Major Histocompatibility Complex Class II-dependent Unfolding, Transport, and Degradation of Endogenous Proteins*

(Received for publication, May 5, 1997, and in revised form, August 14, 1997)

Gerald Aichinger‡∗∗, Lars Karlsson‡, Michael R. Jackson‡, Mikael Vestberg‡, John H. Vaughan∗∗, Luc Teyton†, Robert I. Lechler‡, and Per A. Peterson‡

From the ‡R. W. Johnson Pharmaceutical Research Institute, San Diego, California 92121, the ∗Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, the †Department of Medicine, School of Medicine, University of California at San Diego, La Jolla, California 92039, and the ∗Department of Immunology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, W12 ONN London, United Kingdom

We have analyzed the ability of major histocompatibility (MHC) class II molecules to capture proteins in the biosynthetic pathway and whether this may be associated with MHC class II-dependent antigen processing. When coexpressed with HLA-DR 4 molecules in HeLa cells, influenza hemagglutinin was inhibited from folding and trimerization in the biosynthetic pathway, targeted to endosomal compartments, and rapidly degraded. Due to the interaction with MHC class II molecules, therefore, unfolded forms of hemagglutinin were bypassing the quality control mechanism of the secretory pathway. More important, however, the transport, endocytosis, and rapid degradation of unfolded hemagglutinin in the presence of MHC class II molecules suggest that proteins captured in the endoplasmic reticulum by class II molecules may become substrates for antigen processing and presentation to CD4-positive T cells. In insect cells we show that this phenomenon is not restricted to a few proteins such as hemagglutinin. A highly heterogeneous mixture of proteins from the endoplasmic reticulum including coexpressed hemagglutinin can form stable complexes with soluble HLA-DR α and β chains that were transported into the supernatant. This mechanism may gain biological significance in abnormal situations associated with accumulation of unfolded or malfolded proteins in the endoplasmic reticulum, for example during viral infections.

The function of MHC class II molecules is to present antigenic peptides derived from exogenous antigens to CD4-positive T cells. For MHC class II molecules to serve this function the invariant chain (Ii chain) has evolved as the key molecule that guides assembly of MHC class II α and β chains in the endoplasmic reticulum (ER) (1–3) and their transport to endo-

somal peptide loading compartments (4). At the same time, the Ii chain inhibits loading of peptides by MHC class II molecules in the ER (5, 6). Although antigen presentation and the selection of a functional CD4-positive T cell repertoire by MHC class II molecules are markedly impaired in Ii chain knockout mice (2, 7), mature MHC class II complexes can be generated in the absence of the Ii chain (8–10).

MHC class II molecules require occupancy of the peptide-binding groove for proper folding into a native conformation and transport to the cell surface (11, 12). Normally this requirement is met in the ER by binding of the Ii chain-class II-associated invariant chain peptide region in the peptide-binding groove of class II (13, 14). In contrast, when MHC class II α and β chains are expressed in Ii chain-negative cells they are largely retained in the ER (2, 7, 15). Nevertheless, dependent on the cell line and class II allele, a significant amount of MHC class II molecules can be expressed at the cell surface (3, 16, 17). ER-retained class II chains may be malfolded or associated with unfolded or partially folded proteins in the ER of Ii chain-negative cell lines (2, 18–20); however, transported class II molecules can serve as restriction elements for the presentation of an altered set of peptides to CD4-positive T cells (9, 10, 21).

Although the physiological ligands for mature MHC class II molecules are short peptides (22), it is possible that the initial interaction between class II molecules and peptides occurs while the peptide sequence is still part of a protein or polypeptide chain, and that protein-MHC class II complexes represent a substrate for antigen processing. The ability of MHC class II molecules to bind nonprocessed proteins has been described in different systems (8, 20, 23–27). MHC class II molecules expressed in the absence of the Ii chain were originally considered "empty" (28). However, Busch et al. demonstrated that MHC class II molecules can form allele-specific high molecular weight complexes with various proteins in Ii chain-deficient HeLa cells (20).

In our present report, we have addressed the question whether proteins, captured by HLA-DR4 molecules (DR) in the biosynthetic pathway, can become substrates for class II antigen processing. Our experiments were performed in the absence of the Ii chain because Ii chain inhibits protein binding in the ER (20). Influenza hemagglutinin (HA) was selected as a model protein for these studies for several reasons. HA has been well characterized as an immunogen (29), and many T cell epitopes, such as the immunodominant 306–318 epitope that binds to many different HLA-DR alleles including HLA-DR4 (30) have been defined. Both Ii chain-positive and -negative cell lines present endogenous HA to CD4-positive T cells, whether infected with live virus (9) or transfected with constructs for wild type or ER-retained mutants of HA (31). Presentation of
endogenous HA to CD4-positive T cells was inhibited by chloroquine, suggesting endosomal processing (31). Most important for our analysis, the folding pathway and intracellular transport of HA has been well characterized (32–35) and antibodies specific for different folding intermediates and native conformations were available (34). Folding, transport, and turnover of HA may be altered in MHC class II-expressing cells due to an association of newly synthesized partially unfolded HA with MHC class II molecules in the ER. Unfolded HA has been shown to be retained in the ER by the quality control mechanism (36, 37). However, unfolded HA may be excluded from this mechanism if bound by MHC class II molecules and transported out of the ER, similar to high molecular weight protein-MHC class II complexes, formed in the ER of Ii chain-to-endosomal processing of ER proteins. To study whether protein-MHC class II complexes, formed in the ER of Ii chain-negative cell lines and transported to the cell surface, represented a special case or were part of a more general phenomenon, we expressed soluble forms of HA and DR α and β chains in insect cells and analyzed proteins purified from the culture supernatant. Our results suggest that a direct association with class II molecules is responsible for the changes in the conformation and transport of HA observed in HeLa cells and that many endogenous proteins can be captured and transported out of the ER to endosomes in a similar way and become substrates for MHC class II processing and presentation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**HeLa cells were grown in DMEM (Life Technologies, Inc.) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 10% FCS (complete DMEM) (38) in the presence of 5% CO2 at 37 °C. Drosophila melanogaster SC-2 cells (39) were cultured in Schneider's Drosophila medium (Life Technologies, Inc.) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 7% FCS (all supplements from Life Technologies, Inc.) at 26 °C. Stable SC-2 cell transfectants, secreting truncated soluble HLA-DR αp heterodimers, soluble HA (sHA), complexes of HLA-DR αp with soluble HA or DR α chains were selected and maintained in the presence of a 500 μg/ml active concentration of Gentamicin (G418, Life Technologies, Inc. (40). Antibodies—Monoclonal antibodies, i.e. DA6.147 (41), L243 (42), LB3.1 (43), HB10a (44), 12CA5 (45), and anti-198 (46), were purified from ascites or culture supernatants. Antibodies to HA, 12CA5, anti-198, and rabbit antisera raised against native HA of influenza virus type A/PR8/34 [monoclonal antibodies (anti-HA), sHA (32, 34), or reacting with native HA only (anti-NHA)] or raised against SDS-denatured HA, F4 (34) were generous gift from Dr. I. Wilson, Dr. A. Helenius, Dr. M.-J. Gething, Dr. S. Wharton, and Dr. D. B. Thomas.

**cDNA Constructs—**Constructs coding for wild type and soluble influenza hemagglutinin type III from x31 strain (Hawt, sHA, or HA chain fragment) were cloned into the pCMU II expression vector (38) or pRMHA-3, containing the copper sulfate-inducible Drosophila metallothionein promotor (39). Briefly, a fragment between the bluntized unique ClaI site, 5' of the start codon, and the SalI site, downstream of the stop codon, containing the full-length coding sequence of HA (47), was cloned into pCMU II, opened with StuI and SalI. A similar fragment was cloned into pRMHA-3, opened with Accl and blunt, and cut with SalI. sHA in pCMU or pRMHA-3 was prepared by introducing a stop codon after the BamHI site of the extracellular domain of HA. Constructs coding for sHA with a C-terminal hexahistidine (6H) tag for purification by nickel affinity chromatography were prepared by ligating the cDNA coding for the extracellular portion of HA, into a modified pRMHA-3 plasmid, containing an oligonucleotide coding for a hexahistidine tag. Constructs for the soluble HA 1 fragment (Hawt, sHA, or HA chain fragment) were cloned into pCMU II or pRMHA-3 and religating the vector. A stop codon was introduced immediately downstream.

**DNA constructs for full-length or truncated soluble DR α*0404 and DR α chains were prepared by introducing a 5' NcoI and 3' BamHI cloning site by the polymerase chain reaction method. Full-length constructs were cloned into pCMU II for the expression in HeLa cells. To prepare secreted DR α and β chains, the transmembrane domains were deleted by fusing the C-terminal amino acid of the DR α2 domain or the connecting peptide of the DR β chain with the N-terminal amino acid of the DR α chain. This trimetric tail was attached to appropriate primers and polymerase chain reaction. DR α and β constructs with a C-terminal 6H were prepared by replacing the stop codon of the DR α or β cDNA with an in frame BglII site. These constructs were then ligated into the Drosophila expression vector, pRMHA-3 (39), modified with an oligonucleotide containing a 5' in frame BglII site, followed by the restriction enzyme sequence for sHA on the DR α chain, and religating the vector. The cDNA construct for soluble II chain modified with a N-terminal histidine tag was described elsewhere (6). All DNA constructs were sequenced using an automated DNA sequencer and fluorescent terminator method.

**Transient Transfections and Analysis of HeLa Cells—**HeLa cells were transiently transfected, using the calcium phosphate method as described (36), with cDNA constructs for human DR α and β *0404 chains and wild type or mutant HA, i.e. HAWt or sHA or HA1 as indicated.

**Indirect Immunofluorescence—**Transiently transfected HeLa cells were trypsinized and split 48 h after transfection onto coverslips coated with Cel-Tak (Collaborative Biomedical Research, Bedford, MA). After culturing the cells overnight, they were fixed in 4% paraformaldehyde-PBS for 20 min, treated with 50 mM NH4Cl for 10 min, permeabilized with 0.1% Nonidet P-40 for 30 min, and washed three times with 3% gelatin in PBS (blocking buffer). The cells were then stained with antibodies to DR α or β chains (DA6.147, L243 or HB10a) and rabbit antisera to HA (anti-HA specific for unfolded and folded HA) or F4 (specific for unfolded HA). Affinity-purified fluorescein isothiocyanate-labeled goat anti-rabbit or anti-mouse IgG sera (both from Cappel, West Chester, PA) were used as secondary reagents. The coverslips were washed with PBS and mounted on glass slides with Mowiol as a mounting medium (48).

**Flow Cytometry—**HeLa cell transfectants were harvested from tissue culture plates 72 h after transfection, using 5 mM EDTA in PBS. For staining, transfectants were washed twice with cold PBS containing 2% FCS and 0.04% sodium azide and then incubated with antibodies to DR α chains, L243, rabbit anti-HA serum (anti-HA), antibody to epitope B of the HLA DQ1 domain (anti-198), or rabbit antisera to SDS forms of HA (unfolded HA), F4. Fluorescein-labeled sheep anti-mouse IgG serum (Sigma) or goat anti-rabbit IgG serum (DAKO, Denmark) was used as secondary antibody. Stained cells were analyzed using a Coulter Epic XL flow cytometer. Viable cells were selected by gating on forward and side scatter.

**Native PAGE—**Cell lysates from HeLa cell transfectants were prepared 72 h after transfection in 1% Triton X-100 and a mixture of protease inhibitors (Complete™, Boehringer Mannheim). Lysates were spun at 14,000 rpm for 20 min, and equal aliquots were separated in parallel 6% native PAGE as described by Fourie et al. (49). Native PAGE was performed overnight at 4 °C and blotted onto polyvinylidene difluoride (Millipore, Bedford, MA). Parallel blots were stained with antibody 12CA5, antisera to native HA (anti-NHA), antiserum to denatured HA (F4), and antibody to DR α chains (DA6.147). Blots were developed by enhanced chemiluminescence (ECL, Amersham Corp.) and exposed on film.

**Pulse-chase Experiments and Immunoprecipitations—**Transfectants and untransfected control cells were labeled 72 h after transfection for 20 min in l-cysteine and l-methionine-free DMEM (ICN, Costa Mesa, CA) with 0.2 μCi of [35S]methionine (ICN, Costa Mesa, CA) per dish and lysed in 1 ml of 1% digitonin in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 mM EDTA (lysis buffer) or chased in the presence of complete DMEM for 2 h and then lysed in lysis buffer for 30 min. Cell lysates were spun in a microcentrifuge to remove cell nuclei and debris and precleared for 1 h with 0.1 ml of 10% protein A-Sepharose (Pharmacia Biotech Inc.), prewashed in lysis buffer. The lysates were then incubated for 1–4 h with antibodies to the DR α cytoplasmic tail, DA6.147, or rabbit antiseraum to HA (anti-HA). Immune complexes were precipitated with 30 min of 10% protein A-Sepharose, prewashed in lysis buffer. The precipitates were washed twice with 0.2% digitonin 50 mM Tris-HCl, 150 mM NaCl, twice with 0.2% digitonin, 50 mM Tris-HCl, 300 mM NaCl; once again in 0.2% digitonin 50 mM Tris-HCl, 150 mM NaCl; and finally washed in 50 mM Tris-HCl, 150 mM NaCl. All fluid was removed from the precipitates after the final wash, and the precipitates were resuspended in sample buffer. The samples were boiled for 5 min and loaded onto a 10–15% gradient gel. The gel was dried, and the bands were visualized by autoradiography.
Stable SC-2 Transfectants. Purification of Soluble Molecules from the Culture Supernatant, and Analysis—Appropriate cDNA constructs in the Drosophila expression vector pRMHA-3 were cotransfected with a plasmid containing a neomycin resistance gene, pUCHneo (50) into SC-2 cells, using the calcium phosphate technique (38). Stable transfected SC-2 cells were selected and maintained in the presence of 500 μg/ml active concentration of Geneticin (G418) as described (40). For large scale protein purification from culture supernatants, stable SC-2 transfectants were expanded up to several liters in complete Schneider's Drosophila medium (Life Technologies) or serum free Insect X-Press medium (BioWhittaker, Walkersville, MD).

As-HA complexes (the β chain modified with a histidine tag, DR αβ-6H, soluble HA with a histidine tag (HA-6H), and complexes of soluble DR4 with soluble HA (either DR β chains or soluble HA were modified with a histidine tag, HA-DR αβ-6H or DR αβ-6H, respectively) or soluble HLA-DR αβ-HA chain complexes (the β chain was modified with a histidine tag, DR αβ-6H) were purified from the supernatant of stable SC-2 transfectants. For that purpose, transfected proteins were expanded in serum-free Insect X-Press medium (BioWhittaker) or complete Schneider's Drosophila medium (Life Technologies). Cell cultures were grown up to several liters in roller bottles or tissue culture flasks. Protein synthesis was induced with 1 mM copper sulfate, and the supernatant was harvested after 72 h, 10-fold concentrated, dialyzed against PBS to remove copper sulfate, and loaded onto a nickel-nitrotetrazolium blue resin (Qiagen, Chatworth, CA). The column was washed with PBS and 20 mM imidazole and eluted with 100 mM imidazole in PBS, pH 7.5. Further purification included anion exchange chromatography using a MonoQ HR 5/5 column (Pharmacia), hydrophobic interaction chromatography using a phenyl-Superose HR 5/5 column (Pharmacia), and size exclusion chromatography using a Superose 6 or Superdex 200 column (Pharmacia) and a Pharmacia FPLC system. Chromatography was performed according to the recommendations by Pharmacia. Briefly, material purified from the nickel affinity column was dialyzed against the loading buffer for anion exchange or hydrophobic interaction chromatography or concentrated for size exclusion chromatography. Proteins were loaded onto the MonoQ column in 50 mM Tris-HCl, pH 8.0, and eluted with a 1 M NaCl in 50 mM Tris-HCl, pH 8.0. For hydrophobic interaction chromatography on a phenyl-Superose column, 1 M ammonium sulfate in 20 mM sodium phosphate buffer, pH 7.4, was used as a loading buffer, and 20 mM sodium phosphate buffer, pH 7.4, was used for elution. The size exclusion chromatography was performed in PBS at 0.3 to 0.5 ml/min. The protein concentration was monitored at 280 nm. Protein purification was monitored after each step by SDS-PAGE using 12.5% gels and Coomassie Blue staining or Western blotting or by an ELISA for HLA-DR αβ complexes.

ELISA for MHC Class II—Flat bottom 96-well plates (Corning, Cambridge, MA) were coated with 5 μg/ml purified antibody DA6.147 specific for the cytoplasmic tail of the DR α chain. Antibody-coated plates were washed with 0.02% Tween 20 in PBS (wash buffer) and incubated with 200 μl of 5% FCS, 0.1% bovine serum albumin, and 0.1% gelatin in PBS for 30 min at room temperature. Samples of 100 μl of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 mM cysteine, 5 mM g/ml of preactivated papain in 10 M
MHC Class II-dependent Transport and Degradation of Proteins

FIG. 1. Folding of HA is inhibited by coexpressed MHC class II molecules, and unfolded HA is transported to the cell surface. Panel A shows the subcellular distribution of HA in the absence (a) or presence (c) of coexpressed DR or chains in transfected HeLa cells. The expression of total HA (unfolded and folded forms of HA; a and c) is compared with the expression of unfolded forms of HA in the absence or presence of DR (a and b versus c and d). Transfectants were grown on coverslips overnight and then fixed with 4% formaldehyde and stained with conformation-independent anti-HA serum (a and c) or F4 (specific for unfolded HA) (b and d). Panel B compares the cell surface expression of folded or unfolded forms of wild type or soluble HA (HAWt or sHA) in the absence or presence of DR and chains, as analyzed by flow cytometry (no DR versus +DR, a-f versus g-l). HeLa cells transfected with constructs for wild type or soluble HA with or without DR were stained with a conformation-sensitive antibody to epitope B of the HA1 domain (anti-198; a, d, g, and j), F4 (specific for unfolded HA (b, e, h, and k) or L243 (specific for DR aβ dimers (c, f, i, and l)). Parts a-f (no DR) were overlaid with parts g-l (+DR) to illustrate the DR-dependent change of the expression of native or unfolded HA at the cell surface (+/−DR overlay). Changes of MFI are given under “Results.”

1 and 2), antiserum to native HA, anti-NHA (lanes 3 and 4), antiserum to unfolded HA, F4 (lanes 5 and 6), and anti-DR α chain antibodies, DA6.147 (lanes 7 and 8). HeLa cells transfected with HA alone are shown in lanes 1, 3, 5, and 7, cotransfected with HA and DR in lanes 2, 4, 6, and 8. Consistent with our hypothesis, 12CA5 recognized HA only in the presence (lane 2) but not the absence of DR (lane 1). In contrast, native HA was detected with anti-NHA serum in cell lysates of DR-negative (lane 3) but not DR-expressing transfectants (lane 4), suggesting that binding of DR to HA interfered with the formation of HA trimers. The level of unfolded forms of HA in cell lysates stained with F4 was not significantly different whether DR was present or not (lane 6 versus lane 5), suggesting that coexpression of DR did not affect expression of HA. Unfolding of HA in the presence of DR was not associated with significant aggregation because the amounts of HA that did not enter the stacking or resolving PAGE was not different whether DR was coexpressed or not (lane 6 versus lane 5). The expression level of DR in HA-DR cotransfected cells is shown in lane 8 as stained with DA6.147. Cell lysates of transfectants expressing HA but no DR were negative with DA6.147 (lane 7).

These results indicate that MHC class II molecules interfere with folding and trimerization of HA monomers. Previous analysis of the biosynthetic pathway of wild type and folding mutants of HA demonstrated that unfolded or malfolded HA was retained in the ER by the quality control mechanism of the secretory pathway (36, 53, 54). Here, however, we demonstrated that unfolded forms of membrane-bound or soluble HA were displayed at the cell surface in an MHC class II-dependent manner, suggesting that binding of unfolded HA to MHC class II molecules can bypass the quality control mechanism. Additional coexpression of HLA-A2 with HA (instead of DR) did not alter cell surface levels of native HA, suggesting that overexpression of a second protein did not interfere with the expression and maturation of HA (not shown).

MHC Class II Molecules Enhance the Proteolysis of Coexpressed Proteins—Both denaturation of HA in the ER and transport of unfolded HA to the cell surface may enhance degradation of HA. To analyze the turnover of HA, pulse-chase experiments were performed in transfected HeLa cells (Fig. 3). HeLa cells, transiently transfected with constructs for HAWt or soluble HA1, with or without constructs for DR α and β chains, or transfectants expressing DR but no HA are shown in a, b, and c, respectively. HA (top parts) and DR (bottom parts) were immunoprecipitated after the pulse (lanes 1, 3, and 5) and 2-h chase period (lanes 2, 4, and 6) with an antiserum to HA, reactive with unfolded and native HA (αHA (34), lanes 1 and 2), or monoclonal antibody DA6.147 (41) (lanes 3 and 4) followed by αHA (lanes 5 and 6). When expressed on its own, HA was transported out of the ER during the chase period. This is
indicated by the reduced mobility in SDS-PAGE of terminally glycosylated HA (b in lane 2; compare with a in lane 1) (55). In contrast, when HA (either HAwt or HA1) was coexpressed with class II molecules, HA was present after the pulse (lane 5) but not the chase period (lane 6). Lanes 3 and 4 show proteins coprecipitating with DR α and β chains. However, protein-bands corresponding to ER or glycosylated forms of HA could not be identified above the background of many other coprecipitating protein-bands (compare with lanes 1 and 2). As expected, the half-life of DR was not changed whether HA was present in the transfection or not (bottom parts, compare lanes 3 and 4). Only a small portion of DR was transported out of the ER during the chase period, as suggested by the lack of carbohydrate modifications of the major portion of DR α and β chains. This has been reported previously (2, 7, 20). Loss of immunoprecipitable HA during the chase period may be the result of protein degradation or aggregation. No significant aggregation was noticed on top of or in the stacking gel of the native PAGE in Fig. 2.

**MHC Class II-dependent Protein Degradation Occurs in an Endosomal Compartment**—Although the ER may contribute to MHC class II-dependent degradation of HA, the presence of unfolded forms of HA at the cell surface in class II-expressing cells suggested that a significant part of the degradation could occur in endosomal or lysosomal compartments under these conditions. Chloroquine has been shown to interfere with endosomal functions, including proteolysis of MHC class II-associated invariant chain (56, 57). To test whether HA was transported into a vesicular compartment in a DR-dependent manner, HeLa cells, transfected with constructs for HA with or without constructs for DR α and β chains, were cultured overnight in the presence of 25 μM chloroquine. Fig. 4 shows the immunofluorescence staining pattern of transfectants stained with a conformation-independent antiserum specific for HA (α and c) or with HB10a, specific for DR β chains (b and d). In the absence of DR, HA staining was largely confined to the cell surface, although some vesicular staining could be detected (a). This subcellular distribution is expected, since HA itself lacks endosomal targeting information (58). In contrast, in the presence of DR, vesicular staining for HA was prominent in addition to ER staining (c). Vesicular staining was also observed for DR α and β chains whether HA was coexpressed (d) or not (b), consistent with recent evidence for an endosomal targeting motif in the cytoplasmic tail of the DR β chain (59). The staining pattern of HeLa cells transfected with HA with or without DR in the absence of chloroquine is shown in Fig. 1A. No significant vesicular staining for HA was observed in the absence of chloroquine whether DR was present or not. Taken together, these results suggest that a substantial amount of MHC class II-dependent degradation of HA takes place in endosomal compartments.

**Soluble DR αβ Chains Associate with a Highly Heterogeneous Mixture of Endogenous Proteins Including Coexpressed HA in Insect Cells**—To analyze whether complexes of HA with DR represent a special case or were part of a more general phenomenon, we purified and analyzed soluble DR αβ complexes expressed in *Drosophila* SC-2 cells and secreted into the supernatant. Constructs for sDR α and β (∗0404) chains and sHA with or without C-terminal histidine tags and constructs for soluble Ii chain modified with an N-terminal histidine tag (6) were expressed individually or coexpressed in *Drosophila* SC-2 cells. When expressed together, only one of the chains contained a histidine tag. This expression system, combined with the addition of histidine tags on one of the cotransfected chains (sDR β, sHA, or Ii chain), allowed purification of large amounts of proteins using nickel affinity chromatography under neutral conditions, without acid or alkaline elution steps, previously used for affinity purification of MHC class II molecules (28, 60). This was important, since soluble MHC class II molecules purified from insect cells under neutral conditions aggregated and precipitated when exposed to acid or alkaline pH, due to their interaction with multiple unfolded proteins (see below; Fig. 5). In contrast, peptide-loaded or “empty” MHC class II molecules purified from an affinity column at pH 11.5 were stable over a wide pH range. Fig. 5 shows soluble DR αβ complexes eluted in multiple peak fractions together with many other proteins from anion exchange column (peak fractions 6–8, 11, 12, and 18–20 in A and B). Large amounts of DR αβ complexes, reactive with conformation-sensitive antibodies to DR α chains (L243 (C) or LB3.1 (not shown)) or an antibody to DR β chains (HB10a, not shown) were detectable along the salt gradient by ELISA (C). Similarly, high molecular weight complexes separated by size exclusion chromatography (D and E) contained SDS-stable and -unstable DR αβ complexes identified in Western blots stained with HB10a and DA6.147 (D) and were also reactive with L243 in ELISA (F). In contrast to peptide-loaded or “empty” MHC class II molecules, high molecular weight sDR aggregated and precipitated if exposed to acid pH as shown by the loss of high molecular weight L243-reactive DR αβ complexes at pH 5.5 in ELISA (F). As expected, purified complexes of sIi-6H with sDR did not bind proteins and were homogenous by charge and size (G and H, respectively).

**Protein-DR Complexes Were Revealed by Limited Proteolysis with Papain**—MHC class II molecules have been shown to resist limited proteolysis with papain (28), whereas unfolded proteins complexed with DR αβ dimers would be predicted to be highly sensitive to proteolysis. Fig. 6 shows our results of limited protease digestion of soluble DR αβ complexes with papain analyzed by SDS-PAGE and Coomassie Blue staining (a and b) and parallel Western blots stained for DR α and β chains.

Figure 2. Trimer Formation of HA Is Inhibited in the Presence of MHC Class II Molecules. Cell lysates from HeLa cells transfected with constructs for wild-type HA (HAwt) without or with constructs for DR α and β chains (lanes 1, 3, 5, and 7 versus lanes 2, 4, 6, and 8, respectively) were analyzed in 6% native PAGE, blotted onto poly(vinylidene difluoride) membrane, and stained with conformation-sensitive antibodies to HA, 12CA5 (HA monomers, lanes 1 and 2), anti-NHA (native HA, lanes 3 and 4), F4 (unfolded HA, lanes 5 and 6), or DA6.147 (DR α chains, lanes 7 and 8). Horseradish peroxidase-labeled antibodies to mouse or rabbit IgG (Bio-Rad) were used as secondary reagents, and blots were developed by enhanced chemiluminescence (ECL kit, Amersham). The top of the stacking PAGE is indicated by an arrow.
Table 1: SDS-PAGE Analysis of Membrane-Associated and Secreted Proteins.

| Protein | Membrane-Associated | Secreted |
|---------|---------------------|----------|
| HA      | Yes                 | Yes      |
| DR      | Yes                 | No       |
| Membrane-Associated | Lane 1 | Lane 2 |
| Secreted | Lane 3 | Lane 4 |
| HA      | Yes                | Yes      |
| DR      | No                 | No       |

(c and d). Boiled samples are shown in a and c, and nonboiled samples are shown in b and d. SDS-stable protein-DR complexes were undetectable in nonboiled samples in Coomassie-stained gels (lane 1 in b; compare with boiled samples, a). However, a smear of protein bands in untreated nonboiled samples (lane 1 in d) reacted with antibodies to DR α and β chains in parallel Western blots (compare with c). Limited proteolysis of class II bound proteins with papain uncovered SDS-stable protein-class II complexes of decreasing size in DR αβ bands (lanes 2–5 in d; compare with c) and Coomassie-stained gels (lanes 2–5 in b; compare with a). The relative resistance of DR αβ chains to proteolysis with papain suggests that DR were properly folded.

Complexes of Soluble DR with Soluble HA Are Secreted by Transfected Insect Cells—Purified proteins secreted by stable SC-2 transfected expressing either HA alone or HA with DR were analyzed in parallel SDS gels and Western blots (Fig. 7). One of the cotransfected chains (HA in A or DR β in B) was modified with a histidine tag for purification by nickel affinity chromatography. Fig. 7A shows purified proteins from transfected expressing HA-6H alone (lane 1) or with DR (lane 2). Parallel blots were stained with 12CA5 (lanes 1 and 2) or DA6.147 and HB10a (lanes 3 and 4). sHA monomers (70 kDa), multimers that did not dissociate in the presence of 25 μM chloroquine were fixed and stained for HA (a and c) or DR β chains (b and d) by indirect immunofluorescence. Rabbit anti-HA serum was used for HA staining (a and c); HB10a (DR β chains) was used for DR staining (b and d). Immunofluorescence staining for HA in the absence of chloroquine is shown in Fig. 1A.
FIG. 5. Highly heterogeneous soluble protein-DR αβ complexes form in insect cells. Soluble DR αβ or β2m chain-DR αβ complexes expressed in SC-2 cells were purified from the culture supernatant by nickel affinity chromatography, ion exchange (MonoQ column; A–C and G), or size exclusion chromatography (Superose 6 column, D, E, and F; Superdex 200 column, H). Protein elution profiles are shown as absorption at 280 nm (Abs. 280 nm; B, E, G, and H). Fractions from the MonoQ or Superose 6 column were analyzed by SDS-PAGE stained with Coomassie Blue (A), Western blotting and staining with DA6.147 and HB10a (D), or sandwich ELISA for class II αβ dimers (C and F). Purified soluble DR αβ complexes exposed to acid pH for 10 min (pH 5.5) or left untreated (pH 7.5) were separated by size exclusion chromatography on a Superose 6 column and analyzed by ELISA (F). The positions of bands, corresponding to DR α and β chain (α, β) and low range size markers (14 kDa, lysozyme; 21 kDa, trypsin inhibitor; 31 kDa, carbonic anhydrase; 45 kDa, ovalbumin; 66 kDa, serum albumin; and 97 kDa, phosphorylase b; Bio-Rad) are shown (M). The elution volume of albumin (66 kDa) in size exclusion chromatography is indicated in E, F, and H.
Papain (μg/ml)

|       | 0   | 10  | 3   | 1   | 0.3 |
|-------|-----|-----|-----|-----|-----|
| **boiled** | 1   | 2   | 3   | 4   | 5   |
| a     |     |     |     |     |     |
| b     |     |     |     |     |     |
| **non-boiled** | 1   | 2   | 3   | 4   | 5   |
| a     |     |     |     |     |     |
| b     |     |     |     |     |     |

FIG. 6. Soluble DR αβ dimers but not bound proteins resist limited protease digestion with papain. Purified soluble DR was treated with different concentrations of papain as indicated (lanes 2–5) or left untreated (lane 1). Boiled (α and c) and nonboiled samples (b and d) were separated on parallel SDS-PAGE, stained with Coomassie Blue (α and b), or Western blotted and stained with antibodies to DR-α and β chains (DA6.147 and HB10a; c and d). Protein bands corresponding to DR α or β monomers (α, β), DR αβ dimers (αβ dimers), high molecular weight class II complexes (DR-C), proteolytic fragments derived from all proteins (PF), or DR (PFDR) are shown.

stained with HB10a (lanes 1 and 2) or 12CA5 (lanes 3 and 4). Multiple SDS-stable protein-class II complexes (DR-C) were visible in nonboiled samples (lane 2; compare with lane 1). Consistent with the requirement for partial unfolding for binding to MHC class II molecules, HA monomers (HA), but not multimers, were associated with sDR-6H (lanes 3 and 4). The absence of copurified HA multimers and similar amounts of sHA in boiled versus nonboiled samples (lane 4 versus lane 3) suggest that copurified HA monomers were not released from aggregates. Copurified sHA did not change its mobility in nonboiled versus boiled samples, suggesting that SDS-stable DR αβ complexes were not formed with full-length sHA. It is worth noting that several SDS-stable protein-class II complexes reacted with different anti-HA antibodies in parallel Western blots, suggesting that they contain fragments of sHA (not shown).

Several conclusions can be drawn from these results with insect cell-derived sDR. First, similar to Busch et al. (20) in HeLa cells, we demonstrated endogenously formed stable protein MHC class II complexes and their transport and secretion into the culture supernatant of insect cells. Second, we show that protein binding can involve a large number of properly folded MHC class II αβ dimers binding a highly heterogeneous mixture of proteins. Coexpressed sHA monomers represented a small fraction of this mixture. Third, we show that SDS-stable protein-class II complexes were undetectable in this mixture unless uncovered by charge or size fractionation or protease treatment. Thus, the absence of visible SDS-stable class II complexes in our situation reflected occupancy of the peptide-binding groove with heterogeneous proteins. Fourth, the mutually exclusive nature of Ii chain-versus protein-class II binding is demonstrated by the homogenous size and charge of Ii chain-class II complexes.

DISCUSSION

It is well established that endogenously synthesized proteins can be presented as peptides by MHC class II molecules (9, 22, 31, 61–63), although the classical MHC class II presentation pathway favors the presentation of exogenous antigens. The data presented here provide direct evidence for the involvement of MHC class II molecules in the capturing of endogenous proteins in the biosynthetic pathway leading to their transport to endosomal compartments and subsequent processing. The ability of MHC class II molecules to bind nonprocessed proteins has been demonstrated by several laboratories (8, 20, 23–27). Peptides bound in the peptide-binding groove of MHC class II molecules can extend out of the groove at both ends (22, 64, 65) and can be part of an exposed flexible loop of a native protein such as fibrinogen (23) or partially denatured proteins (24, 25, 27). No physiological role has been attributed to protein binding by MHC class II molecules, but it has been speculated that peptides may be captured by MHC class II molecules when part of the nonprocessed antigen, protecting it from degradation during subsequent processing (66–68). Peptides or proteins may be loaded in one compartment but processed and trimmed in another compartment of the cell. Lindner et al. (27) recently demonstrated that partially denatured hen egg white lysozyme (HEL) can be captured at the cell surface by mature I-A<sup>k</sup> molecules. SDS-unstable HEL-I-A<sup>k</sup> complexes formed at the cell surface were endocytosed, processed, and converted into SDS-stable I-A<sup>k</sup> αβ dimers loaded with HEL peptides of...
different length. HEL-I-A\(^k\) complexes were then presented to CD4-positive T cells after recycling to the cell surface. Proteins may be captured not only in endosomes (69) and at the cell surface (27) but also in the ER (20). High molecular weight protein-MHC class II complexes formed in the ER of HeLa cells have previously been shown to be long lived and transported out of the ER at a very slow rate (20). Compared with the rapid li chain-dependent transport of MHC class II \(\alpha\beta\) dimers out of the ER, this pathway appeared to be rather inefficient. Functional experiments showed, however, that ER-retained proteins, such as HA and other proteins modified with ER retention motifs, were efficiently presented to CD4-positive T cells (31, 61, 62).

In the present study, we found that while assembly and intracellular transport of DR molecules were not affected by the presence of HA, the folding and assembly of HA were distinctly altered by the coexpression of DR. Cell surface expression of native HA was significantly reduced (more than 50%), while folding and trimerization was inhibited due to the association with DR. Furthermore, the association of DR with HA monomers inhibited the formation of trimers and cell surface expression of native HA. This was paralleled by the appearance of significant amounts of unfolded HA at the cell surface in a class II-dependent manner, suggesting a direct involvement of DR in the transport of unfolded HA to the cell surface. This was unexpected, since unfolded proteins are normally retained in the ER by the "quality control" mechanism of the secretory pathway (36, 54) and degraded by ER degradation (70–72), a process that is now believed to occur in the cytoplasm in a proteasome-dependent manner (72, 73). However, our results show that unfolded proteins can be excluded from the quality control mechanism of the secretory pathway and exit the ER if associated with MHC class II molecules.

Not only did the coexpression of DR mediate the transport of unfolded HA to the cell surface, but it also led to rapid degradation of HA as shown in pulse-chase experiments. Although some degradation of HA may occur in the ER, the significant portion of unfolded HA transported to the cell surface and vesicular compartments, as visible after treatment with chloroquine, suggested that part of the degradation occurred in endosomes. HA is not endocytosed on its own, as previously shown (58), suggesting that unfolded forms of HA were endocytosed as a complex with DR similar to what has been reported to occur with partially unfolded HEL (27). These experiments suggest that for some endogenous proteins such as HA a minor but efficient MHC class II-dependent pathway exists that transports unfolded proteins out of the ER to endosomal compartments, implying that endogenously synthesized HA may become a substrate for endosomal antigen processing and presentation by MHC class II molecules.

The binding of the partially folded protein by MHC class II molecules in the ER is likely to involve exposed sequences that may preferentially be presented following proteolytic digestion of the unprotected regions of the protein in endosomes. This could lead to the presentation of a quantitatively or qualitatively different set of peptide epitopes from those presented when the same proteins are processed by the conventional endocytic pathway. Consistent with this hypothesis, alloreactive T cell clones raised against HLA-DR1 in the absence of the li chain were poor responders to DR1 when coexpressed with the li chain (21).

The experiments using MHC class II molecules expressed in insect cells demonstrated that the majority of soluble DR formed high molecular weight protein-DR complexes and did not represent "empty" MHC class II molecules. The large heterogeneity of complexes (as shown by ion exchange or size exclusion chromatography) was due to the large mixture of proteins associated with sDR. sDR reacted with conformation-sensitive antibodies to HLA-DR molecules and was resistant to proteolysis, consistent with it having acquired a native conformation, whereas associated proteins were highly sensitive to limited proteolysis with papain. In addition, sDR formed many SDS-stable complexes of different sizes that became apparent only after separating the complex mixture by charge or size or after limited proteolysis with papain. Our results therefore suggest that the ability of MHC class II molecules to bind and transport unfolded proteins is not restricted to a limited set of ER proteins.

A key question is whether this pathway is operative in normal cells in vivo. Protein-DR complexes were not detected in HeLa cell transfectants coexpressing DR and li chain (20). T cells, however, often require no more than 1–200 peptide-MHC molecules/antigen-presenting cell (74). Thus, the described mechanism could be active below the limits of detection by gel analysis. More importantly, we argue that in circumstances where high levels of unfolded or malfolded proteins accumulate in the ER of MHC class II expressing cells, such as may occur in virus-infected cells, this pathway may gain functional significance for the presentation of endogenous antigens to CD4-positive T cells. This hypothesis is supported by experiments with li chain-positive antigen-presenting cells transfected with ER-retained forms of HEL or HA, showing that ER-retained proteins were presented to CD4-positive T cells only if expression levels were high but not if they were low (31, 62). In addition, it is tempting to consider autoimmunity as a possible outcome of this mechanism. Most interestingly in this context, in patients with Graves’ disease T cells were found that responded to cryptic epitopes of thyroid peroxidase if it was presented as an endogenous protein by thyroid epithelial cells but not if it was added as an exogenous antigen to antigen-presenting cells (63).

These examples suggest that mechanisms of endogenous antigen presentation by MHC class II molecules, distinct from the classical endosomal pathway, are biologically important particularly in abnormal or pathologic situations.

Acknowledgments—We are grateful to Dr. M.-J. Gething and Dr. A. Helenius for providing constructs for wild type and soluble influenza hemagglutinin; Dr. M.-J. Gething, Dr. A. Helenius, Dr. I.A. Wilson, and Dr. D.B. Thomas for antibodies to influenza hemagglutinin; and Dr. M. Liljestad and Dr. Anne Fourie for discussion.

REFERENCES
1. Kvist, S., Wiman, K., Claesson, L., Peterson, P., and Dobberstein, B. (1982) Cell 29, 61–69
2. Bikoff, E. K., Huang, L.-Y., Episkopou, V., Van Meerwijk, J., Germain, R. N., and Robertson, E. J. (1993) J. Exp. Med. 177, 1699–1712
3. Bikoff, E. K., Germain, R. N., and Robertson, E. J. (1995) Immunity 2, 301–310
4. Lotteau, V., Teyton, L., Peleriaux, A., Nilsson, T., Karlsson, L., Schmid, S. L., Quaranta, V., and Peterson, A. P. (1990) Nature 348, 600–605
5. Roche, P. A. and Cresswell, P. (1990) Nature 345, 615–618
6. Teyton, L., O’Sullivan, D., Dickson, P. W., Lotteau, V., Sette, A., Fink, P., and Peterson, P. A. (1990) Nature 348, 39–44
7. Viville, S., Neefjes, J., Lotteau, V., Dierich, A., Lemeur, M., Ploegh, H., Benoist, C., and Mathis, D. (1993) Cell 72, 635–648
8. Anderson, M. S., Swier, K., Arnesson, L., and Miller, J. (1993) J. Exp. Med. 178, 1599–1699
9. Dolfi, A. I., Brett, S., Nordeng, S., Siddhu, S., Batchelor, R. J., Lombardi, G., Nakke, O., and Lechler, R. I. (1984) Eur. J. Immunol. 14, 1623–1629
10. Bodmer, H., Viville, S., Benoist, C., and Mathis, D. (1984) Science 223, 1258–1268
11. Sadegh-Nasseri, S. and Germain, R. N. (1991) Nature 353, 167–170
12. Germain, R. N., and Rinderer, A. G. J. (1991) Nature 353, 725–728
13. Ghosh, P., Amaya, M., Mellins, E., and Wiley, D. C. (1995) Nature 378, 457–464
14. Cresswell, P. (1996) Cell 84, 505–507
15. Claesson-Welsh, L., and Peterson, P. A. (1985) J. Exp. Med. 159, 3551–3557
16. Miller, J., and Germain, R. N. (1985) J. Exp. Med. 164, 1478–1489
17. Sekaly, R. P., Tonnelle, C., Stubin, M., Mach, B., and Long, E. O. (1986) J. Exp. Med. 164, 1490–1594
18. Bonnerot, C., Marks, M. S., Cosson, P., Robertson, E. J., Bikoff, E. K., Germain, R. N., and Bonifacino, J. S. (1994) EMBO J. 13, 934–944
19. Marks, M. S., Germain, R. N., and Bonifacino, J. S. (1995) J. Biol. Chem. 270, 29135
