arginine are three other amino acids (Ala-53, Arg-56, and Glu-59) that *k* shares with only one other haplotype. Yet, despite the fact that it has Ala-53 and Arg-56 like A<sub>α</sub><sup>k</sup>, the A<sub>α</sub> chain from the *u* haplotype does not exhibit Ia.2.

On the basis of these observations, we propose that Arg-57 and Glu-75 are the molecular bases of Ia.2 and of Ia.19, respectively. It may be that these residues are actually part of the epitopes recognized by the mAbs, together with amino acids directly adjacent or quite distant in the primary sequence (for a review of antigenic determinants on proteins, see ref. 26 and refs. therein). Alternatively, it is quite possible that Arg-57 and Glu-75 alter, by allosteric effects, a distant portion of the molecule, or even an associated chain. None-

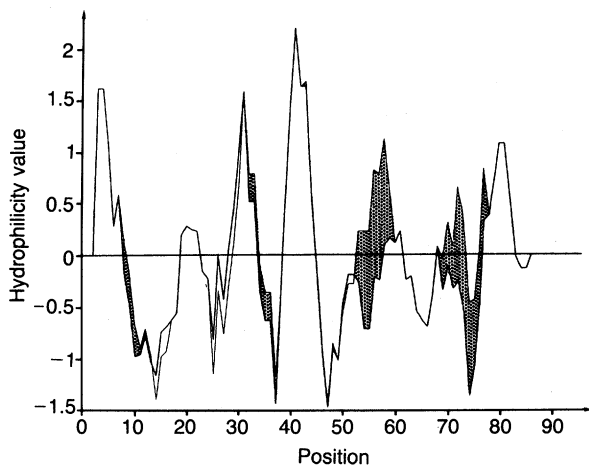


FIG. 4. Hydrophilicity plots of the  $A_{\alpha}^k$  and  $A_{\alpha}^s$  first external domains. The graphs depict the hydrophilicity value, calculated according to Hopp and Woods (23), for the first domain of the  $A_{\alpha}$  chain in the  $k$  and  $s$  haplotypes. A thick line traces the  $A_{\alpha}^k$  plot, and  $A_{\alpha}^s$  appears as a thinner line. Peaks indicating a higher hydrophilicity in  $k$  than in  $s$  are enhanced by dots.

theless, whatever forces may be involved in creating the Ia.2 and Ia.19 determinants, Arg-57 and Glu-75 are crucial to their existence.

**Are Arg-57 and Glu-75 Recognized as Restriction Elements by T Cells?** Because, as discussed above, Ia.2 and Ia.19 may coincide with sites on the Ia complex recognized by some T cells, we are tempted to propose that Arg-57 and Glu-75 lie within T-cell restriction sites on the  $A_{\alpha}$  chain. This speculation is encouraged by the fact that both these residues lie within previously delineated "allelically hypervariable" regions (see Fig. 3 and ref. 15) and by the probability that they occur within exposed regions of  $A_{\alpha}$ , as deduced from hydrophilicity plots (Fig. 4).

Such speculation should, of course, be viewed with caution. T cells, like antibodies, may recognize complicated epitopes resulting from configurations dictated by the allosteric modulation of distant regions or from the juxtaposition of nonadjacent amino acids by protein folding. This point is well illustrated by analyzing the pattern of cross-reactivity of cytolytic T cells specific for mutant MHC class I molecules (27–29). It is clear in this case that the linear sequence alone does not dictate T-cell restriction. On the other hand, the ability to isolate helper T-cell clones that respond to both  $E_{\beta}^b$  and  $A_{\beta}^{\text{bm}12}$  (30) [which probably derives from an  $E_{\beta}$ – $A_{\beta}$  gene conversion event (30–33)] suggests that the primary structure may still be important for class II T-cell restriction.

We have identified two amino acids that are critical to the existence of Ia.2 and Ia.19. Whether these residues are also important in T-cell restriction can now be tested by *in vitro* mutagenesis experiments.

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