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Immunology

Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules

(antigen processing/major histocompatibility complex)

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ABSTRACT **HLA-DR molecules are heterodimeric transmembrane glycoproteins that associate intracellularly with a polypeptide known as the invariant (I) chain. Shortly before expression of the HLA-DR $\alpha\beta$ dimer on the cell surface, however, the I chain is removed from the intracellular $\alpha\beta$I complex by a mechanism thought to involve proteolysis. In this report, we show that treatment of purified $\alpha\beta$I with the cysteine proteinase cathepsin B results in the specific proteolysis of the HLA-DR-associated I chain *in vitro*. As a consequence of this, the I chain is removed and free $\alpha\beta$ dimers are released from $\alpha\beta$I. Although $\alpha\beta$I fails to bind an immunogenic peptide, the released $\alpha\beta$ dimers acquire the ability to bind the peptide after proteolysis of the I chain. These results suggest that the I chain inhibits immunogenic peptide binding to $\alpha\beta$I early during intracellular transport and demonstrate that proteolysis is likely to be the *in vivo* mechanism of I chain removal.**

Major histocompatibility complex (MHC) molecules are polymorphic heterodimeric glycoproteins that function by presenting immunogenic peptides to MHC-restricted T cells. Recent evidence suggests that newly synthesized class I MHC molecules bind immunogenic peptides very early in transport, perhaps as early as the endoplasmic reticulum (1). Binding of peptides to class II MHC molecules is also thought to occur intracellularly; however, in this case the peptides are generated after endocytosis and proteolytic degradation of foreign antigens (2, 3).

The human class II molecule that has been most intensively investigated is HLA-DR (DR). Immediately after synthesis, the DR $\alpha\beta$ heterodimer associates with a nonpolymorphic polypeptide termed the invariant (I) chain (for review, see ref. 4). The $\alpha\beta$–invariant chain ($\alpha\beta$I) complex is then transported through the Golgi apparatus and, at least in human B-lymphoblastoid cell lines, the complex is delayed in a post-Golgi compartment for 2–4 hr. The DR-associated I chain is then removed from $\alpha\beta$I and soon thereafter the $\alpha\beta$ dimer is expressed on the cell surface (5).

Biochemical and electron microscopic evidence suggests that after transport through the Golgi apparatus, $\alpha\beta$I enters a proteinase-containing compartment also intersected by the endocytic pathway (6–8). Consistent with these findings are the results of pulse–chase analyses of class II molecules isolated from B-lymphoblastoid cells grown in the presence of the proteinase inhibitors chloroquine, leupeptin, antipain, and L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane (E-64) (refs. 9–11; unpublished data). These reagents inhibit I chain proteolysis and/or dissociation from $\alpha\beta$I *in vivo*, suggesting that it is the action of endosomal proteinases that leads to the liberation of the I chain from intracellular $\alpha\beta$I.

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We have proposed that the compartment in which foreign antigen-derived peptides are generated and the compartment in which the class II-associated I chain is degraded is the same and that it is only after proteolysis of the I chain that the newly liberated class II $\alpha\beta$ dimer is capable of binding an immunogenic peptide (4, 10). In support of this hypothesis are recent *in vitro* data that demonstrated that whereas $\alpha\beta$ dimers could bind an immunogenic peptide with high affinity, $\alpha\beta$I complexes could not bind this peptide unless the I chain was dissociated from the complex with SDS (12, 13). Although the use of SDS to remove the DR-associated I chain is physiologically irrelevant, this data is consistent with the hypothesis that association with the I chain prevents peptide binding to $\alpha\beta$I.

We now demonstrate that the DR-associated I chain can be specifically degraded and dissociated from purified $\alpha\beta$I by incubation *in vitro* with the endosomal cysteine proteinase cathepsin B. After this treatment, the newly released $\alpha\beta$ dimer acquires the ability to bind an immunogenic peptide, suggesting a physiological mechanism whereby functional $\alpha\beta$ dimers can be liberated from intracellular $\alpha\beta$I complexes *in vivo*.

MATERIALS AND METHODS

Proteins. The B-lymphoblastoid cell line Swei was cultured with monensin (to allow the intracellular accumulation of class II $\alpha\beta$I complexes) and [^{35}S]methionine as described (14, 15). Class II $\alpha\beta$I complexes were isolated from Swei cell lysates by immunoaffinity chromatography and size-exclusion HPLC (12, 13). The peptides corresponding to residues 306–320 of influenza virus A/Texas/1/77 hemagglutinin (HAp) or residues 378–398 of the *Plasmodium falciparum* circumsporozoite protein were labeled with ^{125}I using the chloramine-T method (12). Bovine cathepsin B and E-64 were obtained from Sigma.

Cathepsin B Treatment of $\alpha\beta$I. HPLC-purified $\alpha\beta$I was incubated with cathepsin B in a buffer of 125 mM sodium phosphate, 5 mM cysteine, and 1% *n*-octyl β-D-glucopyranoside (pH 6.0) at 37°C. After various times of incubation, cathepsin B was irreversibly inhibited by the addition of the potent cysteine proteinase inhibitor E-64 (10 μM; ref. 16), and the sample was maintained on ice until further analysis.

Binding of ^{125}I-Labeled HAp (*HAp) to Cathepsin B-Treated $\alpha\beta$I. After the addition of E-64 to the reaction mixtures containing $\alpha\beta$I, *HAp was added to a final concentration of 200 nM. After an additional 8-hr incubation at room temperature, DR-bound and free *HAp were separated by HPLC. The amount of liberated DR $\alpha\beta$ dimer occupied by *HAp was estimated using the known specific activity of the *HAp

Abbreviations: I chain, invariant chain; E-64, L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane; DR, HLA-DR; HAp, synthetic peptide representing amino acids 306–320 of influenza virus A/Texas/1/77 hemagglutinin; *HAp, ^{125}I-labeled HAp; 2D, two dimensional; V_e, elution volume; MHC, major histocompatibility complex.

preparation (25,000 cpm/pmol of *HAp) and the amount of the $\alpha\beta$ dimer present in the sample was determined by the methods described below.

HPLC and Electrophoresis. HPLC was performed using a TSK-G3000 SW size-exclusion chromatography column (7.5 × 600 mm; Pharmacia-LKB) equilibrated in 50 mM sodium phosphate/1% *n*-octyl β-D-glucopyranoside, pH 7.0 (12, 13). Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min. The amount of protein present in the $\alpha\beta$I preparations was estimated by HPLC by comparing the peak area of a sample of the $\alpha\beta$I preparation (monitored at 214 nm) to the peak area of a sample containing a known amount of ovalbumin. The amount of DR released from $\alpha\beta$I was estimated as a percentage of the total β chains present in the sample by immunoblot analysis of HPLC fractions with the class II β-chain-specific monoclonal antibody XD5.A11 (13).

One-dimensional reducing SDS/PAGE and two-dimensional (2D) PAGE (nonequilibrium pH-gradient electrophoresis followed by reducing SDS/PAGE) were performed as described (14). The molecular weight of unknown proteins was determined based on a standard curve obtained using ^{14}C-labeled molecular weight standards (Amersham). HPLC fractions and all samples for 2D PAGE were precipitated with ethanol at −20°C prior to electrophoresis and lyophilized. The radioactivity present in the samples was visualized by fluorography using Kodak X-Omat AR film and Enlightning (DuPont), and the intensity of the bands was determined by automatic integrating densitometry.

RESULTS AND DISCUSSION

Treatment of $\alpha\beta$I with Cathepsin B. Since endosomal cysteine proteinases have been implicated in the proteolytic degradation of the DR-associated I chain (10, 11), we investigated the susceptibility of highly purified DR $\alpha\beta$I to digestion with the cysteine proteinase cathepsin B. $\alpha\beta$I was incubated with various concentrations of cathepsin B for 1 hr and the reaction products were analyzed by SDS/PAGE. Fig. 1*A* demonstrates that whereas the intensity of the DR β chain (27 kDa) remained relatively constant during the incubation, there was a concentration-dependent loss of protein in the 35-kDa range (containing the DR α and I chains). Concomitant with this loss was the accumulation of proteolytic fragments of 24 kDa, 22 kDa, 15 kDa, and lower molecular mass material that migrated at the dye front.

The generation of $\alpha\beta$I fragments by cathepsin B (50 μg/ml) was next studied as a function of time. Fig. 1*B* demonstrates that, after as little as 0.5 hr, significant amounts of the 24-kDa and 22-kDa species had accumulated, but by 2 hr the levels of these two species had dropped dramatically. By contrast, the levels of the 15-kDa material and lower molecular mass material did not begin to increase appreciably until 1 hr, suggesting that the generation of these fragments was the result of proteolysis of the 24-kDa and 22-kDa species.

2D PAGE of Cathepsin B-Treated $\alpha\beta$I. To determine if the lower molecular mass fragments were derived from the DR-associated I chain, $\alpha\beta$I was incubated alone (Fig. 2*A*) or with cathepsin B at 25 μg/ml (Fig. 2*B*) or 50 μg/ml (Fig. 2*C*) for 1 hr and the reaction products were analyzed by 2D PAGE. As a control, $\alpha\beta$I was incubated with cathepsin B in the presence of the cysteine proteinase inhibitor E-64 (Fig. 2*D*). Fig. 2 demonstrates that there was a concentration-dependent loss of intact I chain from $\alpha\beta$I after treatment with cathepsin B. By contrast, the DR α and β chains appear relatively resistant to proteolysis by this enzyme. The species indicated by the long arrowhead has an apparent molecular mass of 24 kDa and migrates with the same mobility as the I chain fragment commonly referred to as "p25" (11, 17, 18). There are also a number of basic fragments of 22 kDa (indicated by brackets) that probably account for the diffuse

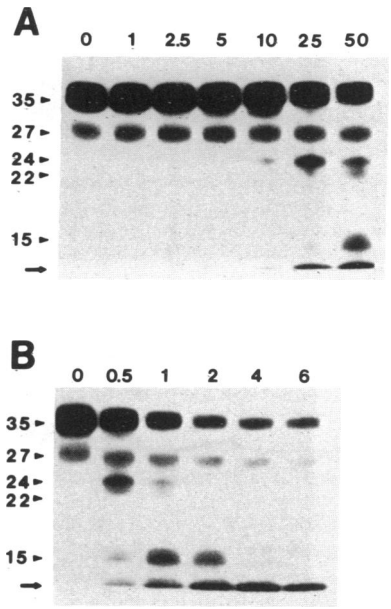

FIG. 1. Proteolysis of $\alpha\beta$I complexes with cathepsin B. DR5 $\alpha\beta$I complexes were obtained from detergent lysates of the B-lymphoblastoid cell line Swei by immunoaffinity chromatography and were purified to apparent homogeneity by size-exclusion HPLC (12, 13). [^{35}S]Methionine-labeled $\alpha\beta$I complexes were treated at 37°C with various concentrations (μg/ml) of cathepsin B for 1 hr as indicated (*A*) or for various lengths of time (hr) with cathepsin B (50 μg/ml) prior to the addition of the cathepsin B inhibitor E-64 to each sample (*B*). The samples were then boiled in SDS/2-mercaptoethanol and analyzed by SDS/PAGE and fluorography. The molecular mass (in kDa) of the DR α and I chains (35 kDa), DR β chain (27 kDa), and the DR-derived major proteolysis products (24 kDa, 22 kDa, and 15 kDa) are indicated by arrowheads. The position of the bromophenol blue dye front is indicated by an arrow.

appearance of this material in SDS/PAGE. In addition to these larger fragments, there are also low molecular mass proteolysis products consisting of a group of very acidic 15-kDa fragments as well as a group of very basic 12-kDa fragments. Proteolysis also led to the production of small ethanol-insoluble polypeptides that migrated at the dye front of the gels (≤10 kDa). Whereas the intensity of the intact I chain decreased with increasing concentrations of cathepsin B, the intensity of the 24-kDa, 22-kDa, 15-kDa, 12-kDa, and lower molecular mass fragments increased under these conditions. These data, with the data shown in Fig. 1, suggest that these lower molecular mass proteolysis products are derived from the intact I chain.

Although the DR α chain appears totally resistant to cathepsin B, the β chain appears to migrate with a slightly decreased molecular mass at a more acidic position after treatment with this enzyme. This is almost certainly due to the well-established carboxyl-terminal peptidyldipeptidase activity of cathepsin B (19). Examination of the carboxyl-terminal sequence of the DR β chains reveals that this region contains basic amino acid residues (20), and cleavage of one (or more) of these residues would result in the observed acidic shift in the mobility of the remaining β chain. Since the carboxyl terminus of the β chain is cytoplasmic in intact cells, this cleavage would not be expected to occur *in vivo*.

Identification of $\alpha\beta$I Digestion Products Isolated by HPLC. $\alpha\beta$I was incubated with cathepsin B (50 μg/ml) in the presence or absence of the cathepsin B inhibitor E-64. After 4 hr of incubation, additional E-64 was added to each sample and the reaction products were separated by HPLC. Fig. 3*A* shows that $\alpha\beta$I incubated with inactivated cathepsin B was eluted as a single high molecular mass peak [elution volume

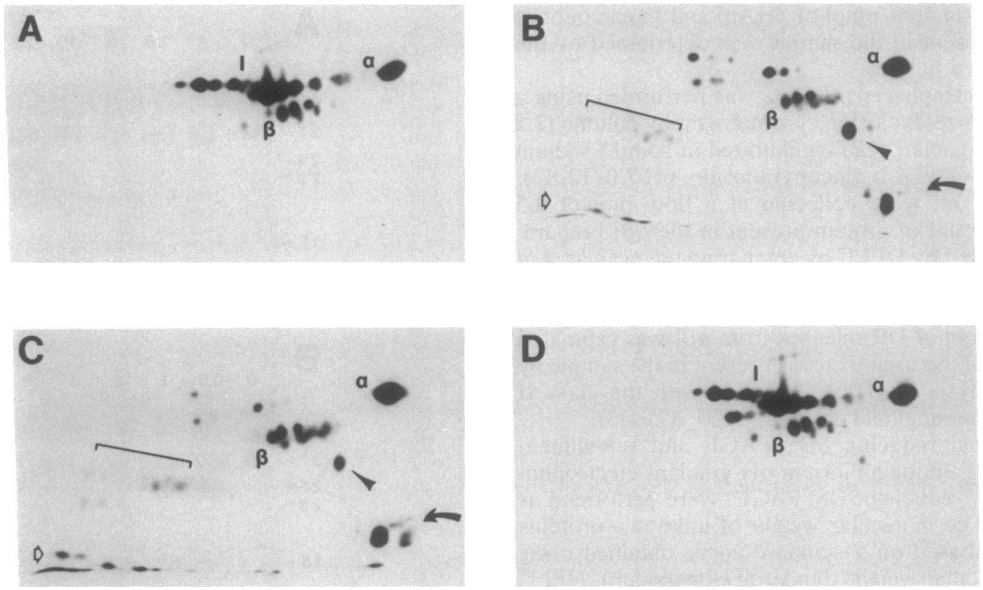

FIG. 2. Specific proteolysis of the class II-associated I chain by cathepsin B. [^{35}S]Methionine-labeled $\alpha\beta$I complexes were incubated at 37°C alone (*A*) or with cathepsin B at 25 μg/ml (*B*) or at 50 μg/ml (*C*) for 1 hr prior to the addition of the cathepsin B inhibitor E-64. [^{35}S]Methionine-labeled $\alpha\beta$I complexes were also incubated at 37°C for 1 hr in the presence of cathepsin B (50 μg/ml) that had been pretreated with E-64 (*D*). The samples were precipitated with ethanol and analyzed by 2D PAGE [nonequilibrium pH-gradient electrophoresis from right to left (acidic to basic) followed by reducing SDS/PAGE (top to bottom)]. The positions of the DR α, β, and I chains are indicated. The major I chain proteolysis products at 24 kDa (long arrowhead), 22 kDa (bracket), 15 kDa (solid arrow), and 12 kDa (open arrow) are also indicated and are discussed in the text.

(V_e), 11 ml]. The high molecular mass of $\alpha\beta$I is almost certainly related to the basic trimeric structure of the I chain (21). Fig. 3*B* shows that there was a dramatic loss of this high molecular mass material after incubation of $\alpha\beta$I with cathepsin B. This loss was accompanied by an increase in the amount of radiolabel eluting in peak II (V_e, 15 ml), peak III (18 ml), peak IV (21 ml), and peak V (25 ml).

To identify the products released by the incubation of $\alpha\beta$I with cathepsin B, samples of the protein present in each HPLC fraction were analyzed by SDS/PAGE and the material in each peak was pooled and analyzed by 2D PAGE. Fig. 4*A* and 2D PAGE (data not shown) confirm that $\alpha\beta$I incubated with inactivated cathepsin B was eluted as a single peak at 11 ml and contained the DR α, β, and I chains. By contrast, Fig. 4*B* and Fig. 5*I* demonstrate that after incubation of $\alpha\beta$I with cathepsin B, the high molecular mass material in peak I contains DR α and β chains associated with the very basic 12-kDa I chain fragment and other small ethanol-insoluble polypeptides. Additional experiments demonstrated that any residual 24-kDa and 22-kDa I chain fragments also eluted in this peak (data not shown).

Fig. 4*B* shows that the loss of the DR α and β chains from the high molecular mass $\alpha\beta$I complex is accompanied by the

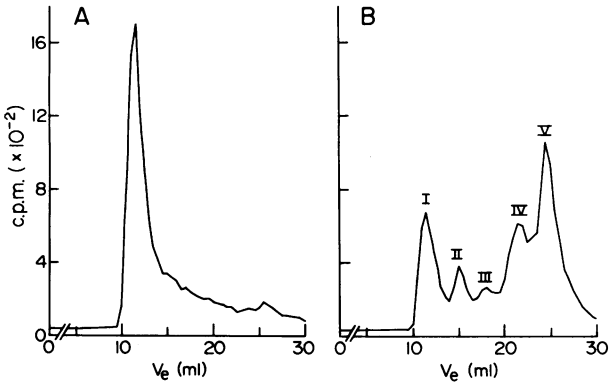

FIG. 3. HPLC of the products of the $\alpha\beta$I–cathepsin B reaction. [^{35}S]Methionine-labeled $\alpha\beta$I complexes were incubated at 37°C with cathepsin B (50 μg/ml) in the presence (*A*) or absence (*B*) of the cathepsin B inhibitor E-64. After 4 hr, additional E-64 was added to both samples and each reaction mixture was separated by size-exclusion HPLC. The radioactivity present in 50 μl of each 0.5-ml fraction was determined in a liquid scintillation counter and is plotted as a function of the V_e. The void volume of the column used in these studies was 10 ml and the included volume was 25 ml (as determined using Blue dextran 2000 and Na^{125}I, respectively).

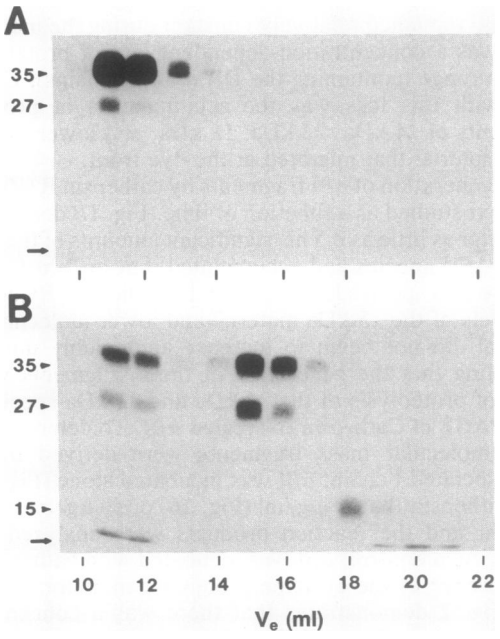

FIG. 4. Cathepsin B releases $\alpha\beta$ dimers and an I chain fragment from $\alpha\beta$I complexes. Samples of the HPLC fractions used to generate the data shown in Fig. 3 were precipitated with ethanol and visualized by SDS/PAGE and fluorography. The fluorographs (*A* and *B*) correspond to the material present in Fig. 3 *A* and *B*, respectively. The molecular mass (in kDa) of the major radioactive species present in the samples and the V_e of the individual fractions analyzed is indicated.

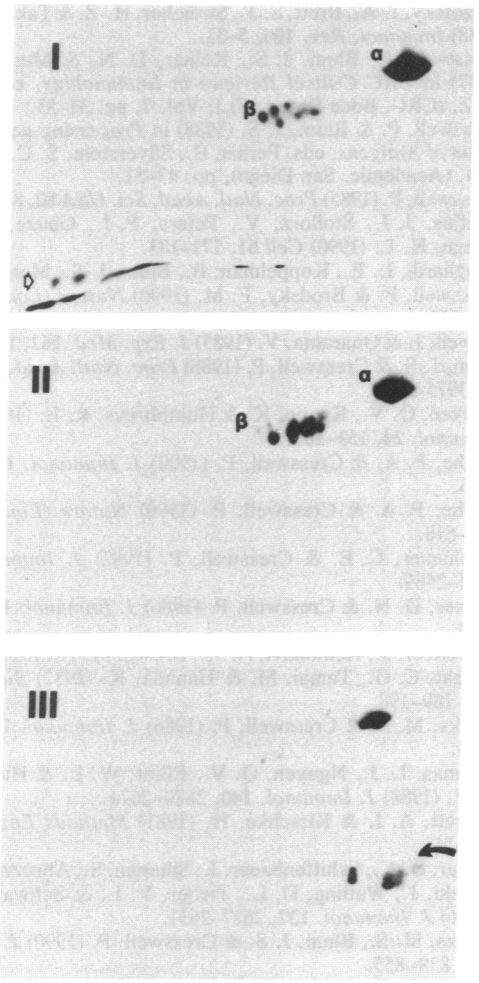

FIG. 5. Cathepsin B treatment of $\alpha\beta$I complexes releases pure $\alpha\beta$ dimers. The material in peaks I, II, and III of the HPLC profile shown in Fig. 3*B* was pooled, precipitated with ethanol, and visualized by 2D PAGE and fluorography. The material in *I–III* correspond to peaks I–III in Fig. 3*B*, respectively. The positions of the DR α and β chains as well as the major I chain proteolysis products at 15 kDa (solid arrow) and 12 kDa (open arrow) are indicated. Note the many small I chain-derived polypeptides (which migrate with the dye front) specifically associated with the material in peak I.

appearance of these chains in peak II (V_e, 15 ml). This is the exact elution position of affinity-purified $\alpha\beta$ heterodimers (12, 13). PAGE (2D) confirmed that peak II contains essentially pure DR α and β chains (Fig. 5*II*) and chemical cross-linking with dithiobis(succinimidylpropionate) demonstrated that these chains were physically associated as $\alpha\beta$ heterodimers (data not shown). The effect of the peptidyl-dipeptidase activity of cathepsin B on the DR β chain is readily apparent in this figure. Automatic integrating densitometry of the fluorograph demonstrated that the α and β chains were present in every fraction of peaks I and II in an equimolar ratio (actual estimate = 1.1 to 1, by assuming that the DR α and β chains have three and two labeled methionine residues, respectively; refs. 20 and 22). This analysis also revealed that 56% of all α chains and 52% of all β chains were present in peak II (see also Fig. 6). Fig. 4*B* and Fig. 5*III* shows that the material in peak III (V_e, 18 ml) contains the very acidic 15-kDa I chain fragment as well as small amounts of DR α and β chains. Peaks IV and V (V_e, 21 and 25 ml, respectively) contain small ethanol-soluble polypeptides, as very little [^{35}S]methionine-labeled material can be identified in the fluorograph of Fig. 4*B*. These results demonstrate that cathepsin B treatment of $\alpha\beta$I leads to the extensive proteolysis of the DR-associated I chain and the subsequent liberation of $\alpha\beta$ dimers that appear (by HPLC analysis) indistinguishable from cell surface $\alpha\beta$ dimers.

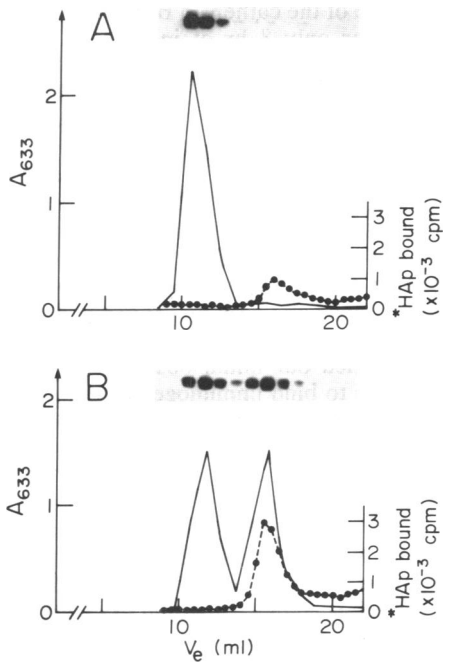

FIG. 6. Cathepsin B-induced $\alpha\beta$ dimers acquire peptide binding ability. Unlabeled $\alpha\beta$I complexes were treated in the absence (*A*) or presence (*B*) of cathepsin B (50 μg/ml) at 37°C for 2 hr prior to the addition of the cathepsin B inhibitor E-64 and the peptide *HAp (200 nM). After an additional 8-hr incubation at room temperature, DR-bound and free *HAp were separated by HPLC and the amount of *HAp present in each fraction was determined in a γ-counter (dashed line). Adjacent pairs of fractions were then precipitated with ethanol and analyzed by SDS/PAGE, and the amount of DR in each fraction was determined by immunoblot analysis with the class II β-chain-specific monoclonal antibody XD5.A11 (13). The amount of DR β chain present in each pair of fractions was then determined by automatic integrating densitometry and is shown as a function of V_e. A parallel immunoblot of these fractions using the DR-specific antiserum 247_{HSB} (13) showed an essentially identical elution profile for the DR α chain (data not shown). The β chain immunoblots used to generate the curves are shown above each chromatogram.

Binding of an Immunogenic Peptide to Cathepsin B-Treated $\alpha\beta$I. To determine if $\alpha\beta$ dimers released by cathepsin B treatment of $\alpha\beta$I were capable of binding immunogenic peptides, unlabeled $\alpha\beta$I was treated with cathepsin B for 2 hr and incubated with *HAp for an additional 8 hr, and DR-bound *HAp was separated from free *HAp by HPLC. Fig. 6*A* shows that untreated $\alpha\beta$I eluted as a single distinct peak and confirms our previous report that $\alpha\beta$I is unable to bind *HAp (13). The small peak of *HAp eluting at 15 ml corresponds to $\alpha\beta$ dimers spontaneously released from $\alpha\beta$I. This very small pool of material is generated during incubation of $\alpha\beta$I at room temperature and is essentially saturated with *HAp (13). As anticipated, Fig. 6*B* demonstrates that the $\alpha\beta$ dimers released from $\alpha\beta$I by treatment with cathepsin B were also capable of effectively binding this peptide. Nearly identical results were obtained using a radiolabeled peptide corresponding to residues 378–398 of the *Plasmodium falciparum* circumsporozoite protein (data not shown). Control experiments demonstrated that the inclusion of a 100-fold excess of unlabeled HAp or unlabeled circumsporozoite peptide to the incubation mixture completely inhibited *HAp binding, confirming that these two peptides were competing for the same binding site on the $\alpha\beta$ dimer (data not shown). Based on the densitometric scan showing the amount of class II β chains present in the two peaks, we estimate that

approximately 6% of the cathepsin B-induced $\alpha\beta$ dimers had bound *HAp after only 8 hr of incubation. This level of binding is comparable to the binding of *HAp to cell surface $\alpha\beta$ dimers at equilibrium (t = 60 hr; ref. 12). The inability of cathepsin B-induced $\alpha\beta$ dimers to completely saturate with *HAp in 8 hr may be due to nonideal reaction conditions or may be a consequence of either I chain- or cathepsin B-derived peptides competing with *HAp for binding. Nevertheless, these data suggest that removal of the I chain from $\alpha\beta$I by a cathepsin B-like proteinase generates class II $\alpha\beta$ dimers that are capable of binding immunogenic peptides.

Two other groups have directly examined the role of the I chain in peptide binding to class II molecules. Teyton *et al.* (23) have confirmed our initial observation that $\alpha\beta$I complexes are unable to bind immunogenic peptides by demonstrating that soluble I chain can inhibit peptide binding to purified DR. On the other hand, Viguier *et al.* (24) have proposed that the I chain does not inhibit peptide binding to class II molecules. In the latter study, however, the specificity of peptide binding was not addressed and the association of I chain with the class II α and β chains was not definitively shown. For these reasons, we believe the direct biochemical evidence suggesting that association with the I chain inhibits peptide binding to class II molecules.

The results of this investigation demonstrate that treatment of $\alpha\beta$I with the cysteine proteinase cathepsin B results in the relatively specific proteolysis of the DR-associated I chain. These data thus strengthen the hypothesis that cysteine proteinases with acidic pH optima are responsible for I chain degradation *in vivo* and that proteolysis most likely occurs in acidic endosome-like compartments. As a consequence of I chain proteolysis, $\alpha\beta$ dimers are released from the $\alpha\beta$I complex, which is capable of very effectively binding immunogenic peptides. These results also support the idea that association with the I chain inhibits peptide binding to immature DR molecules, thus preserving the functional distinction between class I and class II molecules (25). Exceptions to this generally observed dichotomy (26–28) may reflect competition of peptides for I chain binding to class II molecules in the early stages of the assembly of the $\alpha\beta$I complex.

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