Major histocompatibility complex (MHC) class II molecules are present at the plasma membrane of antigen-presenting cells and consist of a dimer of an α and a β chain (1). MHC class II molecules present antigenic peptides to CD4-positive T cells, and these peptides are usually derived from antigens that are internalized and processed in the endosomal/lysosomal pathway (2, 3). Directly after translation and insertion into the endoplasmic reticulum membrane, the αβ dimers associate with a third molecule, the invariant chain (Ii). Ii is known to trimerize, and three αβ dimers associate with these trimers to form a nonameric complex (4, 5). The major form of Ii consists of a 33-kDa form (6); an additional form of Ii, IiP41, arises through alternative splicing and contains an additional exon encoding 64 amino acid residues at the C-terminal end of the molecule (7, 8). In addition, in human cells, an alternative initiation site upstream gives rise to a 35- and a 43-kDa form, which are retained in the endoplasmic reticulum due to the presence of an endoplasmic reticulum retention signal (9). These multiple forms of Ii are usually co-expressed within the same cell and associate with MHC class II molecules (1).

The invariant chain is known to regulate the range of peptides that can be presented by MHC class II molecules in various ways. First, it assists in the folding and export of MHC class II molecules in the endoplasmic reticulum (10). Second, Ii provides a targeting signal for endosomal/lysosomal compartments (9, 11, 12). This targeting signal directs MHC class II/Ii complexes to so-called MHC class II compartments, specialized organelles of the endosomal/lysosomal pathway that are present in antigen-presenting cells (13). In these organelles, the associated Ii is degraded from its lumenal, C-terminal side, thus liberating the peptide binding site in the class II complex (17, 22). Degradation of Ii is most probably carried out by endosomal cathepsins (14, 15) and results in the generation of several Ii fragments. These fragments retain the cytoplasmic, N-terminal portion, but lack different segments of the lumenal, C-terminal domain (16, 17). After Ii degradation, antigenic peptides can be loaded onto the class II dimer, a process that may be assisted by the recently described HLA-DM molecules (18–21). From the MHC class II compartments, peptide loaded class II dimers are transported to the plasma membrane for triggering T cell receptors of CD4+ T cells (20, 22, 23).

As MHC class II molecules present peptides derived from antigens that are usually internalized into the endocytic pathway of antigen-presenting cells, they should be prevented from binding peptides at early stages after biosynthesis. Indeed, a third function of Ii is to prevent peptide association with class II molecules. This function is carried out by the C-terminal, luminal portion of Ii, precisely that part that is degraded upon arrival in endocytic structures prior to peptide loading (1, 16, 17, 22, 24, 25). The region that is responsible for preventing peptide association with MHC class II molecules has been mapped to the membrane-proximal domain of the invariant chain (26–28). These fragments are termed CLIPs (for class II-associated invariant chain peptides). Although a core fragment including methionines 99 and 104 has been shown to bind class II dimers in a manner similar to antigenic peptides (29), the precise Ii fragments binding to class II molecules vary considerably and depend partially on the class II haplotype (30, 31).

In addition to the Ii fragments generated at endosomal sites, in both human and murine cell types, other Ii proteolytic fragments have been observed (25, 32, 33). Presently, it is unknown where in the cell these fragments are generated and what the physiological relevance is of the generation and presence of these fragments. In this paper, we describe the expression, intracellular transport, location, as well as the biological activity of an invariant chain fragment, IiP25, that was associated with MHC class II molecules in the endoplasmic reticulum of...
human melanoma cells. IiP25 included part of the CLIP region but lacked the Ii N-terminal domain responsible for targeting to endocytic organelles. In accordance with such a sequence, IiP25 was not targeted to endocytic compartments but was secreted into the culture medium. Furthermore, IiP25 was biologically active, in that its presence inhibited T cell activation by MHC class II molecules. We propose that the secretion of a luminal Ii fragment extracellularly may contribute to the regulation of class II-restricted T cell responses by competing with antigenic peptides for MHC class II molecules.

**EXPERIMENTAL PROCEDURES**

**Cells, Viruses and Antibodies**—The human melanoma cell line Mel JuSo (34) was maintained in RPMI 1640 medium supplemented with 5% fetal calf serum. Chinese hamster ovary cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The following antibodies were used: monoclonal antibody L243, a kind gift from Dr. T. Johnson; anti-MHC class II rabbit antiserum, a kind gift of Dr. H. L. Ploegh; anti-Ii rabbit antisemur MDDQ, raised against the N-terminal fragment of Ii and monoclonal antibodies Bu43 and Bu45, recognizing the Ii C-terminal domain, a kind gift from Dr. I. McLennon. The clonal tissue culture insect cell line Spodoptera frugiparda line High Five (derived from *S. frugiparda*—cgcgcatgcaggcgctg-3') and 5'-aacgcatgccatttgtgggaatgac-3') and EcoRI and BamHI fragments and ligated into the eukaryotic expression vector pVL1392 digested with the same enzymes. For the generation of recombinant AcNPV, an IiP25 fragment from IiP25pGEM was cloned into the vector pVL1392 digested with the same enzymes. The 89–101 myelin basic protein (MBP)-specific T cell hybridoma 12.3 was a kind gift from Dr. D. Wraith (Cambridge, United Kingdom). B and T cell hybridomas were grown in Iscove’s modified Dulbecco’s medium supplemented with 5% fetal calf serum.

**Plasmid Constructions**—cDNA encoding IiP25 was constructed by cloning the signal sequence of hemagglutinin from fowl plaque virus (strain A/fowl plaque virus/Rostock/34/67/1N (35)) in front of methionine 99 of Ii. Briefly, cDNA encoding amino acid 1–18 of fowl plaque virus-hemagglutinin was amplified by polymerase chain reaction using 5'-gggatccgctgaatgac-3' and 5'-aagcagctgtcgggatgctc-3' as primers and recombinant AcNPV virus encoding fowl plaque virus-hemagglutinin (kindly provided by Dr. H.-D. Klenk) as a template. These primers introduced an EcoRI site 5' and a SpHI site 3' of the coding region. cDNA encoding the 18% paraformaldehyde. After 16 h at 37 °C, production of IL-2 in the supernatant was determined using the IL-2-dependent cell line CTLL-2 (ATCC) and the MTS/PMS reagent (Promega). The protein was determined using anti-Ii antibodies. Antigen Presentation Assays—Presentation of the 89–101 MBP peptide was assayed as follows. The H-2b and H-2a expressing B cell hybridoma LS102.9 (a fusion of B10.S (7R) spleen cells and the Balb/c lymphoma A20.2J (ATCC)) was used as a source of APC at 5 × 104 cells/well in 96-well plates. The MBP peptide 89–101 (0.2 mg/ml) was added simultaneously with the 89–101 MBP-specific T cell hybridoma 12.3 (kindly provided by Dr. D. Wraith, Cambridge, United Kingdom) in the presence or absence of IiP25. Fixation of APC was at room temperature. IL-2 was added at 100 units/ml of IL-2 in the supernatant was determined using the IL-2-dependent cell line CTLL-2 (ATCC) and the MTS/PMS reagent (Promega). Presentation of peptides derived from intact MBP was analyzed by adding whole MBP (Sigma) at 3 μg/ml to 5 × 104 SJL/J irradiated spleen APCs and 5 × 104 cells/well of the T cell hybridoma 6F112 in the presence of IiP25 or bovine serum albumin. T cell activation was determined as described above. Cells are expressed as the ratio *A* of 330/370 nm (peak wavelength of Ii, 330 nm and of A20.2J, 370 nm).

**Immunofluorescence Microscopy**—Cells were grown on glass coverslips and incubated on ice with the proteins and antibodies indicated. Subsequently, cells were fixed in 3% paraformaldehyde, mounted in Fluoroguard Antifade mounting reagent (Bio-Rad), and analyzed using a confocal laser scanning microscope system (MRC-1024, equipped with 522/32 nm and 605/32 nm band pass filter for FITC and Texas Red, respectively; Bio-Rad) attached to an Axiovert 100 microscope (Carl Zeiss, Inc., Thornwood, NY) as described (12, 39). Images were collected using the 488 nm (FITC) and 568 nm (Texas Red) lines of an Argon/Krypton laser, with pinhole settings at 2.0 and 2.5 for FITC and Texas Red, respectively.

**RESULTS**

The human melanoma cell line Mel JuSo expresses MHC class II molecules, as well as all known Ii isoforms, and is a suitable model cell line for studying the biology of MHC class II-restricted antigen processing and presentation (20, 22). When Mel JuSo cells were metabolically labeled with [35S]methionine/cysteine, several polypeptides co-immunoprecipitated with MHC class II molecules (Fig. 1, anti-MHC class II). In addition to the MHC class II α and β chains, the 33-kDa form of Ii was present, as was a protein with a molecular mass of 25

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2 E. E. Eynon, unpublished data.
kDa. To analyze the origin of this 25-kDa protein, the anti-class II immunocomplexes were denatured, diluted in lysis buffer, and reprecipitated using antibodies against either the Ii N-terminal or the Ii C-terminal region. The 25-kDa protein could only be recovered using the anti-IiC terminal antibodies (Fig. 1, anti-IiC), and not the anti-Ii N-terminal antibodies (Fig. 1, anti-IiN). The immunoreactivity of P25 with the anti-Ii antibodies and the difference in molecular mass between Ii and P25 indicates that P25 represents a form of Ii (IiP25) that lacks the N-terminal cytoplasmic region.

In Mel JuSo cells, as in other class II/Ii-positive antigen-presenting cells, various Ii fragments are generated at late stages of biosynthesis, following transport of class II/Ii complexes to post-Golgi endocytic organelles (16, 17). In contrast to IiP25, these fragments represent N-terminal segments that lack different portions of the luminal, C-terminal region (17). To analyze the organelles in which IiP25 resided, subcellular fractionation by organelle electrophoresis was performed. During such electrophoresis, endosomal and lysosomal organelles shift toward the anode, whereas most other subcellular membranes, including the endoplasmic reticulum, remain unshifted (Fig. 2a and Refs. 22 and 38). To reveal the presence of IiP25, cells were metabolically labeled and homogenized, and the membrane fraction was subjected to organellar electrophoresis as analyzed under “Experimental Procedures.” After fractionation, Ii-related molecules were immunoprecipitated from the different fractions and separated by SDS-PAGE. As can be seen in Fig. 2, endosomal and lysosomal organelles were well separated from the endoplasmic reticulum, as analyzed by the presence of β-hexosaminidase reactivity and the presence of radiolabeled molecules after a 4-min [35S]methionine/cysteine pulse (○). b, the resulting fractions after electrophoresis were pooled as indicated, and Ii-related proteins were immunoprecipitated using anti-IiC antibodies. Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

The fact that IiP25 was found to be located in the endoplasmic reticulum raised the possibility that it originated from signal peptidase cleavage. Indeed, the invariant chain has been shown to contain a potentially cleavable signal sequence in its membrane-spanning domain (40). Cleavage by signal peptidase results in an Ii form that lacks the first 42 amino acid residues (from the 33-kDa form) and starts at position 43 with leucine (40). To determine whether the IiP25 described here results from signal peptidase cleavage during translocation across the endoplasmic reticulum membrane, the N terminus of IiP25 was determined. Ii immune complexes from [35S]methionine/cysteine-labeled cells were resolved by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane. IiP25 immobilized on the membrane was subjected to automated Edman degradation, and amino acid-containing fractions were analyzed for their [35S]content, indicative of the presence of methionine and/or cysteine. Radioactivity was eluted after 1, 6, 14, and 23 cycles (Fig. 3). Elution of methionine at these positions is predicted by an IiP25 protein starting at position 99 (see Fig. 3) and in agreement with the observed apparent molecular weight after SDS-PAGE. Thus, IiP25 represents a form of Ii lacking the cytoplasmic and transmembrane domains and therefore did not result from signal peptidase cleavage. In addition, IiP25 includes the CLIP region that is responsible for binding to MHC class II molecules (26).
One important function of Ii is to target the MHC class II complex to endocytic organelles (1, 3). The targeting sequences responsible for endosomal location reside in the N-terminal cytoplasmic tail (9, 11, 12). The absence of this N-terminal domain in IiP25 might thus result in transport of IiP25 through the secretory pathway, rather than being targeted to and retained in endocytic compartments. In addition, the absence of the membrane anchoring domain would result in IiP25 being secreted. To follow the transport and possible secretion of newly synthesized IiP25 molecules, cells were metabolically labeled with [35S]methionine/cysteine and chased in the absence of radiolabel for the times indicated in Fig. 4. At the different chase times, the medium was collected, and anti-Ii immunoreactive proteins were precipitated from the media. After 2 and 4 h of chase, a protein of ~27 kDa was present in the culture medium (Fig. 4). This increase in molecular mass might have resulted from glycosylation during transit through the Golgi compartments; indeed, incubation of the immunoprecipitates with neuraminidase to remove sialic acid residues reduced the apparent molecular mass to 25 kDa (Fig. 4, NANAse). We conclude that IiP25 is transported via the endoplasmic reticulum and Golgi complex to the plasma membrane and secreted into the culture medium.

The lumenal domain of Ii is responsible for blocking peptide assembly to MHC class II molecules, and methionine 99 and 104 (which are part of the CLIP sequence) serve as anchor residues that allow this fragment to bind efficiently to MHC class II molecules (26, 31, 41). The fact that IiP25 represents the Ii lumenal domain, including these anchor methionines, together with its secretion in the culture medium, prompted us to investigate the possibility that class II-restricted antigen presentation could be modulated by the presence of IiP25.

As a source of IiP25, a recombinant form of IiP25 was generated by fusing the coding sequence for IiP25 (amino acid residues 99–216) to the signal sequence of the hemagglutinin molecule of fowl plague virus (see under “Experimental Procedures”) (35). When Chinese hamster ovary cells were stably transfected with this construct, IiP25 could readily be detected in the culture medium of metabolically labeled and chased cells, indicating transport through the secretory pathway (data not shown). In addition, recombinant baculovirus producing IiP25 protein was generated as described under “Experimental Procedures,” and the supernatant from infected insect cells was analyzed for the presence of IiP25. Ii is known to form trimers upon proper folding, and the domain responsible for trimerization is located within amino acid residues 163–183 (42, 43). As this region is present in IiP25, the ability of IiP25 expressed in insect cells to trimerize was analyzed. Following the addition of the chemical cross-linking reagent BS3, IiP25 monomers, dimers, and trimers were detected after SDS-PAGE and immunoblotting (Fig. 5), indicating its proper folding and transport through the secretory pathway in insect cells (44).

To investigate the effect of IiP25 on antigen presentation, we made use of splenic antigen-presenting cells that can internalize and process myelin basic protein and are able to present the 89–101 peptide from MBP in the context of MHC class II molecules to a T cell hybridoma (see under “Experimental Procedures”). When irradiated spleen cells were incubated with whole MBP, T cell activation could be readily measured via IL-2 production (see Fig. 6a). Addition of increasing amounts of IiP25, but not bovine serum albumin, resulted in up to ~80%
inhibition of IL-2 production, indicating that IiP25 can compete with peptides for MHC class II molecules (Fig. 6, a and b). To investigate whether IiP25 acted at the cell surface or an intracellular site, viable (Fig. 6c) or fixed (Fig. 6d) antigen-presenting cells were incubated with the 89–101 MBP peptide, and the effect of IiP25 on the ability to trigger a T cell hybridoma was analyzed. In the case of both nonfixed and fixed antigen-presenting cells, T cell activation was inhibited in a dose-dependent manner by IiP25, up to a maximum inhibition of 85–90% (Fig. 6). Immunodepletion of IiP25 abolished the inhibition of T cell activation. Therefore, the inhibitory effect of IiP25 most probably reflects its ability to compete with antigenic peptides for MHC class II molecules at the surface of antigen-presenting cells.

Binding of IiP25 to the cell surface was analyzed directly by incubating living cells on ice with purified IiP25, followed by an incubation with anti-IiC antibodies. As shown in Fig. 7, a and b, IiP25 can be readily detected at the cell surface. To analyze whether binding of IiP25 to the cell surface occurred via the CLIP sequence that is present in IiP25, binding of IiP25 to living cells was performed in the presence of synthetic CLIP peptide (amino acid residues 81–104). Inclusion of CLIP blocked IiP25 binding to the cell surface, indicating that IiP25 binds to cell surface MHC class II molecules via its CLIP sequence (Fig. 7, c and d).

DISCUSSION

The MHC class II-associated invariant chain acts as an important regulator of antigen processing and presentation (1, 45). Besides functioning as a chaperone in the folding of class II molecules early during biosynthesis, Ii directs the class II complex to MHC class II compartments in the endosomal/lysosomal pathway (3). In addition, Ii prevents the binding of antigenic fragments to MHC class II molecules prior to their deposition in the peptide loading compartment (20). In this report, we describe the transport and extracellular secretion of a lumenal invariant chain form, IiP25, and present evidence that IiP25 can function as a modulator for MHC class II-restricted antigen presentation.

A 25-kDa form of Ii has been detected in several human and mouse cell types (9, 25, 32, 33, 46). In which cellular compartment this form is generated is not clear. Although in some cases, the 25-kDa Ii form might be generated in the endoplasmic reticulum from the full-length 33-kDa Ii form (25, 32), in other cells, IiP25 was found to be generated at the site of
peptide loading (33, 46). The IiP25 form, as detected here, was generated in the endoplasmic reticulum and readily transported through the early secretory pathway in association with MHC class II molecules in a manner similar to wild type Ii. The protease responsible for generation of IiP25 is currently unknown. The membrane spanning segment of Ii does contain a potential signal peptide cleavage site residing in the middle of the membrane spanning region (40). The N terminus of IiP25 was found to be represented by methionine 99, which is located behind the membrane spanning region at the luminal side, but we cannot exclude the possibility that signal peptide cleavage generates a partially N-terminal truncated Ii form. Such a partially truncated form might then be fully translocated into the endoplasmic reticulum and further acted upon by different proteolytic activities. The cotranslational assembly of Ii isoforms within the class II complex might prevent further cleavage of IiP25 due to inaccessibility of the Ii luminal domain to protease activity.

The precise oligomeric state of the Ii isoforms within the MHC class II complex is not known. One possibility is that in IiP25-expressing cells, mixed trimers containing IiP25 assemble with MHC class II molecules. As it has been reported that efficient targeting of Ii molecules from the trans-Golgi network to endosomes requires multimerization of the N-terminal cytoplasmic targeting sequence (47), IiP25 containing trimers may be predominantly transported to the plasma membrane, rather than then being targeted to the endocytic pathway. Following release of IiP25, these Ii/class II complexes may then become internalized from the plasma membrane by virtue of the internalization sequence present in the cytoplasmic tail of Ii (12, 47, 48).

Following transport of IiP25 through the biosynthetic pathway, IiP25 was secreted in the culture medium. Sequence analysis revealed that IiP25 contained methionine 99 of Ii at its N terminus, and it thus includes part of the sequence that has been implicated both in association with class II molecules and in preventing antigenic peptides from binding to class II molecules (1, 5). Both of these properties were retained in IiP25: first, IiP25 was associated with MHC class II molecules, and second, when added exogenously to cells presenting peptides in the context of class II, IiP25 inhibited T cell activation, indicating that IiP25 competes with peptides for MHC class II molecules.

Inhibition of T cell activation by secreted IiP25 occurred directly at the cell surface of antigen-presenting cells through the binding of IiP25 via its CLIP sequence. In cells secreting IiP25, inhibition of peptide presentation to T cells by IiP25 may locally influence the type of T cell response that is generated. This effect may be important in those cases in which the peptide/MHC class II concentration at the cell surface is around the threshold required for T cell activation (49); the presence or absence of IiP25 may dictate in that case whether or not a T cell response will be generated. Interestingly, the Mel JuSo cells used here are derived from melanoma cells, some of which have been reported to be deficient in antigen presentation (50). It is therefore possible that the production of IiP25 may be one of the ways by which tumor cells can evade immune recognition in vivo.

The MHC class II-associated invariant chain has been shown to perform a variety of different functions at various stages of the intracellular pathway of MHC class II molecules. The inhibitory effect of a secreted form of Ii as described here represents a novel mode of regulation exerted by Ii and may contribute to the type of T cell responses that are induced by MHC class II-peptide complexes.