

## Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell

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ALTHOUGH many peptides are generated during the intracellular processing of protein antigens, only a few are selected for recognition by the immune system<sup>1-5</sup>. The immunodominant epitope of hen egg white lysozyme (HEL) for H-2<sup>k</sup> mice is contained in a tryptic fragment of amino-acid residues 46-61 (refs 6, 7). The core of this T-cell epitope, from amino acids 52 to 61 (DYGILQINSR), contains those residues required for binding to the class II molecule I-A<sup>k</sup> (ref. 7). Most of the naturally processed fragments recovered from I-A<sup>k</sup>-bearing antigen-presenting cells (APCs) cultured with HEL contained this 52-61 core sequence, presented as a nested set of peptides with extensions at both the amino and carboxyl termini<sup>8</sup>. We now compare the handling by APCs of peptides containing HEL 52-61 to establish whether there is an advantage for the APC in selecting extended peptides: different complexes between peptides and major histocompatibility complex (MHC) molecules varied greatly in the amount of time associated with the APC, and in their immunogenic strength. This difference in persistence is one of the factors contributing to the selection and immune recognition of peptide-MHC complexes by T cells.

The half-lives of peptide-I-A<sup>k</sup> complexes were measured in APCs pulsed with radiolabelled peptides. After binding, the 46-61 peptide remained associated with I-A<sup>k</sup> without much loss, yielding a half-life of greater than 25 h in the three B-cell lymphomas tested, CH27 (ref. 9), TA3 (ref. 10), and M12C3.F6 (ref. 11) (abbreviated to C3.F6) (Fig. 1). A similar long-lived association was observed for the 48-62 peptide, which represents the most abundant fragment recovered from APCs after culture in HEL<sup>8</sup> (Table 1). By contrast, the 52-61 core peptide bound to I-A<sup>k</sup> but was lost with a half-life of about 7 h in all three APC lines (Fig. 1, Table 1). These differences in half-lives between the 46-61 and the 52-61 peptides were also reflected in the number of biologically relevant complexes at the APC surface (Table 2). Previous studies of half-lives of peptides bound to HLA-DR molecules of B-lymphoblastoid cells had indicated that the peptide-MHC complex was practically irreversible<sup>12</sup>. These results agree with the finding of long-lived complexes but indicate that differences in half-life exist among peptides.

The half-lives of the labelled 46-61 and 52-61 peptides were unchanged by incubation in excess unlabelled HEL 46-61 or HEL, which should discourage the reassociation of any released labelled peptide with I-A<sup>k</sup> (Fig. 1b). Therefore, the persistence of the 46-61 or 48-62 peptides cannot be explained by exchange of this peptide between class II molecules; and the at least 25-h half-life must represent that of the peptide-associated class II molecule. As the half-life of leucine-labelled I-A<sup>k</sup> molecules in these B-cell lymphomas ranged over 14-18 h (medium only in Fig. 2), the 46-61 peptide must extend the life of the class II molecule to which it is bound. Clearly the loss of the 52-61 peptide is not accelerated by excess unlabelled peptide, either through a mechanism of ligand-induced dissociation<sup>13</sup> or competitive exchange<sup>14</sup>. The short half-life of the 52-61 peptide represents either the dissociation of this peptide from the class II molecules of the APC or the selective degradation of the class II molecules to which this peptide is bound. Notably, that

although the long 46-61 and the short 52-61 peptides differ in affinity (30-fold)<sup>15</sup>, this difference is not reflected in dissociation times measured *in vitro*. A low dissociation rate was also observed for the release of these peptides from complexes in cell lysates, done either at 21 °C or 37 °C (Fig. 1c).

The half-life of the I-A<sup>k</sup> molecule in C3.F6 cells was altered by replacing the endogenously presented peptides with peptides generated from HEL (Fig. 2). C3.F6 cells, metabolically labelled with leucine, were cultured in HEL, and after 4 h the cells were washed and placed back in culture with medium alone. The average half-life of the I-A<sup>k</sup> molecules for these cells was prolonged from 12.6 h to 23 h (Fig. 2; total I-A<sup>k</sup>). For the experiment described in Fig. 2, we also compared half-lives of the stable and unstable I-A<sup>k</sup> molecules as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)<sup>16-21</sup>. The half-lives of the stable molecules from APCs grown in medium were longer than those of the unstable molecules (20.3 h compared with 10.2 h) (Fig. 2; medium only). This difference between the stable and unstable half-lives was even more pronounced for cells incubated in HEL (43.0 h and 10.9 h) (Fig. 2; media + HEL). The addition of HEL did not significantly alter the half-life of the unstable molecules. Therefore, the increase in half-life of the total I-A<sup>k</sup> after loading with HEL was due directly to an increase in the relative amount of the SDS-stable molecules, which was evident after culture with HEL.

Table 1 examines the half-life in APCs and the SDS-PAGE stability properties of different MHC-peptide 52-61 complexes. Peptide 51-61 (threonine is the natural residue at position 51) bound to I-A<sup>k</sup> and induced a mixed pattern in SDS-PAGE. The half-life in APC was similar to that of the average I-A<sup>k</sup> molecules. When the threonine residue at 51 was changed to alanine, the complexes produced were unstable and yielded a short half-life. Substitution at residue 51 with lysine, arginine or leucine all resulted in half-lives in the cell close to the mean half-life of I-A<sup>k</sup>. But substitution at this position with a negatively charged glutamic-acid residue resulted in peptides that formed mostly

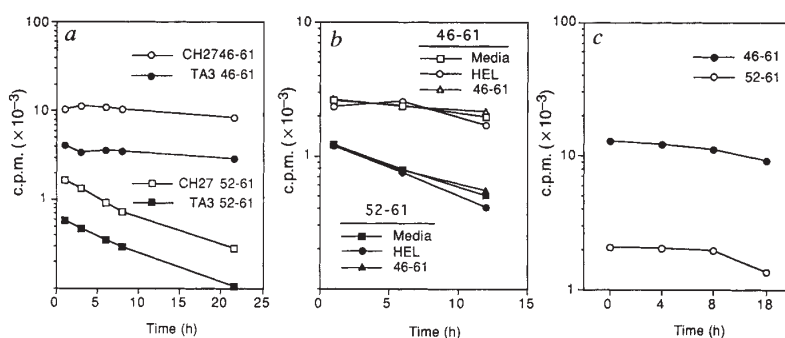
TABLE 1 Time of persistence of I-A<sup>k</sup>-labelled peptides in APCs

Peptide	T <sub>1/2</sub> (h)	n	% Stable
52-61 (DYGILQINSR)	6.8(±2.1)	10	12
52-62 (52-61-W)	8.0	1	16
A-52-61	9.6(±4.4)	4	24
L-52-61	11.5	1	10
R-52-61	12.8(±4.6)	4	12
D-52-61	13.2(1.7)	3	60
K-52-61	13.5(±3.5)	3	45
T-52-61	15.5(±7.7)	5	60
E-52-61	21.8(4.4)	4	>90
48-62 (DGST-52-61-W)	22.3(2.9)	3	>90
46-61 (NTDGST-52-61)	22.6(2.6)	11	>90

Summary of results of all experiments on the persistence of the indicated <sup>125</sup>I-labelled peptides. Experiments using all three lymphoma cells are included, although most were done in C3.F6 cells, as described in the legends for Figs 1 and 2. With a given peptide, no differences in half-life were found among the three cell lines. Indicated are the mean values, with s.d., and the number of individual experiments (n). '% Stable' is the fraction of labelled peptide bound to I-A<sup>k</sup> that ran in the position of a stable dimer on SDS-PAGE. The results of % stability have been published previously<sup>15</sup>. For a given peptide-I-A<sup>k</sup> complex in APCs, the percentage found in the stable or unstable forms at different times in culture did not vary significantly. The data from three representative experiments are: peptide K-52-61 gave a half-time (t<sub>1/2</sub>) of persistence of 11.5 h. The percentage stable at 1, 3, 6, 9 and 12 h was 54, 59, 60, 65 and 61%, respectively. Peptide T-52-61 (t<sub>1/2</sub> 11.5 h in this experiment) showed % stable, at the same times, of 45, 37, 35, 44 and 45%, respectively. Peptide A-52-61 (t<sub>1/2</sub> of 12 h) showed % stable, at the same times, of 20, 23, 30, 32 and 30%, respectively. This indicates to us that stable and unstable complexes do not represent two independent populations.

FIG. 1 Half-lives of 46-61 and 52-61 bound to I-A<sup>k</sup> of TA3 and CH27 cells (a), in C3.F6 in the presence of excess unlabelled peptide (b) and in cell lysates (c). a, Differences in life time of various peptide-MHC class II complexes were quantified by immunoprecipitation at different time points from cells cultured in <sup>125</sup>I-labelled peptides. Results for 46-61 and 52-61 in CH27 cells or TA3 cells in one representative experiment of 10 carried out with the various B lymphomas are shown (see Table 1 for a summary). b, The persistence of <sup>125</sup>I-labelled 46-61 or [<sup>125</sup>I]-52-61 bound to I-A<sup>k</sup> of C3.F6 is not changed by culturing in a vast excess of HEL or unlabelled 46-61. C3.F6 were incubated first with labelled peptides, followed by an excess of HEL or unlabelled 46-61. c, The half-life of I-A<sup>k</sup> peptide complexes in cell lysates is long. The cells were lysed at 4 h, unlabelled peptides were added and the lysate was incubated; I-A<sup>k</sup>-labelled peptides were immunoprecipitated at the indicated times.

METHODS. a, Synthetic peptides were labelled with <sup>125</sup>I to a specific activity of  $2-4 \times 10^9$  c.p.m.  $\mu\text{g}^{-1}$  by the chloramine-T method and then purified on C<sub>18</sub> reverse-phase HPLC. Cells were exposed to 1,000 rad of gamma irradiation then washed in DMEM containing 2% FCS and resuspended at  $5 \times 10^6$  cells  $\text{ml}^{-1}$ . A total of  $4-8 \times 10^6$  c.p.m. of labelled peptide was added to each set of  $5 \times 10^7$  cells, at 37 °C, for 4 h. After washing in medium the cells were resuspended at  $1 \times 10^6$  cells  $\text{ml}^{-1}$  in 10-ml flasks. The total uptake of peptide was  $\sim 1-5\%$  of the input c.p.m.; the amount precipitable with I-A<sup>k</sup> ranged from 1 to 10% of the cell-associated radioactivity. At different times, cells from individual flasks were lysed and complexes immunoprecipitated as previously described<sup>15</sup>. Briefly, the cells were collected, washed and then lysed in 0.5 ml 1% Triton X-100, 10 mM iodoacetamide, 1 mM phenylmethylsulphonyl fluoride, and 20 mg  $\text{ml}^{-1}$  leupeptin. After a 40-min pre-clear with 25  $\mu\text{l}$  50% Protein-A-Sepharose in PBS containing 1% Triton-X-100, the peptide-I-A<sup>k</sup> complexes were immunoprecipitated, using 50 mg  $\text{ml}^{-1}$  final of monoclonal antibody specific for the I-A<sup>k</sup>  $\beta$ -chain. In this case the antibody was 40.F<sup>22</sup>, although in similar experiments we have used 10.3.6.2 (ref. 23) with essentially identical results. Lys-



ates were incubated with antibody for 2 h with Protein-A-Sepharose added for the last hour of incubation. The complexes were spun, washed and eluted for 1 h at room temperature in 2% SDS loading buffer before analysis by SDS-PAGE. The amount of labelled peptide at each time point was determined by direct gamma counting of excised gel fragments. Half-lives were determined by regression analysis. In b, C3.F6 were incubated with <sup>125</sup>I-labelled 46-61 or 52-61 for 4 h as described for a. We estimated that at 1 h,  $10^7$  C3.F6 cells contained 1 fmol of [<sup>125</sup>I]-46-61 or [<sup>125</sup>I]-52-61 bound to I-A<sup>k</sup>. The  $10^7$  cells were then washed and incubated with 700 nmol of HEL and with 1  $\mu\text{mol}$  of 46-61 peptide, expecting to contain 7 nmol of HEL and 1 nmol of 46-61 respectively; this represents a 7 and 1 million-fold excess over the 1 fmol of peptide bound to I-A<sup>k</sup> respectively. Aliquots of  $10^7$  C3.F6 were taken at the indicated times and the I-A<sup>k</sup> immunoprecipitated with 10.3.62 monoclonal antibody. In c, C3.F6 were incubated with [<sup>125</sup>I]-46-61 or [<sup>125</sup>I]-52-61 or 4 h, as detailed above. After 4 h, the cells ( $16 \times 10^7$ ) were lysed in 16 ml of lysis buffer. The amounts of [<sup>125</sup>I]-52-61 or [<sup>125</sup>I]-46-61 contained in the lysate were about 30 pmol and 10 pmol respectively. A 10,000-fold excess of nonradioactive peptides was then added to the lysate which was incubated for the times indicated. Aliquots of lysate corresponding to  $10^7$  cells were collected, immunoprecipitated with 10.3.62 antibodies and analysed as detailed in a. Similar experiments at 37 °C resulted in half-dissociation times of 18 and 20 h for 46-61 and 52-61, respectively.

FIG. 2 Culture of C3.F6 with HEL increases the half-life of I-A<sup>k</sup>. C3.F6 cells were pulse labelled with [<sup>3</sup>H]-leucine in the absence or presence of HEL. After a 30-min pulse the cells were cultured in regular media (with or without HEL), and at the indicated times the I-A<sup>k</sup> molecules precipitated from aliquots with 10.3.62 monoclonal antibody. The amount of immunoprecipitated I-A<sup>k</sup> was estimated from eluates on Protein-A-Sepharose beads. The eluates were also analysed by SDS-PAGE. The radioactivity in the stable (that is,  $\alpha\beta$ -dimer) and unstable ( $\alpha$ -plus  $\beta$ -bands) was measured by counting excised gel fragments, and the percentage in each was calculated. The figure shows the mean results of four different experiments. In each experiment duplicate or triplicate observations were made at each time point. The indicated point represents means of two to four experiments. Variations of the mean ranged over 5-20%. Because the degree of uptake of [<sup>3</sup>H]-leucine varied among the four experiments, the first time point (3 h) was normalized to 100%. a, Half-life of total [<sup>3</sup>H]-leucine-labelled I-A<sup>k</sup>. b and c, Half-lives of the individual stable and unstable components in cells cultured without or with HEL, respectively. The percentage of stable molecules in C3.F6, not cultured with HEL, was 40, 49, 50, 48 and 51% at 3, 6, 8, 9 and 12 h, respectively. For C3.F6 cultured with HEL the percentage of stable molecules, at the same times, was 66, 70, 78, 76 and 77% respectively. The  $t_{1/2}$  values for I-A<sup>k</sup> calculated by linear regression analysis were: for C3.F6 cultured in regular media without HEL 12.6 h, with 10.2 h for the unstable and 20.3 h for the stable components. For C3.F6 cultured in media with HEL 23.0 h with 10.9 h for the unstable and 43.0 h for the stable component.

METHODS. C3.F6 cells were cultured at  $2 \times 10^7$  in leucine-deficient DMEM for 1 h, after which [<sup>3</sup>H]-leucine (Amersham, 150 Ci  $\text{mmol}^{-1}$ ) was added for 30 min. Aliquots contained 800  $\mu\text{Ci}$  per  $1.2 \times 10^7$  cells in 6 ml, in T-25 flasks. One aliquot of the cells contained 2 mg  $\text{ml}^{-1}$  of HEL. After 30 min nonradioactive leucine was added, cells were collected, washed and resuspended in media (without or with HEL, as above), and incubated at 37 °C. At the times indicated, aliquots of  $2 \times 10^7$  were collected, washed and immunoprecipitated as indicated in Fig. 1.

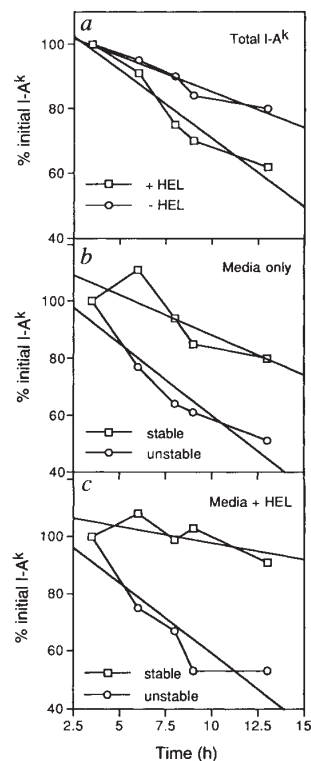


TABLE 2 Loss of immunogenicity of peptide 52-61

Time (h)	IL-2 induced with:	
	46-61 (IL-2 units)	52-61 (IL-2 units)
1	40	18
6	35	1
20	45	0

The immunogenicity of peptide 46-61 persists for long periods whereas that of 52-61 rapidly decays. Irradiated C3.F6 cells were incubated with 46-61 ( $10 \times 10^6$  cells in 5 ml with  $1 \mu\text{M}$ ) or with 52-61 ( $10 \times 10^6$  in 5 ml with  $50 \mu\text{M}$ ) for 4 h at  $37^\circ\text{C}$ . After 4 h the cells were washed, resuspended in medium without peptide and incubated for the indicated time periods. The cells were then fixed in 1% paraformaldehyde (at room temperature for 15 min, then incubated in 0.4 M lysine for 15 min, washed and stored at  $4^\circ\text{C}$ ). Cells were assayed with the BD-4 T-cell hybridoma and tested for interleukin-2 (IL-2) production. The figures indicate units of IL-2 assayed by conventional methods on the IL-2 indicator CTLL cells<sup>6</sup>. The BD-4 hybridoma was derived from lymph nodes of CBA mice immunized with 52-61 peptide in complete Freund's adjuvant. Before this experiment identical protocols were evaluated using input amounts of 46-61 of  $10 \mu\text{M}$ ,  $1 \mu\text{M}$  and  $0.3 \mu\text{M}$  with identical results; there was no drop in the T-cell response during the first 20 h of incubation. The doses of 10 and  $1 \mu\text{M}$  of 52-61 showed similar rapid loss of the T-cell response. The doses of  $10-1 \mu\text{M}$  gave responses in the linear portion of the dose-response curve. In a previous study<sup>24</sup> a shorter persistence of peptide was found but under different conditions of incubations (that is, limiting amounts of peptide for only 30 min).

stable molecules (90%), with markedly prolonged half-life. The contribution of the amino acid at position 51 to both the stability and persistence of the complex can be cancelled if the amino-acid side chain is hydrophobic (Table 1 legend, and our unpublished studies), or contains a positive charge (Table 1 legend).

Our results indicate that APCs discriminate between different I-A<sup>k</sup>-peptide complexes as evidenced by different times of persistence. These differences inevitably select for long-lived peptides and affect both the potency and immunodominance of the complexes recognized by T cells. The differences in persistence may reflect conformational changes in the I-A<sup>k</sup>-peptide complex read out by the propensity of the peptide to form stable SDS-PAGE molecules. Live APCs may sense these differences in structure and handle the complexes in different manners (that is, for example, intracellular turnover rate, recycling, different traffic patterns). For complexes of intermediate stability the correlation to short half-life is not strict. We find a correlation only between peptides having a high propensity to stabilize the I-A<sup>k</sup> dimer and the longer half-life. □

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## Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia

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ACHONDROPLASIA, the most common cause of chondrodysplasia in man (1 in 15,000 live births), is a condition of unknown origin characterized by short-limbed dwarfism and macrocephaly<sup>1,2</sup>. More than 90% of cases are sporadic and there is an increased paternal age at the time of conception of affected individuals, suggesting that *de novo* mutations are of paternal origin. Affected individuals are fertile and achondroplasia is transmitted as a fully penetrant autosomal dominant trait, accounting for rare familial forms of the disease (10%)<sup>3-6</sup>. In contrast, homozygous achondroplasia is usually lethal in the neonatal period and affects 25% of the offspring of matings between heterozygous achondroplasia parents. The gene responsible for achondroplasia has been mapped to chromosome 4p16.3 (refs 7, 8); the genetic interval encompassing the disease gene contains a member of the fibroblast-growth-factor receptor (FGFR) family which is expressed in articular chondrocytes. Here we report the finding of recurrent missense mutations in a CpG doublet of the transmembrane domain of the FGFR<sub>3</sub> protein (glycine substituted with arginine at residue 380, G380R) in 17 sporadic cases and 6 unrelated familial forms of achondroplasia. We show that the mutant genotype segregates with the disease in these families. Thus it appears that recurrent mutations of a single amino acid in the transmembrane domain of the FGFR<sub>3</sub> protein account for all cases (23/23) of achondroplasia in our series.

Individuals with achondroplasia (ACH) have growth cartilage of their long bones that undergoes minimal proliferation<sup>9</sup>, but the cartilage of their remaining epiphyses is normal, implicating a possible abnormality in a growth factor or its chondrocyte receptor<sup>9</sup>.

The gene for ACH has been mapped to chromosome 4p16.3 by linkage in ACH families<sup>7,8</sup> and the disease locus has been subsequently mapped distal to D4S43 (maximum pairwise lod score at D4S111:  $Z_{\text{max}} = 3.01$  at  $\theta = 0$  in six ACH families; results not shown). Interestingly, the gene encoding FGFR<sub>3</sub> maps to the same region<sup>10</sup>. FGFR<sub>3</sub> is a tyrosine kinase receptor with a large glycosylated extracellular ligand-binding domain containing three immunoglobulin-like repeats, a single hydrophobic transmembrane region and a cytoplasmic domain that contains a tyrosine kinase catalytic domain disrupted by a hydrophobic

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