

AN HLA-A2/ β_2 -MICROGLOBULIN/PEPTIDE COMPLEX ASSEMBLED FROM SUBUNITS EXPRESSED SEPARATELY IN *ESCHERICHIA COLI*

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Abstract—The human class I histocompatibility antigen HLA-A2 has been assembled from subunits expressed separately in *E. coli*. A peptide that is known to be recognized by human cytotoxic T lymphocytes (CTLs) in association with HLA-A2 is a necessary component of the reconstitution mixture. The N-terminal extracellular fragment of the HLA-A2 heavy chain is initially synthesised as an insoluble aggregate. The aggregate is solubilized in denaturant, mixed with the influenza nucleoprotein 85–94 decapeptide (NP peptide), and diluted into a solution containing human β_2 -microglobulin (β_2 m) isolated from the *E. coli* periplasm. The HLA-A2 heavy chain becomes soluble in physiological solutions if both β_2 m and the NP peptide are present. The reconstituted HLA-A2 complex is recognised by a monoclonal antibody that is specific for the native HLA-A2/ β_2 m heterodimer, and is also recognised by a monoclonal antibody that recognises β_2 m. When other peptides known from CTL studies to associate with HLA-A2 are used, a significantly lower yield of reconstituted complex is obtained. The isoelectric point of the reconstituted complex depends on which peptide is used, confirming that the peptide is a component of the reconstituted complex.

INTRODUCTION

Class I molecules contain two subunits: the HLA heavy chain, which is an integral membrane protein, and β_2 -microglobulin (β_2 m), which also exists as a monomeric serum protein (Bjorkman and Parham, 1990). The function of Class I molecule is to bind a small peptide derived from any protein present in the same cell that expresses the histocompatibility antigen. The peptide is bound in such a way that the T-cell receptor on cytotoxic T-lymphocytes (CTL) can distinguish it from other peptide/class I complexes (Townsend and Bodmer, 1989). Several lines of evidence suggest that the peptide may be integral to the structure of class I molecules: (A) the crystal structures of the class I molecules HLA-A2 and HLA-Aw68 reveal that something is already present in the peptide-binding cleft (Bjorkman *et al.*, 1987a,b; Garrett *et al.*, 1989). This material presumably corresponds to endogenous peptides that were not separated away during purification; (B) in certain mutant cell lines, stable cell surface expression of class I molecules requires addition of exogenous peptide (Townsend *et al.*, 1990; Ljunggren *et al.*, 1990; Lie *et al.*, 1990); (C) only a small percentage of purified class I molecules can bind radio-labelled peptides in solution (Chen and Parham, 1989), presumably because the endogenous peptides are very tightly associated.

In order to understand how such a wide variety of peptides can bind to each histocompatibility antigen, it would be useful to have a binding assay that is not complicated by the endogenous peptide. A prokaryotic expression strategy was designed that should synthesise the exact same primary structure of the HLA heavy chain as is present in the HLA-A2 complex that can be solubilised from cell membranes by papain.

The X-ray structure of the papain-released HLA-A2 indicates that the C-terminal fragment that is removed by papain digestion does not contribute directly to peptide binding. Although HLA-A2 is a glycoprotein, the single carbohydrate is not essential for folding, because neither mutation of the carbohydrate acceptor sequence (Santos-Aguado *et al.*, 1987) nor growth in tunicamycin (Ploegh *et al.*, 1981) abolishes cell-surface expression. Since the HLA heavy chain contains two disulphide bonds, a secretion cloning plasmid (pIN-III-ompA2) (Ghrayeb *et al.*, 1984) was chosen to direct secretion into the oxidising environment of the periplasm. β_2 m has already been successfully synthesised in *E. coli* using this same vector (Parker and Wiley, 1989).

Here, we report that the HLA heavy chain can be expressed in *E. coli* as an insoluble aggregate. In order to obtain the conformational properties of a native preparation, a major histocompatibility complex (MHC)-restricted peptide and β_2 m must both be added during the reconstitution procedure. The reconstitution process itself can be used to study peptide binding.

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MATERIALS AND METHODS

An 1853 bp restriction fragment (*Bgl*I-*Bgl*I) containing exon 4 was excised from an HLA-A2 genomic clone (van de Rijn *et al.*, 1984), treated with T4 DNA polymerase, and inserted into *Sma*I cleaved M13mp18. The codon which encodes Leu 272, the site of papain cleavage (Orr *et al.*, 1979), was changed to a stop codon (CTG to TAG) using site-directed mutagenesis in M13 (Norlander *et al.*, 1983) with the 17-mer CCCTCACCTAGAGATGG. After subcloning from M13 into pUC18, the 1574 bp fragment (*Bgl*II-*Nco*I) containing exon 4 was reinserted into the genomic A2 clone. An intact HLA-A2 cDNA was isolated from a JY lymphoblastoid cell line cDNA library (Speck and Strominger, 1985) using the partial A2 cDNA clone JY 107 (Arnot *et al.*, 1984). A 643 bp fragment (*Eco*RI-*Nde*I) that contained exon 1 through part of exon 4 (cDNA) and a 1263 bp fragment (*Nde*I-*Sph*I) containing the rest of exon 4, the stop mutation and genomic DNA extending into the intron after exon 7 was then inserted into *Eco*RI/*Sph*I-digested pUC18. The 1912 bp fragment (*Eco*RI-*Hind*III) was then inserted into *Eco*RI/*Hind*III-digested pIN-III-ompA2. The 2022 bp fragment (*Xba*I-*Hind*III) containing the *ompA* signal sequence and the A2 construct was subcloned into M13, and the 30-mer ACCGTAGC-GAGGCCGGATCCCACTCCATG was used to bring the *ompA* signal sequence in frame with the mature N-terminus of HLA-A2, thus deleting the *Eco*RI site and the original HLA-A2 leader peptide sequence. The entire *ompA*2-HLA-A2 coding sequence was confirmed by DNA sequencing. The new 1916 bp

fragment (*Xba*I-*Hind*III) was then reinserted into pIN-III-ompA2 to generate p4037.

p4037 was transformed into strain KS474 (Strauch and Beckwith, 1988), and 1 litre was grown at 30°C in L broth (Luria broth base, Gibco BRL). The cells were pelleted, resuspended in 10 ml of 23% sucrose in 10 mM Tris-chloride pH 8.0, and treated with 0.2 ml 5 mg/ml lysozyme, and then diluted into 20 ml ice-cold 3 mM EDTA. The suspension was passed three times through a French press cell (SLM instruments) set at 10,000 psi. The homogenate was layered onto a cushion of 5 ml 67% sucrose in 10 mM Tris-chloride pH 8.0/1 mM EDTA and centrifuged for 1 hr at 25,000 rpm in a Beckman SW 27 rotor. The cloudy material that floated on the 67% sucrose cushion was placed on a stop gradient consisting of 67% sucrose, 53% sucrose and 40% sucrose (all in 10 mM Tris-chloride pH 8.0/1 mM EDTA) and centrifuged for 18 hr at 18°C as above. Aggregates containing HLA heavy chain were found at the 67%/53% interface, and were used in reconstitution assays without further purification.

An aliquot (0.05 ml) from the sucrose gradient fraction (5.5 ml total volume) that was most enriched in mature, intact HLA heavy chain (e.g. Fig. 1, lane 7) was mixed with 0.45 ml of 10 mM 4-morpholineethanesulfonic acid (MES) pH 6.5/150 mM NaCl, centrifuged for 15 min, and resuspended in 12.5 μ l 8.8 M urea/125 mM MES. A 12.5 μ l aliquot of this solution was added to 2.5 μ l peptide (400 μ M) dissolved in the same buffer, and incubated for 90 min at 25°C. Then 45 μ l of dilution buffer containing β_2 m was added (final concentration 3.0 μ M β_2 m, 20 μ M peptide, 0.88 M urea, 25 mM MES, 150 mM NaCl), and the solution was incubated at 4°C

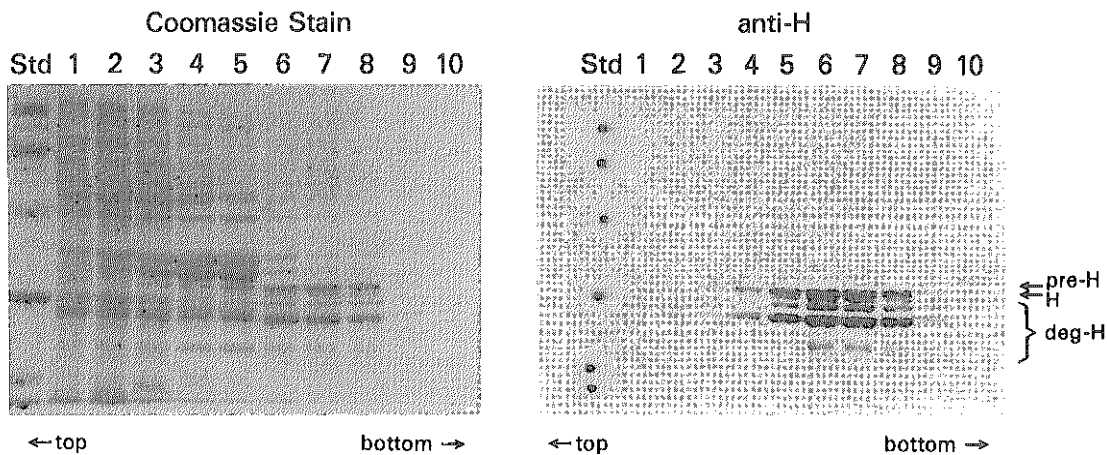


Fig. 1. Purification of inclusion bodies by sucrose density centrifugation. Aliquots of each sucrose gradient fraction were loaded onto a 12% SDS gel. (A) A PVDF membrane, stained with Coomassie blue following SDS PAGE and electroblotting. (B) The same membrane as above was destained, and developed with the MoAB HCA-10 and alkaline phosphatase coupled to anti-Ig. Std refers to BioRad molecular weight standards (phosphorylase B, $M_r = 97,400$; bovine serum albumin, $M_r = 66,200$; ovalbumin, $M_r = 45,000$; carbonic anhydrase, $M_r = 31,000$; trypsin inhibitor, $M_r = 21,500$; lysozyme, $M_r = 14,400$). The position of each standard protein was marked with ink prior to destaining. "anti-H" refers to the MoAB HCA-10, which recognises the HLA heavy chain. "Top" refers to the top and; "bottom" to the bottom of the sucrose gradient. "pre-H" refers to the *ompA* leader peptide/HLA-A2 fusion protein (preprotein). "H" refers to the intact, mature HLA-A2 heavy chain. "deg-H" refers to HLA heavy chain-derived proteins that appear to be degraded. The HLA banded at the 53% sucrose/67% sucrose interface.

for at least 18 hr. In Fig. 2, the samples were diluted an additional 3-fold prior to analysis. The reconstituted HLA is stable for at least a week under these conditions. Alternatively, as in Fig. 3, HLA pelleted from the sucrose gradient as described above was dissolved in 6 M NaSCN/25 mM MES pH 6.5 containing 200 μ M peptide and dialysed (Spectrapor, 500 molecular weight cutoff) into 25 mM MES/150 mM NaCl. Immediately prior to analysis, the samples were centrifuged at 15,000 rpm (19,500 *g*) in a TOMY MTX-150 refrigerated micro-centrifuge to remove non-reconstituted material.

SDS gels were prepared as described (Laemmli, 1970). Native acrylamide gel electrophoresis (NAGE) gels were identical to SDS gels except in that they contained no SDS. In addition, the samples were not exposed to reducing agents or SDS and were not heated to 100°C prior to loading. Native isoelectric focusing (NIEF) gels were performed as described (Silver *et al.*, 1991). A BioRad Trans-Blot apparatus was used to electroblot from all three of the gel systems to polyvinylidene difluoride (PVDF) membranes (Millipore, Immobilon-P). To obtain efficient transfer from native gels, gels were washed three times for 5 min each in 1% SDS/50% methanol prior to equilibration in 20% methanol/Tris glycine transfer buffer (modified from Neffjes *et al.*, 1986). PVDF membranes were stained in Coomassie Blue, and the relevant bands were excised and used directly for protein sequencing as described (Matsudaira, 1987). Protein sequencing was kindly carried out by W. Lane at the Harvard microchemistry facility.

ELISA sandwich assays were performed as follows. First, about 100 ng of Protein A affinity-purified MoAB W6/32 was added to each well of a micro ELISA

plate (Dynatech). Then the plates were blocked with 3% bovine serum albumin, and 100 μ l aliquots of the solutions to be assayed were added and incubated for 1 hour at room temperature. About 100 ng of MoAB BBM.1 labelled with *N*-hydroxysuccinimidylbiotin was added to complete the sandwich. The plate was developed with ExtrAvidin alkaline phosphatase (Sigma) and *p*-nitrophenyl phosphate tablets (Kirkegard and Perry, Gaithersburg, MD, U.S.A.)

The MoAB BBM.1 (Brodsky *et al.*, 1979) was purified from an ascites tumour grown at the Harvard BioLabs hybridoma facility. MoAB W6/32 (Parham *et al.*, 1979) was obtained from the laboratory of J. Strominger (Harvard University). MoAB HC-10 (Stam *et al.*, 1986) was a gift from H. Ploegh (Netherlands Cancer Institute). The anti-HLA antibody M300020 was from Olympus Immunochemicals (Lake Success, NY, U.S.A.). β_2 m was purified from *E. coli* as described (Parker and Wiley, 1989). *E. coli* strain KS474 was from K. Strauch (Strauch and Beckwith, 1988), and strain JM103 (Hanahan, 1983) was from the lab of J. Seidman (Harvard Medical School). The NP peptide and others were a gift from J. Rothbard (ImmuLogic Pharmaceutical Co.). The NP Y \rightarrow F peptide (see Table 1) was synthesised using an Applied Biosystems Model 431A Peptide Synthesizer using Fmoc chemistry.

The HLA-A2 construct encoded by p4037 is downstream of a *lac* operator sequence, and the parent plasmid, pIN-III-ompA2, was designed to be inducible by the *lac* inducer isopropyl-1-thio- β -D-galactopyranoside (IPTG) (Ghrayeb *et al.*, 1984). Nonetheless, when *E. coli* harbouring p4037 are grown in rich medium, a substantial amount of immunoreactive protein can be detected,

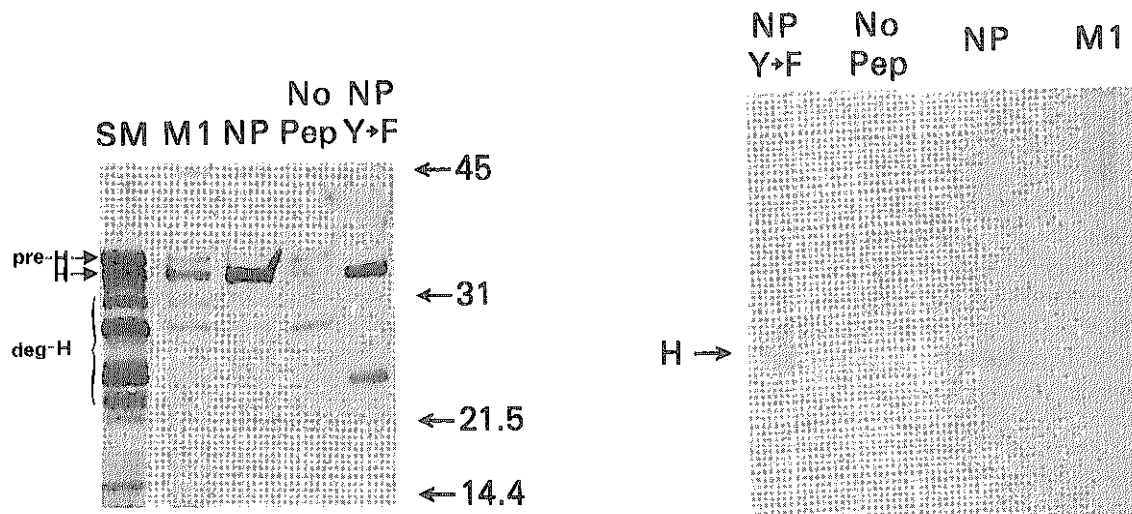


Fig. 2. Selective solubilisation of HLA-A2 from inclusion bodies in the presence of various peptides. (A) A PVDF membrane stained as Fig. 1B following 12% SDS PAGE. The amount of reconstituted HLA that was loaded onto each well was derived from inclusion bodies expressed by 0.6 μ l of *E. coli* at lag phase. "SM" = whole inclusion bodies (starting material); "M1" = supernatant of inclusion bodies reconstituted with β_2 m and the M1 peptide (see Table 1 for exact sequence); "NP", "No PEP" and "NP Y \rightarrow F" = samples identical to "M1" but reconstituted with peptide NP, no peptide, or peptide NP Y \rightarrow F, respectively. (B) A PVDF membrane stained as Fig. 1B following 5% NAGE. Identical samples as in Fig. 2A, except that the equivalent of 0.9 μ l *E. coli* at lag phase was loaded. "H" points to the reconstituted HLA-A2/ β_2 m/peptide complex band, visible only in lanes NP and NP Y \rightarrow F.

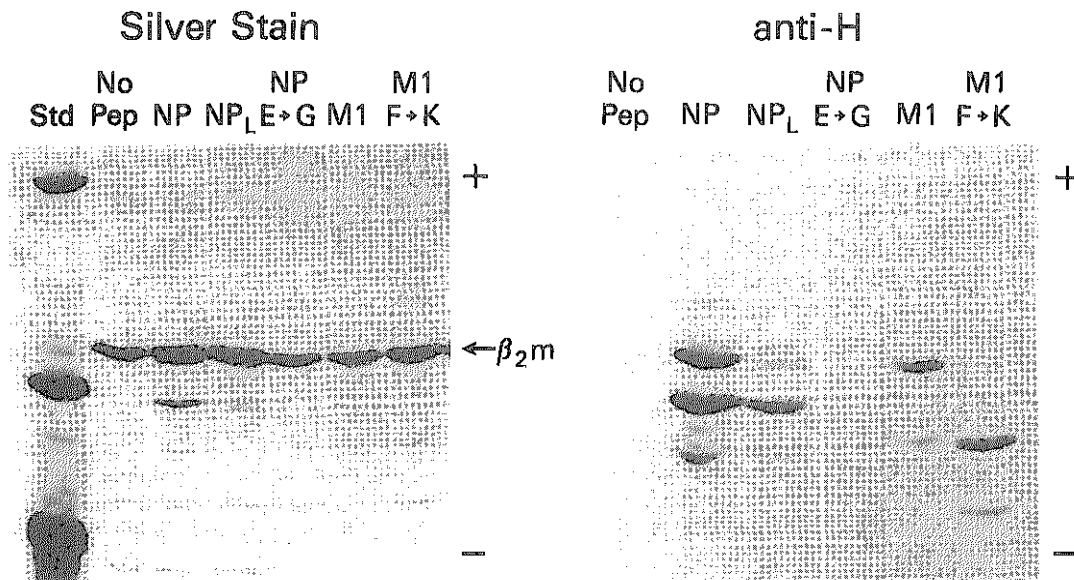


Fig. 3. Analysis of reconstitution by NIEF acrylamide electrophoresis. (A) Reconstitution supernatants as in Fig. 2 were analysed by NIEF gel electrophoresis and silver-stained. "Std" refers to NIEF standards. "No Pep" refers to an aliquot similarly prepared as in the "No Pep" lane of Fig. 2. "NP", "NP_L", "NP E → G", "M1" and "M1 F → K" refer to aliquots reconstituted with the peptides defined in Table 1. + refers to the anode. - refers to the cathode. " β_2m " points to the band that corresponds to free, uncomplexed β_2m . (B) An identical set of samples was run on an identical NIEF gel, electroblotted onto PVDF membranes, and developed with anti-HLA MoAB (Olympus) and alkaline phosphatase coupled to anti-Ig.

whether or not IPTG is added. The M_r of the predominant species varies between different *E. coli* host strains, especially when the cultures are not induced with IPTG. For example, in strain JM103, there are two major immunoreactive species, with M_r s of 35k and 33k (data not shown), whereas in strain KS474, the pattern of immunoreactive material depends on the growth conditions. When a fresh transformation of p4037 in KS474 is prepared, the profile contains four major bands (see Fig. 1, lane 7) with M_r s of 33k ("pre-H" in Fig. 1), 32k ("H" in Fig. 1), 30k and 28k ("deg-H" in Fig. 1). Depending on the experiment, the protein with a M_r of 33k may be nearly absent, as in Fig. 1, lane 7, or it may be one of the major immunoreactive species, as in Fig. 2A, "SM". Induction with IPTG causes a larger percentage of the immunoreactive protein to run at a M_r of 33k. Likewise, when a culture of p4037 is grown up from a frozen stock, the major immunoreactive component has a M_r of 33k.

RESULTS

The plasmid p4037 was designed to express the fragment of the HLA heavy chain that is present in the HLA/ β_2m complex whose crystal structure was determined by X-ray crystallography (Bjorkman *et al.*, 1987). When cultures of *E. coli* harbouring p4037 are analysed by SDS PAGE, several new proteins can be detected compared to control plasmids. These new bands are specifically recognised by antibodies directed against the HLA-A2 heavy chain, confirming that they are derived from the HLA-A2 coding sequence (Fig. 1). When *E. coli* harbouring plasmid p4037 are disrupted and ultracentrifuged, all of the immunoreactive species of HLA are quantitatively found in the pellet accompanied by several other major proteins derived from *E. coli*. The insoluble material was separated on a sucrose step gradient. The major contaminating proteins floated at the 40% sucrose/53% sucrose interface (Fig. 1A, lanes

Table 1

Abbreviations	Descriptive name	Sequence ^a
NP	Influenza B NP peptide 85-94	KLGEFYNQMM
NP _L	Influenza B NP peptide 82-94	MVVKLGEFYNQMM
NP E → G	Influenza B NP peptide 83-94 E88 → G	VVKLGGFYNQMM
M1	Influenza matrix peptide 58-68	GILGFVFTLV
M1 F → K	Influenza matrix peptide 58-68 F62 → K	GILGKVTLV
NP Y → F	Influenza NP peptide 85-94 Y90 → F	KLGEFFNQNor ^b Nor

^aStandard single letter amino acid code.

^bNor designates norleucine.

3–5) as expected for *E. coli* outer membranes (Osborn *et al.*, 1972). Most of the immunoreactive material was concentrated at the 53% sucrose/67% sucrose interface (Fig. 1A, lanes 6–8). This material is reminiscent of the inclusion bodies that are commonly obtained upon overexpression of many proteins in *E. coli*, and we use the term “inclusion body” to refer to this sucrose gradient fraction. Regardless of the strain tested, the most abundant proteins detectable by protein staining in these inclusion bodies corresponded to HLA immunoreactive material. (Compare lanes 6–8 of Fig. 1A with lanes 6–8 of Fig. 1B). Direct sequence analysis of inclusion bodies from strain JM103 harbouring plasmid 4037 yielded a unique sequence which corresponded exactly to the *ompA* leader peptide. This indicates that in JM103, the leader peptide is not clipped off by *E. coli* leader peptidase. In contrast, when inclusion bodies from strain KS474 harbouring plasmid 4037 was transferred to PVDF membranes, three major immunoreactive proteins were detected, with M_r s of 32k (labelled “H” in Fig. 1B), 30k and 28k (labelled “deg H” in Fig. 1B). Each of these proteins was subjected to Edman degradation. In all three cases, the N-terminal sequence of mature HLA-A2 was obtained, indicating that the leader peptide had been cleaved. The 32k protein presumably represents mature HLA-A2 heavy chain as encoded by p4037, whereas the other major immunoreactive species presumably are shortened from the C-terminus.

HLA reconstitution is an effective means of purifying the intact, mature HLA heavy chain from the other immunoreactive proteins. Moreover, successful reconstitution of HLA-A2 requires the presence of a peptide known to be restricted to HLA-A2. When the inclusion bodies are dissolved in urea and diluted into buffer, with or without β_2m , all species containing HLA are recovered in the pellet. Analysis of the supernatant from such a sample by SDS gel electrophoresis followed by immunoblotting demonstrates that very little immunoreactive material is soluble (Fig. 2A, lane “No Pep”); however, when the inclusion bodies in urea are mixed with the influenza B nucleoprotein (NP) peptide 85-94 known to be recognised by CTLs in the context of HLA-A2 (P. Robbins, personal communication; Robbins *et al.*, 1989) and then diluted into β_2m -containing buffer, some of the intact, mature HLA heavy chain is selectively solubilised (Fig. 2A, lane “NP”). The remainder of the intact HLA-A2 heavy chain and all of the other immunoreactive species precipitate. The NP peptide analogue NP Y \rightarrow F is equally active (lane “NP Y \rightarrow F”), whereas the matrix peptide 58–68 (M1) (Gotch *et al.*, 1987, 1988; McMichael *et al.*, 1988) is not very selective at solubilising only the intact, mature heavy chain. The sequence of the NP peptide and all of the other peptides used in this manuscript are shown in Table 1.

The reconstituted HLA-A2 complex reacts both with the conformationally sensitive MoAB W6/32 (Parham *et al.*, 1979) and the anti- β_2m MoAB BBM.1 (Brodsky *et al.*, 1979) in an enzyme-linked immunoassay. An aliquot (0.6 μ l) of sucrose gradient purified inclusion bodies (2.5 ml total volume) derived from 1 litre of

E. coli gave a signal equivalent to 5 ng of HLA-B7 (data not shown). Therefore, the amount of reconstitution-component HLA in the preparation is about 20 μ g per litre, or about 2% of the starting intact mature HLA-A2 heavy chain initially made. This estimate is consistent with the silver staining data in Figs 2 and 3A. The absolute amount of reconstitution was found to vary between different experiments. We are currently investigating the reasons for this variation. When the M1 peptide is used during the reconstitution procedure, the yield is indistinguishable from background by this ELISA assay.

If this reconstitution methodology does generate an HLA-A2 preparation that is similar to the native HLA-A2 as isolated from lymphoblastoid cell lines, then the preparation should run as a single band in nondenaturing polyacrylamide gels (NAGE). When no peptides were present upon reconstitution (Fig. 2B, lane “No Pep”), or when the M1 peptide was used (Fig. 2B, lane “M1”), a smear extending from the sample well throughout the top half of the gel was detected following immunoblotting. Just as with the SDS gel experiment described above, the intensity of this smear could be lessened by prolonged incubation at 4°C followed by centrifugation of the sample prior to electrophoresis. In contrast, when the reconstitution assay was carried out in the presence of the NP peptide (Fig. 2B, lane “NP”) or a NP analog peptide (Fig. 2B, lane “NP Y \rightarrow F”), a distinct band was detected. Thus, the HLA heavy chain preparation appears to be relatively homogeneous following reconstitution with β_2m and the NP peptide. In contrast, preparations of HLA-A2 or HLA-B7 derived from the lymphoblastoid cell line JY appear much more heterogeneous when analysed by NAGE, probably due in part of sialic acid heterogeneity (data not shown).

As a further test of the chemical homogeneity of the reconstituted material, the samples were analysed on native isoelectric focusing gels (NIEF). Significantly greater sensitivity is achieved upon silver staining of these gels, presumably because NIEF concentrates the protein into a smaller area than NAGE. Once again, when the NP peptide is used, a sharp band is obtained in addition to the β_2m band (Fig. 3A, lane “NP”). No such band appears when peptide is not included in the reconstitution mixture. A much less intense band is also obtained when the matrix peptide analog M1 F \rightarrow K is used (Fig. 3A). To confirm that these bands correspond to a complex that contains the HLA heavy chain, the NIEF gel was immunoblotted onto PVDF membranes and developed with anti-HLA antisera. A large number of bands appear which were invisible or barely detectable by silver staining, suggesting that immunoblotting with the anti-HLA antibody is significantly more sensitive than silver staining. Immunoblotting of a parallel NIEF gel with an anti- β_2m MoAB confirms that the NP/HLA-A2 complex also contains β_2m (data not shown). All of the bands in Fig. 3A that are specific to each peptide are detected by immunoblotting in Fig. 3B. In contrast, monomeric β_2m , which is the major protein detected by silver staining in Fig. 3A, is not reactive with

the anti-HLA antibody used for immunoblotting. [There are also minor bands in every lane of Fig. 3A (except the Std lane) that derive from contaminants in the β_2m preparation, since the relative amount of these species is variable between different-preparations of β_2m , data not shown.] There is no evidence that any HLA heavy chain is solubilised when reconstitution is carried out in the absence of peptides (Fig. 3, lane "No Pep") or in the presence of a NP peptide variant that contains a substitution of glycine for glutamic acid (lane "NP E \rightarrow G"). Two bands are visible in Fig. 3B, lane "M1", indicating that the M1 peptide does reconstitute to some degree. The darker of these two bands comigrates almost exactly with β_2m ; therefore, the large amount of free β_2m that is present in the reconstitution solution probably masks this band in Fig. 3A.

Both the pI and the number of different HLA-containing species depend upon the peptide used during the reconstitution. In particular, the pI of the HLA complex shifts significantly when a peptide with a different net charge is used (Fig. 3B, compare "M1" to "M1 F \rightarrow K"). The shift is in the expected direction; the substitution of lysine for phenylalanine causes both bands present in lane "M1" to shift toward the cathode, and by the same amount. Since the isoelectric point of the reconstituted complex is dependent on the peptide, the peptide must actually be present in the reconstituted complex, and cannot merely be playing an accessory role in HLA-A2 solubilisation. The multiple bands that become visible upon immunoblotting (Fig. 3B) presumably arise from heterogeneity in either the peptide or the HLA heavy chain preparation. The more efficiently the peptide complexes to the HLA heavy chain and β_2m , the larger the number of bands that can be detected. In Fig. 3B, lane "NP", at least seven bands are visible in the original figure. The three darkest bands are almost evenly spaced, and probably differ from one another by a single charge, and may originate from deamidation of glutamine or asparagine residues in the HLA preparation or from carbamylation of amino groups by isocyanate ions originally derived from urea. The minor bands in Fig. 3B, lane "NP" are located between the major bands and probably are due to more subtle side-chain modifications. Four much less prominent bands are visible in Fig. 3B, lane "NP_L", and three of these match the darkest three bands in Fig. 3B, lane "NP" both in pI and relative amount. Thus, the addition of three hydrophobic amino acids to the N-terminus of the NP peptide has no effect on the pI of the reconstituted complex, but does cause the peptide to reconstitute less efficiently.

DISCUSSION

Both subunits of HLA-A2 have been successfully synthesised in *E. coli*. β_2m can be purified in milligram amounts in native form from the periplasmic space (Parker and Wiley, 1989). In contrast, HLA heavy chain produced by the same expression vector accumulates as

an insoluble aggregate, as expected since it is not known to be soluble in the absence of β_2m . Under appropriate culture conditions, the signal peptide of HLA heavy chain is cleaved, indicating successful export to the periplasmic space prior to aggregation; however, under these conditions, shorter molecules with the mature HLA-A2 N-terminus are also made in molar excess over intact heavy chain. In spite of the presence of these other species, all of which according to their M_r contain at least the peptide-binding N-terminal 180 amino acids, in the presence of β_2m and an appropriate peptide, the reconstitution procedure results in the specific solubilization of intact HLA-A2 heavy chain (Fig. 2A). Since the reconstituted material is recognised by an antibody that is specific for native HLA-A2, it appears that an immunologically relevant peptide, β_2m , and the class I heavy chain itself are all that is necessary for the class I complex to assemble *in vitro*.

When HLA-A2 is isolated from human lymphoblastoid cell lines, and β_2m is separated from the HLA heavy chain in denaturant, a peptide must be added prior to removal of the denaturant for efficient reconstitution to occur (Silver *et al.*, 1991). According to another recent report, about 10% yield is obtained in the absence of a specifically added peptide (Elliott and Eisen, 1990). The same NIEF assay that we have used in Fig. 3 shows that with HLA-A2 isolated from human cells, different peptides cause the reconstituted complex to focus in a different position (Silver *et al.*, 1991). Moreover, both gel filtration and autoradiography are used to demonstrate that [¹⁴C]-internal labelled NP peptide is a component of the reconstituted HLA complex (Silver *et al.*, 1991). In the *E. coli* system, it has not yet been possible to demonstrate reconstitution with the same labelled peptide preparation, because we lack enough labelled peptide to achieve the high concentrations of peptide that are needed to drive reconstitution in the presence of reconstitution-incompetent HLA heavy chain species.

We find that the use of the NP peptide 85-96 results in a higher level of reconstitution than the influenza matrix peptide 58-68. (Compare lane "M1" to lane "NP" in Figs 2A, 2B, 3A and 3B). This result is unexpected, because it has been reported that the matrix peptide 56-68 can inhibit lysis by NP-specific CTLs at lower concentrations than the NP peptide 82-94 can inhibit lysis by matrix-specific CTLs (Robbins *et al.*, 1989). There are several possible explanations that could account for these results. The NP peptide we have chosen may bind more tightly to the HLA-A/ β_2m complex than the matrix peptides we have studied. Alternatively, the NP peptide may be more effective in the reconstitution assay than the matrix peptide because (for example) it can bind better to an intermediate in protein folding. A possible, trivial explanation is that the NP peptide is present in a higher active concentration than the matrix peptide because it is more soluble in the reconstitution solution. Several papers have recently reported the isolation of peptides derived from isolated class I molecules (Van Bleek and Nathenson, 1990) or

from whole cell extracts (Falk *et al.*, 1990; Rotzschke *et al.*, 1990) that correspond to CTL determinants. Both groups find that the isolated peptides are remarkably homogeneous, indicating that *in vivo* each CTL clone recognises a unique peptide, rather than a series of overlapping peptides with different lengths. Hence, it is possible that for each CTL determinant, a limited set of peptides are able to bind directly to the class I molecule. Experiments are now in progress to discriminate between these possibilities.

Bacterial expression and reconstitution of HLA should be useful for distinguishing between class I mutants that affect the stability of the final HLA-peptide complex and those that exert their effects in other ways on the path to cell-surface expression. The apparent requirement for peptide in the folding and assembly of HLA-A2 from *E. coli* supports recent experiments which conclude that the interaction of ligands and class I histocompatibility antigens is intimately associated with the stability of the complex (Townsend *et al.*, 1990; Ljunggren *et al.*, 1990; Lie *et al.*, 1990). The linkage between subunit assembly and peptide binding may help account for the difficulty of replacing bound peptide with free peptide once the complex is formed, and for the broad binding specificity of histocompatibility antigens for peptides that bear little primary sequence homology.

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