Translocation of Peptides through Microsomal Membranes Is a Rapid Process and Promotes Assembly of HLA-B27 Heavy Chain and β2-Microglobulin Translated In Vitro

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Abstract. We have translated major histocompatibility complex (MHC) class I heavy chains and human β2-microglobulin in vitro in the presence of microsomal membranes and a peptide from the nucleoprotein of influenza A. This peptide stimulates assembly of HLA-B27 heavy chain and β2-microglobulin about fivefold. By modifying this peptide to contain biotin at its amino terminus, we could precipitate HLA-B27 heavy chains with immobilized streptavidin, thereby directly demonstrating class I heavy chain–peptide association under close to physiological conditions. The biotin-modified peptide stimulates assembly to the same extent as the unmodified peptide. Both peptides bind to the same site on the HLA-B27 molecule. Immediately after synthesis of the HLA-B27 heavy chain has been completed, it assembles with β2-microglobulin and

peptide. These interactions occur in the lumen of the microsomes (endoplasmic reticulum), demonstrating that the peptide must cross the microsomal membrane in order to promote assembly. The transfer of peptide across the microsomal membrane is a rapid process, as peptide binding to heavy chain–β2-microglobulin complexes is observed in <1 min after addition of peptide. By using microsomes deficient of β2-microglobulin (from Daudi cells), we find a strict requirement of β2-microglobulin for detection of peptide interaction with the MHC class I heavy chain.

Furthermore, we show that heavy chain interaction with β2-microglobulin is likely to precede peptide binding. Biotin-modified peptides are likely to become a valuable tool in studying MHC antigen interaction and assembly.

NEWLY synthesized class I molecules of the major histocompatibility complex (MHC)[1] present fragments of proteins (peptides) at the cell surface to cytolytic T lymphocytes (CTLs) (Townsend et al., 1985; Townsend et al., 1986; Maryanski et al., 1986; Braciale et al., 1987; Clayberger et al., 1987; Gotch et al., 1987; Taylor et al., 1987; McMichael et al., 1988; Moore et al., 1988; Nixon et al., 1988; Oldstone et al., 1988; Rothbard and Taylor, 1988; Bodmer et al., 1989; Lurquin et al., 1989; Reddehase et al., 1989; Wallny and Rammensee, 1990). During a virus infection the presented peptides are of viral origin, but it is likely that peptides of endogenous origin also can bind to MHC class I antigens under normal physiological conditions (Kourilsky and Claverie, 1989a,b; Margulies, 1989; Falk et al., 1990; Rötzschke et al., 1990). Determination of the three-dimensional structure of the human class I antigen HLA-A2, has revealed a peptide-binding groove, where the walls are composed of two alpha-helices from the alpha-one and -two domains, respectively. The bottom of the groove consists of eight beta-pleated sheets, four each contributed by the $\alpha 1$ and $\alpha 2$ domains (Bjorkman et al., 1987). The

alpha-three domain, located below the alpha one/alpha two domains, and closest to the membrane, interacts noncovalently with the small subunit β2-microglobulin. The HLA heavy chain must assemble with β2-microglobulin in order to be transported from the ER to the cell surface (Arce-Gomez et al., 1978; Ploegh et al., 1979). Several recent reports suggest that the compartment where the assembly of class I molecules with peptides and β2-microglobulin occurs, corresponds to the ER (Nuchtern et al., 1989; Yewdell and Bennink, 1989; Kvist and Hamann, 1990).

It has been demonstrated that presentation of viral peptides is controlled by an additional gene(s) located within the MHC complex (Cerundolo et al., 1990). The existence of such an additional gene(s) involved in MHC class I assembly, was shown by using the human cell lines LBL 721.174 (DeMars et al., 1984, 1985), and two fusion derivatives (T1 and T2) of this line (Salter et al., 1985). The 721.174 and T2 cells are defective in assembly and expression of MHC class I antigens (DeMars et al., 1985; Shimizu et al., 1986; Salter and Cresswell, 1986), but this defect can be overcome, or compensated for, by exposure of the cells to proper peptides (Cerundolo et al., 1990). Further evidence that the MHC class I assembly is not a spontaneous process is supported by the finding that assembly and cell surface expres-

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1. *Abbreviations used in this paper:* CTL, cytolytic T lymphocytes; MHC, major histocompatibility complex.

sion of mouse H-2 antigens can be induced by gamma-interferon treatment in certain murine cell lines (Klar and Hämmerling, 1989). It is not known whether the defect in these cells is due to an inability to generate the correct peptide epitopes, to translocate the peptides across the ER membrane, to interfere directly with the assembly process, or a combination of these events.

There is an increasing body of evidence that suggests that peptides play an essential role in the assembly process. It has been shown that peptide can stimulate assembly and cell surface expression of murine H-2 class I antigens in RMA-S cells (Townsend et al., 1989). This cell line is defective in class I antigen assembly and is likely to be the murine counterpart of the human 721.174 cell line (Ljunggren and Kärre, 1985; Kärre et al., 1986). Recently, it has been shown that assembly of MHC class I antigens can occur in the lysate of detergent-solubilized cells, and that this process is dependent of both peptide and β2-microglobulin concentrations (Townsend et al., 1990). However, it has also been shown that MHC class I heavy chains associated to β2-microglobulin can be transported and expressed at the cell surface in RMA-S cells in the absence of peptide, provided that the culturing temperature of the cells is lowered to ∼26°C (Ljunggren et al., 1990; Schumacher et al., 1990). These results demonstrate that "empty" class I antigens can assemble and be expressed at the cell surface, although they seem to be more sensitive to degradation and it has not been shown that they occur under normal physiological conditions.

Although peptide association to MHC class I antigens must be considered as an established fact, direct binding has not been shown until recently. Chen and Parham (1989) could show by using a gel filtration assay and purified HLA complexes, that two peptides derived from influenza A proteins, directly bind two different HLA specificities. By using a solid phase support to immobilize peptides, Bouillot et al. (1989) could demonstrate peptide binding to class I antigens. Both methods proved useful to show direct peptide-MHC class I antigen interaction. However, neither assay can be used to study the interaction under physiological conditions.

We have recently described a cell free translation system in lysate from rabbit reticulocytes, supplemented with human microsomal membranes to mimic the ER compartment (Kvist and Hamann, 1990). It was shown that mRNA encoding the HLA-B27 heavy chain could be efficiently translated, and that the nascent chain was translocated through and inserted into the microsomal membrane where it interacted with endogenous microsomal β2-microglobulin. Furthermore, the assembly process was stimulated by the presence of a peptide from the nucleoprotein of influenza A (NP384-394), known to induce CTL activity in an HLA-B27 restricted fashion (Huet et al., 1990).

In this paper we describe a novel assay to directly show peptide binding to MHC class I antigens translated in a cell free system. By using peptides modified with a biotin group, we can directly visualize the HLA-B27 heavy chain bound to peptide, by reaction with agarose-immobilized streptavidin, a protein with high affinity for biotin. A biotin assay for detection of MHC class II antigens on whole cells has previously been described (Busch and Rothbard, 1990). We present here a system that can be used to study the early events in the MHC assembly at the molecular level under close to physiological conditions. We believe that this system will be of great importance for dissection of the assembly process and possibly also other ligand–receptor interactions.

Materials and Methods

Construction of Plasmids Encoding HLA-B27, -A2, -B13, and H-2 Kᵏ Heavy Chains, and Human β2-Microglobulin

The complete cDNAs for HLA-B27, -A2, -B13 heavy chain and human β2-microglobulin, generously provided by Drs. E. Weiss and J. Burckardt, were separately inserted into the EcoRI site of pGem-3Zf(-) (Promega Biotec, Madison, WI), so that transcription would be promoted by the SP6 RNA polymerase promoter. The cDNA encoding the H-2 Kᵏ heavy chain was a generous gift from Dr. B. Arnold, and was inserted as a BamHI fragment into the same transcription vector. Upstream of the cDNAs encoding the MHC heavy chains, a translating enhancing fragment from the E3/19K gene of adenovirus type 2 was inserted.

In Vitro Transcription and Translation

Before transcription the cDNAs were linearized and the DNA was purified by phenol extraction and ethanol precipitation. Transcription was carried out according to the protocol by Promega Biotec (Kvist and Hamann, 1990), by using SP6 RNA polymerase. After completion of transcription the DNA was digested with DNase I (Boehringer Mannheim Diagnostics, Inc., Houston, TX) and the mRNA was further purified by ammonium acetate/ethanol precipitation. The remaining mRNA was quantified spectrophotometrically. During transcription 10 units of RNase inhibitor (RNasin; Promega Biotec) was present. The mRNA was dissolved in autoclaved water containing 0.1% of diethylpolycarbonate, and was stored as aliquots at −85°C. Rabbit reticulocyte lysate was prepared essentially as described (Pelham and Jackson, 1976; Jackson and Hunt, 1983). Translation mixture contained: 50–100 ng of mRNA for the HLA-B27 or H-2 Kᵏ heavy chains and (where indicated in Figs. 5 and 6) 200 ng of mRNA for β2-microglobulin; 3 μl of microsomal membranes (60 A_{280} U/ml); 90 μCi of [^{35}S]methionine (>1,000 Ci/mmol, Amersham International, Amersham, UK); RNasin 10 U; 2 μg of PMSF, and 10 U of aprotinin. The peptide was added to a final concentration of 8–32 μM. Total incubation mixture was 70 μl. Translation was started by adding the mRNA and incubation was at 37°C for 60–90 min (or as indicated). Where indicated, cycloheximide was added to a final concentration of 100 μg/ml to block further translation. The reaction was terminated by shifting the temperature to 0°C and by adding 135 μl of TNE buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM EDTA). The membranes were pelleted in an Eppendorf centrifuge (Brinkman Instruments, Inc., Westbury, NY) for 5 min, the pellet was washed once in TNE buffer, and solubilized in a physiological buffer containing 5 mM EDTA and 1% Triton X-100. Nonsolubilized material was pelleted by centrifugation as above and the supernatant was used for either immunoprecipitation or precipitation by streptavidin-agarose.

Preparation of Microsomal Membranes from Tissue Culture Cells

Normally 1–5 × 10⁹ cells were used in each preparation. Cells were washed in STKMM (250 mM sucrose, 50 mM triethanolamine-HCl pH 7.5, 50 mM potassium acetate, 5 mM magnesium acetate, and 0.1% 2-mercaptoethanol) and resuspended in water before homogenization by a Dounce homogenator. Immediately after this treatment the disrupted cells were diluted in STKMM, and intact cells and debris were pelleted by centrifugation at 7,000 g. The supernatant was further centrifuged at 47,000 g for 40 min, and the pellet containing the microsomal membranes was resuspended in RM buffer (250 mM sucrose, 50 mM triethanolamine-HCl pH 7.5, 50 mM potassium acetate, 2 mM magnesium acetate, and 1 mM DTT) at a concentration of 20 A_{280} U/ml and was treated with 150 U/ml of micrococcal nuclease (Boehringer Mannheim Diagnostics, Inc.) for 10 min at 25°C in the presence of 1 mM calcium chloride. The reaction was terminated by addition of 2 mM EGTA-KOH, pH 7.0. The microsomes were further purified by ultracentrifugation through a 0.5 M sucrose cushion in RM buffer at 90,000 g for 30 min. The pellet was resuspended in RM buffer to a final concentration of 60 A_{280} per milliliter, and the membranes were frozen as aliquots in liquid nitrogen and stored at −135°C. All the buffers contained 10 U/ml of aprotinin and 10 μg/ml of PMSF. The recovery from 5 × 10⁹ cells was ∼1 ml of membranes.

Antisera, Monoclonal Antibodies, Immunoprecipitation, and SDS-PAGE

Two different antisera were used in this study. The rabbit anti–H-2 serum has been described previously (Kvist et al., 1978). The rabbit anti–HLA heavy chain serum was prepared in our own laboratory. The mAb W6/32 (Barnstable et al., 1978) reacts with a framework determinant of assembled HLA antigens and the mAb BBM.1 is directed against β2-microglobulin (Brodsky et al., 1979).

Immunoprecipitation and SDS-PAGE have been described in detail previously (Kvist et al., 1982; Burgert and Kvist, 1985). Biotinylated peptides were precipitated by streptavidin immobilized to agarose (Sigma Chemical Co., St. Louis, MO). We used 30 μl per precipitation of a 50% slurry agarose/buffer. Incubation and washing was as for immunoprecipitation.

Proteinase K Treatment of Microsomes

For the experiment shown in Fig. 4 A the translation was terminated after 45 min by adding cycloheximide to a final concentration of 100 μg/ml, followed by addition of proteinase K (Boehringer Mannheim Diagnostics, Inc.) to a final concentration of 200 μg/ml. The samples were incubated for 30 min on ice, followed by addition of PMSF to a final concentration of 40 μg/ml. The membranes were washed in TNE as described above, and solubilized in the presence of PMSF.

Peptides and Biotinylation

Two different peptides were used in this study. The NP384-394 (Huet et al., 1990) has the sequence RYWAIRTRSGG and the NP147-158 (Taylor et al., 1987) has the sequence TYQRTRALVRTG. Peptides were dissolved and kept as stocks of 1 mM at −85°C.

In the biotinylation reaction, the NHS-LC-Biotin reagent (Pierce Chemical Co., Rockford, IL) was dissolved at a concentration of 10 mM in 100 mM sodiumphosphate buffer, pH 7.5, just before use. This reagent contains a 22 Å long spacer arm between the biotin group and the amino acid which it is linked to. The reaction was initiated by adding the reagent in 5 M excess to the peptide solution. The reaction was allowed to continue for 2 h at room temperature under continuous agitation. At the end of the reaction, Tris-HCl, pH 7.4, was added to a final concentration of 10 mM. This treatment consumes completely the excess of NHS-LC-biotin and prevents the reagent to react with other proteins in the translation mixture. The efficacy in the biotinylation reaction as described here ranged between 70 and 90% as measured by iodination and precipitation with streptavidin-agarose. Biotinylated peptides were stored at −85°C.

Other Reagents

Protein A–Sepharose was from Pharmacia (Uppsala, Sweden).

Results

Stimulation of Assembly of HLA-B27 Heavy Chain and β2-Microglobulin by a Biotinylated Peptide

We have previously shown that the NP384-394 peptide (Huet et al., 1990) can stimulate assembly of HLA-B27 heavy chain and β2-microglobulin in a reticulocyte lysate supplemented with microsomal membranes prepared from the lymphoid cell line Raji (Kvist and Hamann, 1990). These microsomes contain large amounts of endogenous β2-microglobulin that form complexes with radiolabeled in vitro translated HLA-B27 heavy chains, making it unnecessary to translate additional β2-microglobulin (Kvist and Hamann, 1990). To examine whether the same peptide would stimulate assembly once it has been modified by a biotin group at its amino terminus, we performed in vitro translations of mRNA for the HLA-B27 heavy chain in the presence of both unmodified and modified peptide.

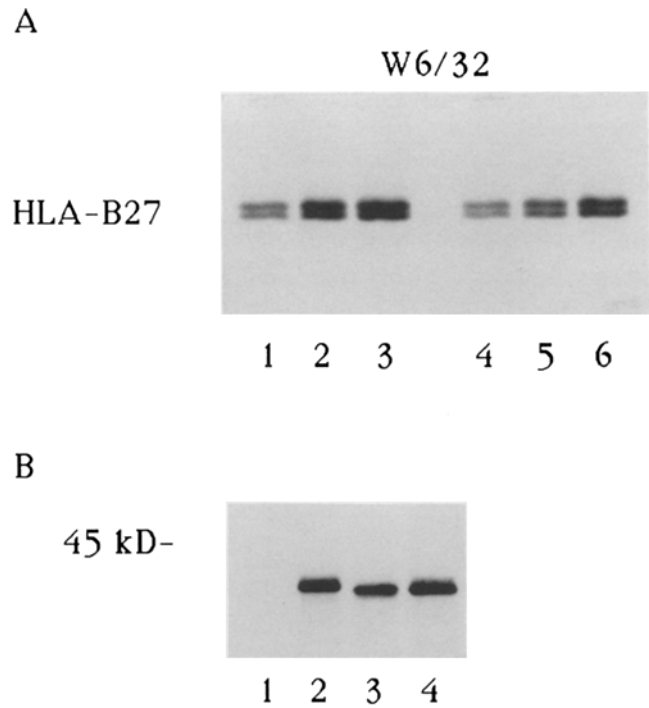Translations were carried out as described in Materials and Methods, the microsomal membranes were detergent

Figure 1. Stimulation of assembly of HLA-B27 antigens by the NP384-394-biotin peptide. (*A*) Messenger RNA for HLA-B27 heavy chain (50 ng) was translated in a reticulocyte lysate for 90 min at 37°C, in the presence of microsomal membranes prepared from Raji cells. The nucleoprotein peptide NP384-394 (lanes *2* and *3*) and its biotin modified derivative NP384-394-biotin (lanes *5* and *6*) were added at the start of translation and their concentrations were: no peptide present (lanes *1* and *4*); 8 μM (lanes *2* and *5*); 32 μM (lanes *3* and *6*). After completion of translation, the microsomal membranes were pelleted, washed once and solubilized. Immunoprecipitation was carried out with mAb W6/32 and the precipitates were analyzed by SDS-PAGE. (*B*) Messenger RNA for HLA-B27 (lane *2*), HLA-A2 (lane *3*), and HLA-B13 (lane *4*) were translated as in *A*, and an aliquot of the total membrane content was analyzed. No mRNA was added to the translation shown in lane *1*. Analysis was as in *A*.

solubilized and the material was immunoprecipitated with the mAb W6/32. This antibody reacts with a framework determinant specific for assembled heavy chain–β2-microglobulin HLA antigens (Barnstable et al., 1978). Finally, the immunoprecipitated material was analyzed by SDS-PAGE. In the absence of exogenous peptides, a doublet of HLA-B27 heavy chains can be seen (Fig. 1 *A*, lanes *1* and *4*). The unmodified NP384-394 peptide stimulates HLA-B27 heavy chain assembly to β2-microglobulin already at the concentration of 8 micromolar (Fig. 1 *A*, lane *2*), and the intensity of the bands increases further at 32 micromolar (lane *3*). The NP peptide modified with biotin (NP384-394-biotin) stimulates assembly in a fashion indistinguishable from the unmodified peptide (Fig. 1 *A*, lanes *4–6*). By densitometric scanning of the gel we found an approximate fivefold stimulation for both peptides at the concentration of 32 μM. Thus, we conclude that the NP384-394-biotin peptide possesses the same ability to stimulate HLA-B27 heavy chain assembly to β2-microglobulin as does the unmodified peptide under the conditions used here.

To rule out the possibility that Raji mRNA would copurify with the microsomes, and subsequently be translated together with the HLA-B27 mRNA, we performed translations both without exogenous mRNA added, and with mRNA of two other HLA class I types, HLA-A2 and HLA-B13. Analysis was done on the total translation products recovered from the microsomal membranes. In the absence of exogenous mRNA no HLA heavy chain band could be detected (Fig. 1 B, lane 1). The presence of mRNA for the HLA-B27 heavy chain gives rise to a band with identical molecular weight as in Fig. 1 A (Fig. 1 B, lane 2), whereas translation of the mRNAs for HLA-A2 and HLA-B13 results in protein bands of similar molecular weight with a slightly faster migration (Fig. 1 B, lanes 3 and 4, respectively). In most experiments the HLA-B27 heavy chain appears as a doublet, but the relative intensity of the two bands varies (cf. Fig. 1, A and B, lane 2). Thus, no mRNA copurifying with the microsomes can be translated under the conditions described here.

The NP384-394-biotin Peptide Binds Directly and Specifically to HLA-B27 Molecules

The highly specific interaction between biotin and avidin or streptavidin ($K_d = 10^{-15}$ M) is the major reason for its usefulness in different biological applications (Bayer and Wilchek, 1980). The fact that the NP384-394-biotin peptide can stimulate assembly of HLA-B27 heavy chain with $\beta 2$-microglobulin suggests that it might be possible to precipitate the heavy chain of HLA with streptavidin immobilized to agarose (see Materials and Methods). We tested this by performing the same type of experiment as described above, but instead of using the mAb W6/32 we carried out the precipitation reaction with streptavidin-agarose.

At concentrations of 8 and 32 μM of NP384-394-biotin peptide, increasing amounts of the two bands representing the HLA-B27 heavy chain were observed (Fig. 2 A, lanes 2 and 3, respectively). No radioactive bands were observed in the absence of peptide (Fig. 2 A, lane 1). Thus, the newly synthesized HLA-B27 heavy chains can be visualized directly via their interaction with the biotinylated peptide. That the interaction actually occurs via the biotin was shown by using the unmodified peptide at 32 μM. No radioactive HLA-B27 bands were seen (Fig. 2 A, lane 4). To exclude that the biotinylation reagent reacted directly with the HLA-B27 heavy chains, and formed heavy chain–biotin complexes without peptide involved, we incubated the translation mixture with the solution used for biotinylating the peptide. No heavy chains could be detected (Fig. 2 A, lane 5). Finally, to prove the specificity of the peptide for HLA-B27 we used the NP384-394-biotin peptide in a reaction where the mRNA for the mouse MHC class I K^k heavy chain was translated under identical conditions. No interaction was observed between the NP384-394-biotin peptide (Fig. 2 A, lane 6), although the K^k heavy chain was efficiently translated and could be immunoprecipitated with a rabbit antiserum against mouse MHC class I molecules (lane 7). We conclude that immobilized streptavidin with affinity for biotin linked to the NP384-394 peptide can precipitate the HLA-B27 heavy chains synthesized in the reticulocyte lysate.

To examine whether biotinylated peptides can be used in general to study class I–peptide interactions in vitro, we analyzed two other class I molecules, HLA-A2 and HLA-B13,

in a similar fashion with specific peptides. Both antigens were specifically precipitated by immobilized streptavidin (data not shown). Thus, this method is not limited to HLA-B27 antigens.

The biotinylation efficiency of the different NP384-394-biotin peptide preparations used in this study varied between 70 and 90%. Thus, 10–30% in every preparation was unbiotinylated peptide. If the unbiotinylated peptide would bind to HLA-B27 much better than the biotinylated peptide, this could account for the majority of stimulation observed. To test this we preabsorbed the biotinylated peptide with streptavidin, and analyzed the supernatant (containing the 10–30% unbiotinylated peptide) for stimulation of HLA-B27 assembly with the mAb W6/32. A clear stimulation of assembly was observed before absorption with streptavidin at peptide concentrations of 0, 8, and 32 μM (Fig. 2 B, lanes 1, 2, 3, respectively). In contrast, no stimulation of assembly was seen for the absorbed peptide when the concentration increased from 0 to 8 μM (Fig. 2 B, lanes 4 and 5, respectively), and from 8 to 32 μM (lanes 5 and 6, respectively). Thus, the stimulation of assembly of HLA-B27 antigen observed for the biotinylated peptide in Fig. 1, cannot be explained by the remaining fraction of unbiotinylated peptide present within the preparation, but must be due to direct binding of NP384-394-biotin peptide to HLA-B27 as shown in Fig. 2 A. The efficiency of absorbtion was demonstrated by examining the binding of peptide to HLA-B27 before and after absorbtion (Fig. 2 B, lanes 7 and 8, respectively).

Also, this experiment made it possible to directly compare stimulation with peptide binding. The intensity in lane 7 of Fig. 2 B should then equal the intensity obtained by subtracting lane 3 with lane 1 (the background assembly). Densitometric analysis revealed that this is actually the case, indicating that almost all stimulation of assembly is caused by biotinylated peptide binding to HLA-B27. However, this does not mean that all assembly observed is due to the exogenously added peptide, as a considerable background is always present. This is presumably caused by endogenous peptides present within the microsomes.

To prove that the NP384-394 peptide and its biotinylated derivative actually bind to the same site of the HLA-B27 molecule we performed a competition experiment. Increasing amounts of unmodified NP384-394 peptide were used to compete a constant concentration of NP384-394-biotin peptide. Analysis was made by streptavidin precipitation and SDS-PAGE analysis. The NP384-394 peptide efficiently competed the biotin-modified peptide, whereas a control peptide (NP147-158) from the nucleoprotein of influenza A, and specific for the mouse MHC class I antigen K^d, could not compete the NP384-394-biotin peptide (data not shown). Thus, the NP384-394 peptide and its biotinylated derivative share the same binding site on the HLA-B27 heavy chain, and their binding cannot be competed by a nonrelated peptide.

HLA-B27 Heavy Chain Forms Complex with $\beta 2$-Microglobulin and Peptide Immediately after Its Synthesis

We next studied the rate of synthesis of HLA-B27 heavy chain and $\beta 2$-microglobulin in the in vitro translation system, and how soon after synthesis complex forms could be

A

HLA-B27

Sa

R218

H-2 K^k

1 2 3 4 5 6 7

B

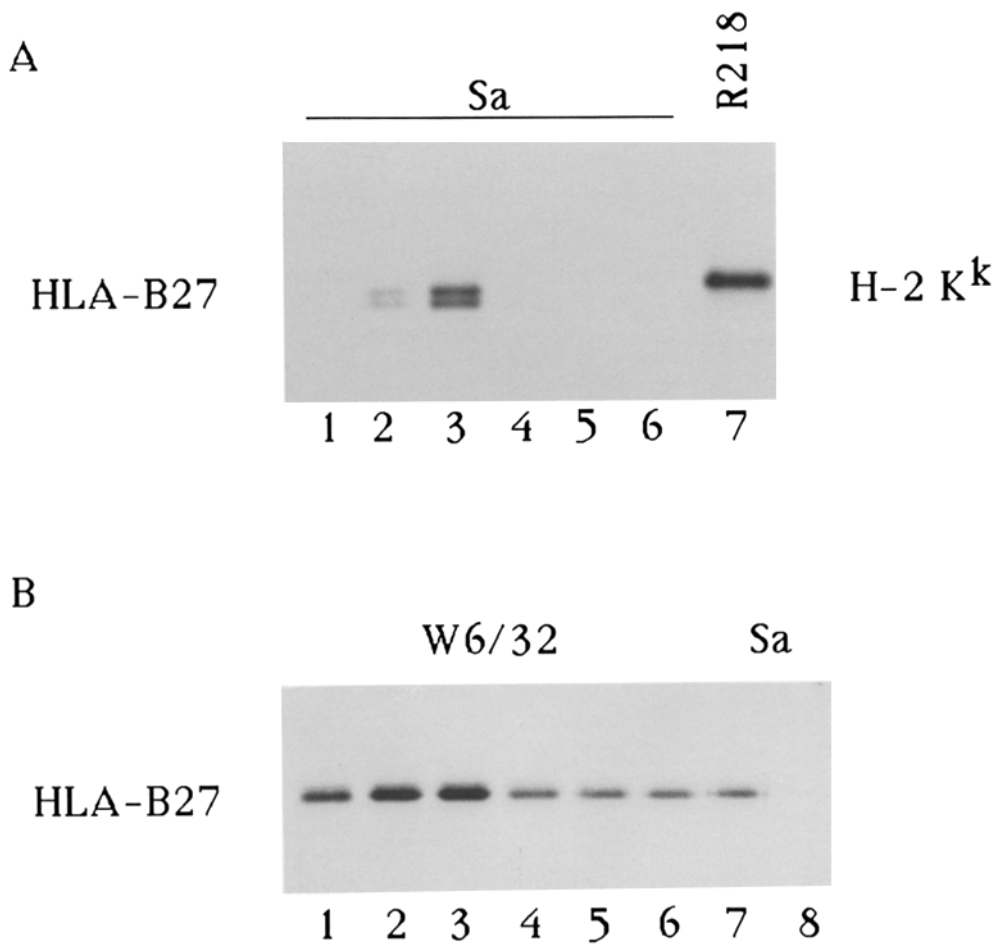W6/32   Sa

HLA-B27

1 2 3 4 5 6 7 8

Figure 2. The NP384-394-biotin peptide interacts directly and specifically with the HLA-B27 heavy chain. (*A*) Messenger RNA for HLA-B27 heavy chain (lanes *1–5*) and for H-2 K^k (lanes *6* and *7*) were translated as described in Fig. 1. Precipitations were performed by streptavidin-agarose (lanes *1–6*) and a rabbit anti-H-2 serum (R218) (lane *7*). The different lanes show: lane *1*, absence of peptide; lanes *2* and *3*, 8 and 32 μM NP384-394-biotin, respectively; lane *4*, 32 μM NP384-394; lane *5*, biotinylation solution without peptide; lanes *6* and *7*, 32 μM NP384-394-biotin. (*B*) Messenger RNA for HLA-B27 heavy chain was translated and incubated with different concentrations of NP384-394-biotin peptide that was either untreated (lanes *1–3*, and *7*) or preabsorbed with streptavidin (lanes *4–6*, and *8*). Concentrations of peptides were 0 (lanes *1* and *4*), 8 (lanes *2* and *5*), and 32 μM (lanes *3* and *6–8*). Precipitations were done by mAb W6/32 (lanes *1–6*), or streptavidin-agarose (lanes *7* and *8*). Analysis was as in Fig. 1. See Materials and Methods for further details.

detected. Messenger RNA for HLA-B27 heavy chain and β2-microglobulin were cotranslated in a reticulocyte lysate supplemented with microsomal membranes prepared from Daudi cells. These cells are unable to synthesize β2-microglobulin due to mutation in its gene, but express HLA heavy chains (Rosa and Fellous, 1982). The microsomal membranes, therefore, contain class I heavy chains but no β2-microglobulin. Translation was carried out in the presence of 32 μM of biotin-labeled NP384-394 peptide. Translation was allowed to proceed for the time indicated in Fig. 3, and was terminated by adding ice-cold termination buffer (see Materials and Methods for details). Each translation was divided into three equal samples and subjected to precipitation with a rabbit antiserum against HLA antigens (HLA heavy chain and β2-microglobulin), the mAb W6/32 (recognizing only heavy chain-β2-microglobulin complexes) and streptavidin (recognizing biotin), respectively. The precipitates were analyzed by SDS-PAGE. To quantitate the amount of HLA-B27 heavy chain and β2-microglobulin we performed a densitometric analysis of the x-ray autoradiogram.

After 6 min of translation a protein band for β2-microglobulin was seen (Fig. 3, lane *2*). By repeating the experiment with shorter time intervals we found that the synthesis of the β2-microglobulin polypeptide is completed after 4 min (data

not shown). As this protein contains $\sim$120 amino acids (including the signal sequence), we calculated a translation rate of $\sim$30 residues per minute. The HLA-B27 heavy chain was observed first after 12 min of translation (Fig. 3, lane *4*). This protein has the length of $\sim$360 amino acids (including the signal sequence), thus confirming a translation rate of 30 residues per minute. The mAb W6/32 detected β2-microglobulin already at 6 min of translation (Fig. 3, lane *9*). As this antibody only recognize HLA complexes, this means that newly synthesized β2-microglobulin forms complex with endogenous microsomal HLA heavy chains immediately after synthesis. The presence of such chains in Daudi microsomes was demonstrated by Western blotting (data not shown). After 12 min of translation the band for HLA-B27 heavy chain was detected with the mAb W6/32, showing immediate complex formation between the two newly synthesized polypeptides (Fig. 3, lane *11*). The intensity of the HLA-B27 heavy chain band increased until it reached a plateau after about 30 min of translation (Fig. 3, lane *13*). This time-point coincided with maximal level of translation (Fig. 3, lane *6*).

Streptavidin was used to examine how soon after synthesis of the HLA-B27 heavy chain and β2-microglobulin, the NP384-394-biotin peptide could be found bound to the complex. After 12 min of synthesis, the same time it took to com-
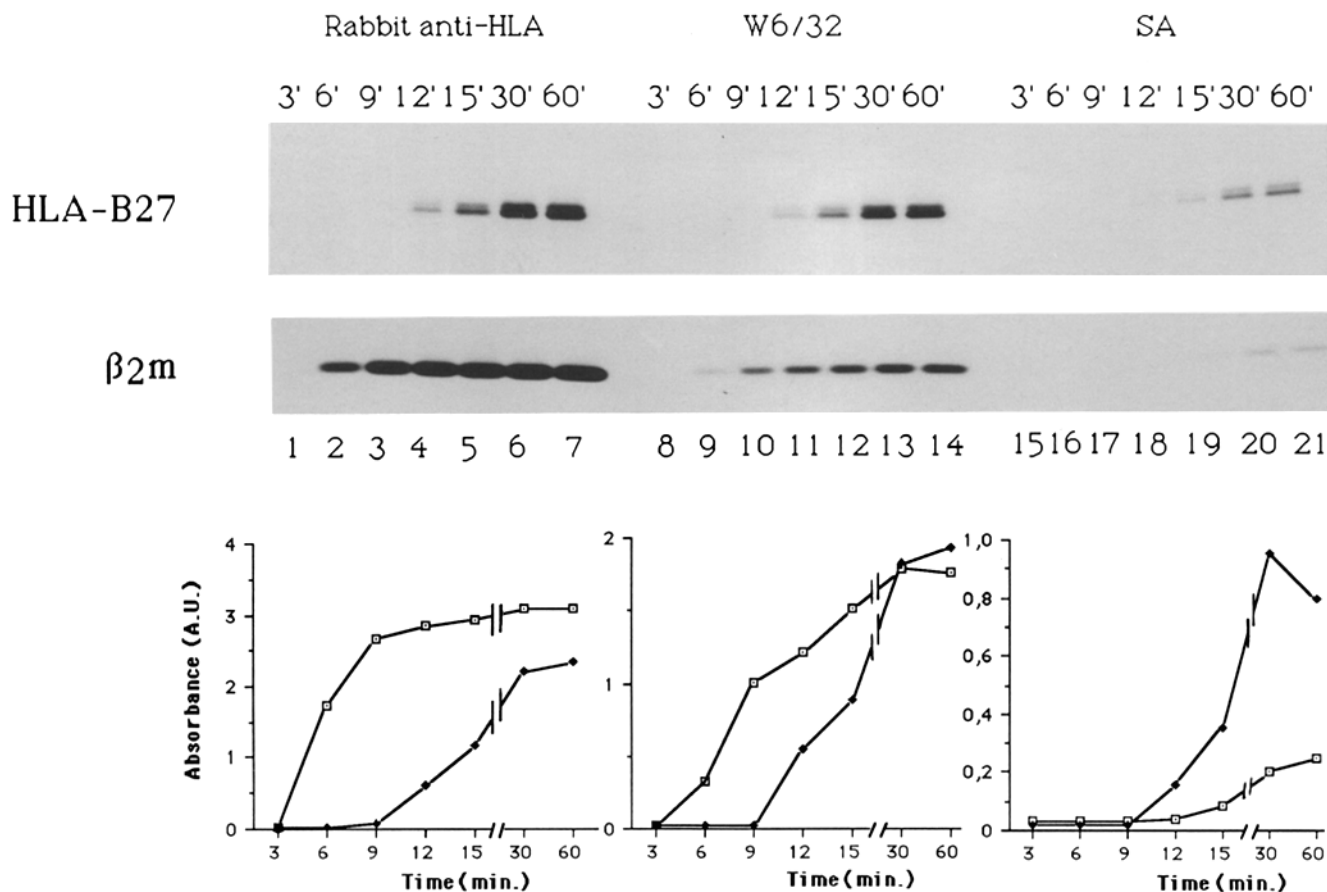
Figure 3. HLA-B27 heavy chain and β2-microglobulin assemble immediately after synthesis. Messenger RNA for the HLA-B27 heavy chain (50 ng) and β2-microglobulin (150 ng) were translated in the presence of a constant concentration of NP384-394-biotin peptide (32 μM) and microsomal membranes prepared from Daudi cells. Precipitations were carried out by a rabbit anti-HLA serum (lanes 1–7), the mAb W6/32 (lanes 8–14) heavy chain and β2-microglobulin are denoted by arrows. In the lower part of the figure a graphic representation of the autoradiogram is shown. Open boxes denote β2-microglobulin and filled boxes denote HLA-B27 heavy chain. See text for further details.

plete synthesis of the HLA-B27 heavy chain, both the heavy chain and β2-microglobulin were visible (Fig. 3, lane 18). The intensity increased till about 30 min, and was observed for both HLA-B27 heavy chain and β2-microglobulin (Fig. 3, lanes 18–20). We were unable to detect β2-microglobulin at early times of synthesis (Fig. 3, lanes 15–17), although its synthesis was completed (lanes 2 and 3). Thus, the NP384-394-biotin peptide associated to the HLA-B27 heavy chain–β2-microglobulin complex as soon as synthesis of the heavy chain was completed, but did not interact with complexes of endogenous microsomal HLA–newly translated β2-microglobulin, although such complexes existed (Fig. 3, lanes 9 and 10). Again, this confirms the specificity of the NP384-394-biotin peptide for HLA-B27 antigen. The HLA-B27 heavy chain band precipitated by streptavidin (Fig. 3, lanes 15–21), was considerably weaker due to the fact that it represents NP384-394-biotin peptide specific binding with a background of zero. This was in contrast to the unspecific background for assembly seen in Fig. 1 A, lane 1.

Peptides Have to Cross the ER Membrane in Order to Interact with the HLA-B27 Molecules

One of the most essential questions in MHC antigen presen-

tation of peptides is where the interaction occurs between heavy chain and peptide (Parham, 1990). As our assay system is easier to manipulate than whole cells, we could approach this question by dissecting the in vitro system further.

Translation of mRNA for the HLA-B27 heavy chain was performed as described above in two identical series in the absence and presence of NP384-394-biotin peptide (two different concentrations). Translation was terminated by addition of cycloheximide. The first series was washed and solubilized in the usual way. To the second series, proteinase K was added in order to digest proteins present in the reticulocyte lysate outside the microsomal membranes. After digestion for 30 min on ice, PMSF was added to stop digestion, and the membranes were washed and solubilized as for the first series. Both series of tubes were incubated with streptavidin-agarose and the precipitates were analyzed by SDS-PAGE.

For the first series we observed the same binding to HLA-B27 as in Fig. 2 (Fig. 4 A, lanes 1–3). The molecular weights of the HLA-B27 heavy chain bands were identical to those in previous figures. However, the second series of tubes, which had undergone proteinase K digestion, showed a decrease in the molecular weights for the HLA-B27 heavy chains, corresponding to loss of the ∼30 amino acids at their

carboxy terminus normally protruding at the cytoplasmic side of the ER membrane (Fig. 4 *A*, lanes *4–6*). Thus, the proteinase K digestion was efficient and degraded the cytoplasmic portion of membrane integrated proteins. Despite this, a similar binding of peptides to HLA-B27 heavy chains and β2-microglobulin was observed for this series as for the first one. If proteinase K was first added to the peptide, no binding to HLA-B27 was observed (data not shown).

To examine whether some assembly of HLA-B27 heavy chain and β2-microglobulin could occur after solubilization of the microsomes we performed the following experiment. Messenger RNA for HLA-B27 heavy chain was translated in the presence of microsomal membranes prepared from Raji cells, in the presence and absence of NP384-394-biotin peptide. In the absence of peptide we observed the background level of assembly of approximately one-fifth compared to 32 μM concentration of peptide by using the mAb W6/32 (Fig. 4 *B*, cf. lanes *1* and *2*). Also, precipitation with streptavidin detects the two heavy chain bands (Fig. 4 *B*, lane *3*). No stimulation of assembly was observed when the peptide was added together with the solubilization buffer (Fig. 4 *B*, cf. lanes *4* and *5*). Under these conditions precipitation with streptavidin did not detect the HLA-B27 heavy chains (Fig. 4 *B*, lane *6*). Thus, in our vitro system assembly of HLA-B27 heavy chain and β2-microglobulin must occur in intact microsomal membranes, and not after solubilization of the microsomes.

The following conclusions can be made from these experiments. (*a*) The interaction between the HLA-B27 heavy chain and the NP384-394-biotin must occur on the luminal side of the ER membrane as it is protected from proteinase K digestion. (*b*) The peptide must be present within the lumen of the microsomal vesicles and, thus, must somehow cross the ER membrane. (*c*) The cytoplasmic tail of the HLA-B27 heavy chain is not required for maintenance of stable complexes.

Transfer of Peptide Across the ER Membrane Is a Rapid Event

We next examined whether translation can be completed before addition of peptide and stimulation of assembly maintained. This was done by first completing and blocking further translation, and then adding the peptide (data not shown). Under these conditions, stimulation of assembly was still obtained. Assembly can thus be accomplished after translation has been completed. This allowed us to investigate peptide transfer across the ER membrane.

Messenger RNA for the HLA-B27 heavy chain was translated as described above. After 45 min translation was terminated by addition of cycloheximide, and the NP384-394-biotin peptide was added to a final concentration of 32 μM. Aliquots were withdrawn at the times indicated in Fig. 5, and were immediately diluted 16 times with ice-cold termination buffer and analyzed for their ability to react with streptavidin (see Materials and Methods for details). At time 0, the NP384-394-biotin peptide was mixed with termination buffer to give a final concentration of 2 μM.

Already 15–30 s after addition of the NP384-394-biotin peptide, an increase of the HLA-B27 heavy chain band is observed (Fig. 5, lanes *2* and *3*). The intensity of this band increased for the first 2 min after peptide addition, and then reached a plateau (Fig. 5, lanes *5–8*). Densitometric analysis
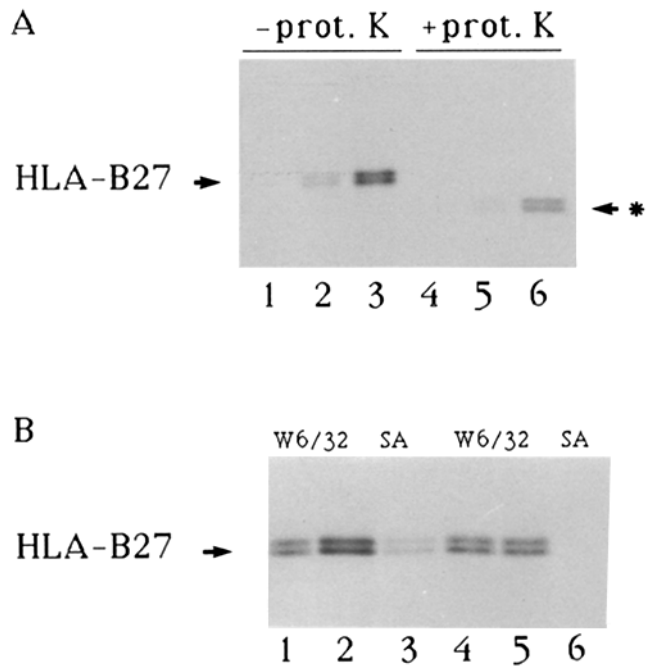

Figure 4. The NP384-394-biotin peptide must cross the microsomal membrane in order to associate with the HLA-B27 heavy chain. (*A*) The HLA-B27 heavy chain was translated in vitro in the absence (lanes *1* and *4*) and presence of NP384-394-biotin peptide (lanes *2* and *5*, 8 μM, lanes *3* and *6*, 32 μM). Samples shown in lanes *1–3* were treated normally as described above. Samples shown in lanes *4–6* were, after completion of translation (45 min), treated with cycloheximide to prevent further translation, and then digested with proteinase K at 0°C for 30 min. Digestion was stopped by adding PMSF and the membranes were recovered and analyzed as for lanes *1–3*. Precipitation was performed with streptavidin-agarose. The starred arrow denotes the position for HLA-B27 heavy chain without its carboxy-terminal cytoplasmic tail. (*B*) Translation of mRNA for HLA-B27 (100 ng) for 60 min was performed in the presence of Raji microsomal membranes. The NP384-394-biotin peptide was present at 32 μM concentration (lanes *2* and *3*) or absent (lanes *1* and *4–6*) during translation. For lanes *1–4* the microsomes were lysed in normal solubilization buffer after translation, whereas for lanes *5* and *6*, solubilized material was incubated for 60 min the the presence of 32 μM NP384-394-biotin peptide before immunoprecipitation. Precipitations were made by mAb W6/32 (lanes *1*, *2*, *4*, and *5*) and streptavidin-agarose (lanes *3* and *6*).

of the bands confirms the rapid uptake and binding of NP384-394-biotin peptide to HLA-B27. We conclude that peptide transfer across the ER membrane, and peptide binding to the HLA-B27 heavy chain–β2-microglobulin complex, is a very rapid process.

Association of HLA-B27 Heavy Chain with Peptide Is Stabilized by β2-Microglobulin

We have examined the role of β2-microglobulin in the assembly process by using microsomes prepared from Daudi cells. These cells do not express β2-microglobulin (Rosa and Fellous, 1982).

Messenger RNA for the HLA-B27 heavy chain was translated either alone, or together with mRNA for human β2-microglobulin, in the presence of 32-μM NP384-394-biotin peptide. The translation mixture was supplemented with
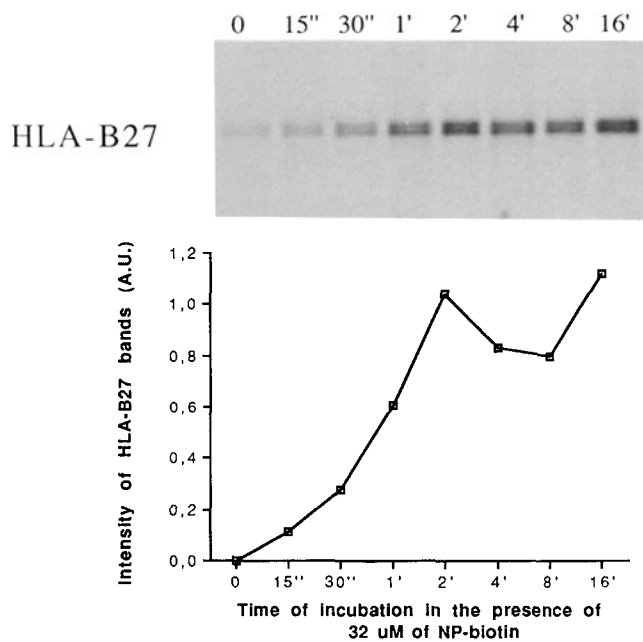
Figure 5. The NP384-394-biotin peptide is rapidly translocated through the microsomal membrane. Messenger RNA for HLA-B27 heavy chain (100 ng) was translated in the presence of Raji microsomal membranes for 45 min. The translation was terminated by addition of cycloheximide (final concentration of 100 μg/ml). The NP384-394-biotin peptide was added and allowed to react with HLA-B27 heavy chain–β2-microglobulin for the times indicated. The reaction was terminated by dilution of the translation mixture (16 times) with TNE-buffer (see Materials and Methods). The samples were immediately lysed, precipitated with streptavidin-agarose, and analyzed by SDS-PAGE. A graphic representation of the bands is shown in the lower part of the figure. The background intensity of the HLA-B27 band in lane *1*, corresponds to 0 absorbance in the graph.

Figure 6. In vitro translation of HLA-B27 heavy chain and human β2-microglobulin in microsomes deficient of endogenous β2-microglobulin. Messenger RNA (50 ng) for HLA-B27 heavy chain was translated either alone (lanes *1* and *2*), or together with mRNA (200 ng) for β2-microglobulin. All incubations contained the NP384-394-biotin peptide at 32 μM concentration. The microsomal membranes were prepared from Daudi cells and were added to the same concentration as were Raji microsomes in Figs. *1–4.* The membranes were recovered as described above. Precipitations were performed by the following reagents: rabbit anti-HLA (lane *1*); streptavidin-agarose (lanes *2* and *4*); mAb W6/32 (anti-HLA; lane *3*); and mAb BBM.1 (anti-β2-microglobulin; lane *5*). See Materials and Methods for details.

microsomes prepared from Daudi cells in the same amounts as described in the previous experiments.

The HLA-B27 heavy chain was efficiently translated as verified by immunoprecipitation with a rabbit antiserum against HLA antigens (Fig. 6, lane *1*). In the absence of translation of β2-microglobulin, we could not detect any precipitation of HLA-B27 heavy chain by using streptavidin-agarose (lane *2*). However, by cotranslation of mRNA for β2-microglobulin the streptavidin-agarose precipitated both HLA-B27 heavy chains and β2-microglobulin (Fig. 6, lane *4*), although considerably less than the mAb W6/32, which recognizes only assembled HLA antigens (lane *3*). Considerably more of β2-microglobulin is however available, as evidenced by immunoprecipitation with the mAb BBM.1 (anti-β2-microglobulin; Fig. 6, lane *5*). From this experiment we conclude that the presence of β2-microglobulin is necessary to stabilize the HLA-B27 heavy chain interaction with peptide. Furthermore, the fact that the mAb W6/32 can precipitate about fivefold more of both the heavy chain and β2-microglobulin, than can the streptavidin, indicates that assembly in Daudi microsomes might occur either with endogenous peptides or without peptides involved (see below).
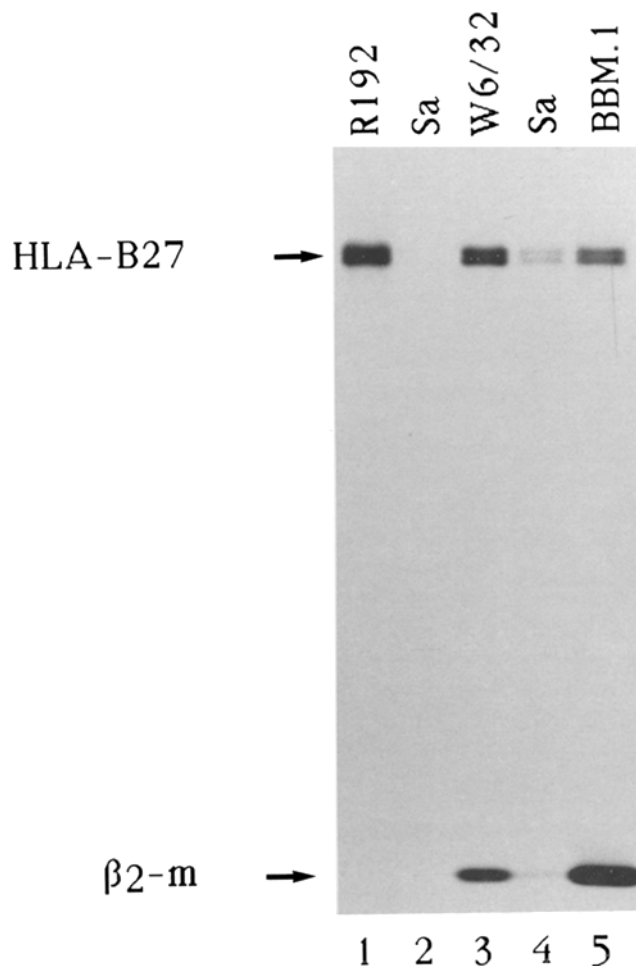
The Order of Assembly

It has recently been shown that mouse MHC class I heavy chains can assemble to β2-microglobulin in the absence of peptide at a temperature of 26°C (Ljunggren et al., 1990; Schumacher et al., 1990). As the in vitro system allows translation at this temperature we examined whether also HLA-B27 heavy chain interaction with β2-microglobulin could be stabilized in a similar fashion at low temperature. Furthermore, by performing the experiment in both absence and presence of peptide, it could be possible to dissect the order of assembly.

Messenger RNAs for both HLA-B27 heavy chain and β2-microglobulin were translated in the presence of microsomal
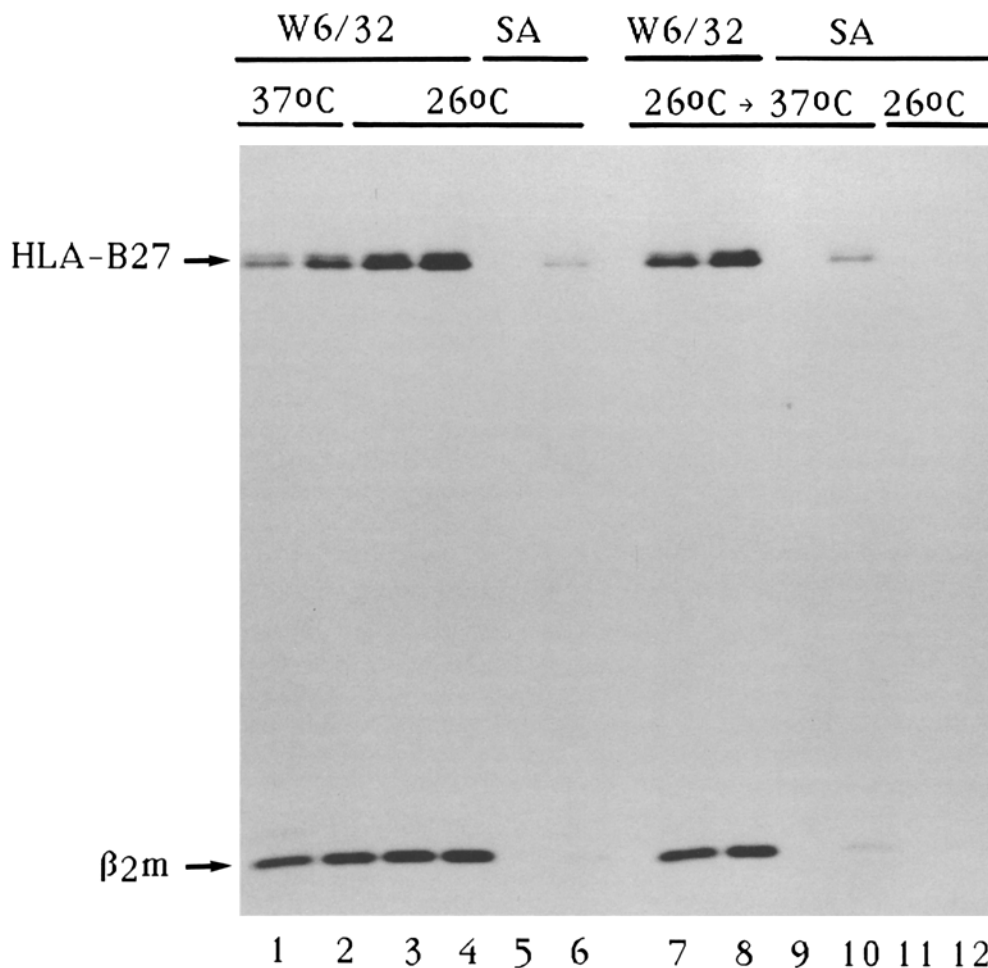
Figure 7. HLA-B27 heavy chain interacts with β2-microglobulin before peptide binding. Messenger RNA for HLA-B27 heavy chain (100 ng) and β2-microglobulin (200 ng) (lanes *1–10*), or only mRNA for HLA-B27 heavy chain (lanes *11* and *12*), were translated in the presence of microsomal membranes prepared from Daudi cells as in Fig. 5. Incubation temperature was either at 37 (lanes *1* and *2*) or at 26°C (lanes *3–12*). In lanes *7–10*, translation was completed at 26°C, blocked with cycloheximide, and further incubated at 37°C for 15 min before precipitation. The NP384-394-biotin peptide was present at the concentration of 32 μM in lanes *2, 4, 6, 8, 10,* and *12.* The membranes were recovered as described in Fig. 1. Precipitations were done with the mAb W6/32 (lanes *1–4, 7,* and *8*) or streptavidin-agarose (lanes *5, 6,* and *9–12*). See Materials and Methods for further details.

membranes derived from Daudi cells. Translation at 37°C in the absence and presence of the NP384-394-biotin peptide shows the usual stimulation of assembly (Fig. 7, lanes *1* and *2*, respectively). When the translation was performed at 26°C, the HLA-B27 heavy chain bands were equally strong independent of the presence of peptide (Fig. 7, lanes *3* and *4*). The intensities of the bands were similar or identical to that found for translation at 37°C in the presence of peptide. Thus, it seems that at low temperature the HLA-B27 heavy chain–β2-microglobulin complex is formed and stabilized also in the absence of peptide. However, when we performed the precipitation with streptavidin instead of the mAb W6/32, we found that the NP384-394-biotin peptide was associated with the HLA-B27 heavy chain–β2-microglobulin complex (Fig. 7, lane *6*). In the absence of peptide streptavidin did not precipitate the heavy chain (Fig. 7, lane *5*). We interpret this to mean that the HLA-B27 heavy chain band seen in Fig. 7, lane *4* must contain peptide, despite the fact that no increase in intensity was observed (Fig. 7, cf., lanes *3* and *4*).

We next performed the identical experiment as described above at 26°C, but before solubilization the temperature in the samples was increased to 37°C for 15 min in order to examine whether the complexes could be destabilized at this higher temperature. In the absence of the NP384-394-biotin peptide, the intensity of the HLA-B27 heavy chain band decreased, indicating dissociation of complexes (Fig. 7, lane *7*). In contrast, when the peptide was present, an increase in temperature did not lower the intensity of the band (Fig. 7, lane *8*), and the peptide was still bound to the heavy chain (lane *10*). Thus, the presence of the peptide does stabilize the ternary complex, whereas the HLA-B27 heavy chain interaction with β2-microglobulin (without peptide) is not a stable complex at 37°C. Translation of the mRNA for HLA-B27 heavy chain only in the presence of NP384-394-biotin peptide (absence of β2-microgloublin), and precipitation with streptavidin, did not demonstrate a heavy chain–peptide interaction at 37 nor at 26°C (Fig. 7, lanes *11* and *12*, respectively). We interpret this to mean that assembly of the HLA-B27 heavy chain with β2-microglobulin precedes binding of the peptide, although we cannot exclude weak interactions between heavy chain–peptide before assembly with β2-microglobulin.

Discussion

In this paper we have described an assay to study direct peptide interaction with MHC class I molecules. We have biotinylated the peptide and performed in vitro translation of the HLA-B27 heavy chain in a rabbit reticulocyte lysate. To detect peptide associated to class I antigens, the complex was precipitated with streptavidin immobilized to agarose. To

mimic the ER membrane, the lysate was supplemented with microsomal membranes. We have previously shown that these microsomes (from Raji cells) contain large amounts of endogenous both class I heavy chains and β2-microglobulin. By translating mRNA for the HLA-B27 chain only, it could be shown that assembly occurs with unlabeled microsomal β2-microglobulin (Kvist and Hamann, 1990).

It is important to note that the biotinylated peptide behaved in our system indistinguishably from the unmodified peptide, i.e., the same degree of stimulation of assembly of HLA-B27 heavy chains and β2-microglobulin was observed (Fig. 1). It was also possible to compete the biotinylated peptide with the unmodified peptide, proving that both peptides bind to the same site of the heavy chain (data not shown). It is likely that the proper behavior of the modified peptide is dependent on two different important factors. First, a biotinylating reagent was used that has a 22-Å-long spacer arm between the biotin group and the site used for coupling to the aminoterminal amino acid. This may be important to avoid steric hindrance when the peptide interacts with the class I molecule. It might also facilitate the interaction of the biotin with streptavidin, as the binding site in this protein is located 9 Å below the surface of the molecule (Green et al., 1971). Second, it seemed important to perform the biotinylation at a neutral pH to avoid modification of aminogroups on the side chains of internal amino acids within the peptide.

It has previously been demonstrated that peptides can be radioactively iodinated and bind to MHC class I antigens (Schumacher et al., 1990; Townsend et al., 1990). The system described here demonstrates interaction between peptide and MHC class I molecules via biotin coupled to the ligand. A similar method has recently been described (Luescher et al., 1991). The interaction seems specific (Fig. 2), and is not limited to the peptide used here (NP384-394), but can be used for several other peptides and MHC class I antigens as well (data not shown; Lévy, F., and S. Kvist, manuscript in preparation). This method is likely to be a useful tool as no specific amino acid seems required for the biotin label. In particular, it is interesting that MHC antigen–peptide interaction can now be studied in an in vitro system, which is close to physiological with regard to ongoing protein synthesis and membrane insertion of the MHC heavy chain.

The early events in the assembly of HLA-B27 heavy chain with β2-microglobulin and the NP384-394-biotin peptide have been studied. Both the heavy chain and β2-microglobulin were translated at a rate of ~30 amino acids per minute (Fig. 3). Translation and insertion into the microsomal membrane was completed after 12 min for the heavy chain, whereas β2-microglobulin was completed already after 4 min. Immediately after its translation, β2-microglobulin could be detected by mAb W6/32, which recognizes only HLA antigen complexes, indicating complex formation between the newly synthesized β2-microglobulin and endogenous microsomal HLA heavy chains. Complexes of HLA-B27 heavy chain and β2-microglobulin were found as soon as synthesis of the heavy chain was completed (Fig. 3, lanes 8–14).

Translations were carried out in the presence of the NP384-394-biotin peptide. This peptide (without its biotin modification) has been shown to induce CTL lysis in an HLA-B27 antigen restricted fashion (Huet et al., 1990). Here the NP384-394-biotin peptide was shown to interact with the HLA-B27 heavy chain–β2-microglobulin complex immediately after synthesis of the heavy chain (Fig. 3, lanes 15–21). No interaction of the peptide with HLA complexes formed between endogenous HLA heavy chains (Daudi microsomal membranes) and in vitro translated β2-microglobulin could be detected, although the existence of such complexes was demonstrated (Fig. 3). Thus, the binding of the NP384-394 peptide seems specific for HLA-B27 antigens. This finding is in agreement with the results presented by Van Bleek and Nathenson (1990), who found a high degree of specificity in peptide binding to the murine H-K^b molecule. This contrasts with the results obtained with the peptide-coated plate assay, which suggested a promiscuous binding of peptide to MHC class I antigens (Bouillot et al., 1989; Chen et al., 1990; Choppin et al., 1990; Frelinger et al., 1990). We believe that the results presented here represent close to physiological conditions, and therefore are likely to reflect the natural situation of peptide binding to MHC class I antigens.

The NP384-394-biotin peptide induced stimulation of assembly of the HLA-B27 heavy chain and β2-microglobulin, and the microsomal vesicles protected the heavy chain from degradation by proteinase K (Fig. 4 A). The interaction must therefore take place on the luminal side of the ER membrane. This means that the peptide must be transferred across the membrane into the lumen. How the peptide reaches the vesicular lumen is unknown. Addition of peptide after completion of translation showed peptide binding within 1 min (Fig. 5). It is possible that saturation with peptide could be due to additional steps other than binding to HLA-B27. Such a saturation must, however, occur before the time points 15 and 30 s, as the HLA-B27 bands then start to increase. If a rate-limiting step for peptide translocation, or peptide release to the HLA-B27–β2-microglobulin complex, would exist, this should be reflected as an increase in the HLA bands at later time points as heavy chain B27 would still be available in an unsaturated form. As a plateau was reached already after 2 min, this possibility is unlikely. Thus, peptide transfer is a very efficient and rapid process. This is compatible with both diffusion and active transport of the peptide. Recently, several groups identified genes within the MHC which encode proteins similar in structure to the ABC superfamily of transporters (Deverson et al., 1990; Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990; Spies and DeMars, 1991). It is likely that these proteins play an important role in the transport of peptides, or by other means, to facilitate their entry into the lumen of the ER.

Townsend et al. (1990), showed that assembly of MHC class I antigens can occur in lysates of detergent-solubilized cells. In our system this does not occur as no assembly could be detected, when the NP384-394-biotin peptide was added together with the solubilization buffer (Fig. 4 B). The reason for this is most likely an effect of concentration of the different constituents. The radioactively labeled HLA-B27 heavy chain in our experiments is not detectable in Western blots (Kvist and Hamann, 1990), and may therefore need the concentrated environment of the microsomal vesicles in order to assemble.

Microsomes prepared from Daudi cells, which are devoid

of endogenous $\beta 2$-microglobulin, were used to examine whether the HLA-B27 heavy chain could be found associated to the NP384-394-biotin peptide in the absence of $\beta 2$-microglobulin (Fig. 6). An absolute requirement was found for the presence of $\beta 2$-microglobulin to detect heavy chain–peptide interaction. By performing the translation at 26°C (instead of 37°C) the findings by Ljunggren et al. (1990), and Schumacher et al. (1990), which show that MHC class I heavy chains form complexes with $\beta 2$-microglobulin also in the absence of specific peptide, could be confirmed (Fig. 7). In the presence of the NP384-394-biotin no further increase in assembly was observed, but the HLA-B27 heavy chain was precipitated by streptavidin. This demonstrated that despite the lack of stimulation of assembly, the complexes contained peptide. These complexes were stable when the temperature was raised to 37°C (Fig. 7). We interpret this to mean that HLA-B27 heavy chain–$\beta 2$-microglobulin complexes first form a loose interaction that is stabilized at 26°C, but not at 37°C. Binding of the NP384-394-biotin peptide to this complex stabilizes the structure and makes it stable also at 37°C. In the absence of translation of $\beta 2$-microglobulin we could not detect any HLA-B27 heavy chain–NP384-394-biotin interaction at 37 nor at 26°C with normal exposure of the x-ray film (Fig. 7). This finding lends further support to the conclusion that heavy chain interaction with $\beta 2$-microglobulin precedes peptide binding. However, after prolonged exposure of the x-ray film, very faint bands corresponding to HLA-B27 heavy chain was observed also in the absence of $\beta 2$-microglobulin. As these bands were detected after extreme exposure times, we are not convinced of their relevance.

It has been discussed previously that assembly of MHC class I–peptide–$\beta 2$-microglobulin complex can be formed in two ways (Townsend et al., 1990). (a) The heavy chain can either form a loose interaction with $\beta 2$-microglobulin that is stabilized by peptide binding to the alpha one/alpha two groove, or (b) that peptide interacts in a first step with the heavy chain to stabilize a structure which can in a second step interact with $\beta 2$-microglobulin to form the final complex. No heavy chain–peptide interaction was observed in the absence of $\beta 2$-microglobulin (Figs. 6 and 7). We interpret this to mean that such formations most likely do not occur, and this favours the first possibility. Alternatively, the heavy chain–peptide complex is not stable enough to be precipitated by streptavidin-agarose. The stability of the complex may also be affected by the nature of the peptide, and influence the order of assembly. It is also possible that peptide and $\beta 2$-microglobulin might stabilize each other's binding to the heavy chain. In the light of two reports demonstrating that MHC heavy chain–$\beta 2$-microglobulin complexes actually do exist without peptide (Ljunggren et al., 1990; Schumacher et al., 1990), and are stabilized in the cold, we favor the first possibility outlined above, i.e., that MHC class I heavy chains exist in a loose interaction with $\beta 2$-microglobulin, and that this complex can be stabilized by either peptides or other means, e.g., low temperature.

This report describes an in vitro system that can be used to further dissect the MHC class I antigen assembly process. It might be useful as a complement to that described by Townsend and colleagues (1990). The relative ease with which the different components (e.g., type of mRNA, membranes, etc.) can be varied will be valuable for the analysis of peptide-binding specificities of different MHC class I alleles.

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