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Immunology

Expression of endogenous peptide–major histocompatibility complex class II complexes derived from invariant chain–antigen fusion proteins

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ABSTRACT **$CD4^+$ T cells recognize major histocompatibility complex (MHC) class II-bound peptides that are primarily obtained from extracellular sources. Endogenously synthesized proteins that readily enter the MHC class I presentation pathway are generally excluded from the MHC class II presentation pathway. We show here that endogenously synthesized ovalbumin or hen egg lysozyme can be efficiently presented as peptide–MHC class II complexes when they are expressed as fusion proteins with the invariant chain (Ii). Similar to the wild-type Ii, the Ii–antigen fusion proteins were associated intracellularly with MHC molecules. Most efficient expression of endogenous peptide–MHC complex was obtained with fusion proteins that contained the endosomal targeting signal within the N-terminal cytoplasmic Ii residues but did not require the luminal residues of Ii that are known to bind MHC molecules. These results suggest that signals within the Ii can allow endogenously synthesized proteins to efficiently enter the MHC class II presentation pathway. They also suggest a strategy for identifying unknown antigens presented by MHC class II molecules.**

T cells recognize antigens as peptides bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). $CD4^+$ and $CD8^+$ T cells recognize antigens bound to MHC class II and MHC class I, respectively. In addition to providing immunity against foreign pathogens, both $CD4^+$ and $CD8^+$ T cells are implicated in the destruction of self tissues that leads to autoimmune disorders (1). Identification of these peptides and their donor proteins that elicit T-cell immunity is essential for development of vaccines and understanding the role of these antigens in autoimmunity.

Antigens that stimulate T cells can in principle be identified by sequencing the purified MHC-bound antigenic peptide. This biochemical approach is, however, made difficult by the fact that many different peptides are bound to MHC on the APC surface, and individual peptides represent only a very small fraction of the total (2–4). We have proposed (5) an alternative genetic strategy for identifying T-cell-stimulating antigens that does not depend upon biochemical purification of peptides but instead, relies upon expression of peptide–MHC complexes in APCs transfected with the antigen genes. The generation of peptide–MHC class I complexes from transiently expressed genes and the ability to detect rare APCs with the single-cell *lacZ* T-cell assay suggested that these methods could serve as an expression cloning strategy to identify antigens recognized by $CD8^+$ T cells (5, 6). Indeed this strategy has allowed the identification of several antigens that are presented by MHC class I molecules (ref. 7 and S. Malarkannan, M. Afkarian, and N.S., unpublished data).

The efficiency of peptide–MHC expression after gene transfer is central to expression cloning strategies. Not only should the genes transferred cause expression of peptide–MHC complexes, but this should occur in a large fraction of APCs to permit screening of cDNA libraries with appropriate T cells. Our earlier studies with the model antigen ovalbumin (OVA) showed that these two requirements were met for expression and detection of the OVA–K^b MHC class I complex in transiently transfected K^b COS cells (5). Extending this strategy to peptide–MHC class II complexes recognized by $CD4^+$ T cells is, however, not straightforward because distinct pathways are used for generating peptides presented by MHC class II vs. MHC class I molecules. Endogenously synthesized proteins that readily enter the peptide–MHC class I pathway are generally excluded from the peptide–MHC class II presentation pathway (8, 9). This dichotomy is evident from studies showing that efficiency of endogenous peptide–MHC class II expression is correlated with cellular localization of the antigen and with the recipient APC type (10–12). The reasons for these differences are not fully understood but are likely to involve the MHC class II-associated invariant chain (Ii) (13–16) and other recently identified MHC-encoded *DM* gene products (17, 18). Available evidence indicates that the Ii blocks loading of the MHC class II molecules during their transit through the endoplasmic reticulum (ER) and is instrumental in targeting the MHC molecules to the endosomal compartment(s) where they can be loaded with processed antigenic peptides (19–23). Introducing endogenously synthesized proteins consistently and with a high efficiency into the MHC class II pathway has, therefore, remained a difficult undertaking.

In this report we analyzed the expression of peptide–MHC class II complexes in transiently transfected cells. We show that MHC class II-expressing COS cells, in the presence of the Ii, efficiently processed and presented exogenous or endogenously synthesized OVA to murine $CD4^+$ T cells. Most significantly, both OVA and hen egg lysozyme (HEL) peptide–MHC class II complexes were efficiently generated from endogenously synthesized fusion proteins containing the Ii and antigen residues. By immunoprecipitation analysis and measurements of relative antigen presentation activity of deletion constructs, we show that signals within the Ii allow intracellular association with MHC class II molecules and generation of endogenous peptide–MHC complexes. These findings provide a method for generating peptide–MHC class II complexes from endogenously synthesized proteins and suggest a general expression cloning strategy for antigens recognized by $CD4^+$ T cells.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Antigens. The *lacZ*-inducible T-cell hybrids KZO (OVA–A^k specific), KZH (HEL–A^k specific), and B3Z (OVA–K^b specific) COS and K^b COS cells have been described (5, 6). The OVA–A^d-specific hybrid DO.11.10 (24) was from H. McConnell (Stanford University). The

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Abbreviations: APC, antigen-presenting cell; HEL, hen egg lysozyme; Ii, invariant chain; OVA, ovalbumin; MHC, major histocompatibility complex; ER, endoplasmic reticulum; CB-OVA, CNBr-digested OVA.

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B-lymphoma LK35.2 (H-2^{kxd}) and the hybridomas 10.2.16 (anti-A^k) and In1 (anti-Ii) were from American Type Culture Collection and R. N. Germain (National Institutes of Health). The polyclonal rabbit anti-OVA antibodies were from Cappel. HEL and OVA were purchased from Sigma and Worthington, respectively. All cell lines were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, 7 mM pyruvate, 50 μM 2-mercaptoethanol, penicillin (200 units/ml), and streptomycin (200 μg/ml) at 37°C in 5% CO_2/95% air.

Plasmid DNA Constructs. The cDNAs encoding $A^k\alpha$, $A^d\alpha$, $A^k\beta$, $A^d\beta$, and murine p31 Ii (from R. N. Germain, National Institutes of Health, and J. Miller, University of Chicago) were subcloned into the *Eco*RI site of the expression vector pcDNA1 (Invitrogen). The full-length OVA (25) and HEL (26) cDNAs were from M. Moore (University of Southern California, Los Angeles) and J. Kirsh (University of California, Berkeley), respectively. The I160–OVA construct encoding Ii-(1–161) and OVA-(28–386) was prepared in the pcDNAI vector by using standard methods (27). The I80–OVA [encoding Ii-(1–80) and OVA-(138–386)], δ22I80-OVA, and δ22Ii constructs were prepared by assembling the fragments in pSP72 vector (Promega) and then subcloning the fusion constructs into the pcDNAI vector. The I80–HEL construct was made by replacing the OVA residues with those of mature HEL generated by PCR amplification of HEL cDNA with the oligonucleotide primers 5′-GCGCAAGCTTAAAGTCTTTGGACGA-3′ and 5′-ATTTAGGTGACACTATAG-3′. The fragment encoding I80–HEL was then subcloned into pcDNA1. Constructs were sequenced to confirm fidelity of their translational reading frames.

Transient Transfections. The DEAE-dextran transfection protocol has been described (28). Briefly, COS cells were transfected in 96-well plates with 3×10^4 cells per well or in 6-well plates with 1×10^6 cells, with the indicated quantity of cDNAs for the antigens, MHC class II $A^k\alpha$ and $A^k\beta$, and with or without Ii. After 48 h the cells were assayed for T-cell stimulation or taken for protein analysis.

Protein Analysis. COS cells were transfected with the Aα and Aβ cDNAs along with the indicated Ii or Ii–OVA constructs. Two days later the cells were labeled with Tran^{35}S-label (ICN, Irvine, CA) at 200 μCi/ml (1 Ci = 37 GBq) for 1 h. The lysates were immunoprecipitated, fractionated by SDS/PAGE on 15% gels, and analyzed on PhosphoImager SF (Molecular Dynamics) as described (27). Each lane represents 1.5×10^5 cell equivalents.

T-Cell Activation Assays. T cells (1×10^5 cells) were added to each well of a 96-well plate containing the indicated quantity of antigen or medium alone and the APCs. After overnight incubation, T-cell activation was measured by conversion of the β-galactosidase (*lacZ*) substrate chlorophenol red β-galactoside for 4 h or by assaying the culture supernatants for interleukin 2 (6). The data shown are mean absorbance of replicate wells.

RESULTS AND DISCUSSION

Role of Ii in Generation of Peptide–MHC Class II Complexes in COS Cells. We analyzed the antigen presentation function of MHC class II molecules in simian COS cells because they do not express endogenous MHC class II or the Ii (29) and can be readily transfected with a high efficiency. This laboratory has demonstrated (5, 28, 30) that COS cells expressing murine MHC class I and antigen genes serve as efficient APCs for murine $CD8^+$ T-cell hybrids. Cotransfection of MHC class II $A\alpha^k$ and $A\beta^k$ genes into COS cells also allowed stimulation of the OVA–A^k-specific KZO.1.17 T-cell hybrid (abbreviated as KZO) (6), in presence of CNBr-digested OVA (CB-OVA) (Fig. 1*A*). The T-cell response was comparable to that obtained with murine A^{k+} B-lymphoma LK35.2 used as conventional APCs (Fig. 1*B*). However, unlike LK35.2 cells, A^k COS cells were inefficient in presenting native OVA, unless they were also cotransfected with the murine p31 Ii gene (Fig. 1*C*). These results demonstrate that COS cells can generate the appropriate peptides for recognition by murine T cells. Thus, as for several other antigens (31, 32), the generation of OVA–A^k complex from exogenous peptides required only A^k molecules but from native OVA required both A^k and Ii. These data and the colocalization of MHC class II and Ii in endosomal vesicles by immunohistology (20) show that COS cells can serve as a model APC for the expression of peptide–MHC class II complexes.

To design constructs for expression of endogenous OVA–A^k complex, we first identified the OVA peptide presented by A^k MHC to KZO T cells as the OVA-(243–274) fragment by HPLC fractionation of CB-OVA and sequencing the single active fraction. Based upon the location of the antigenic peptide, cDNA constructs were prepared (Fig. 2) and tested for expression and generation of OVA–A^k complex. Again, in

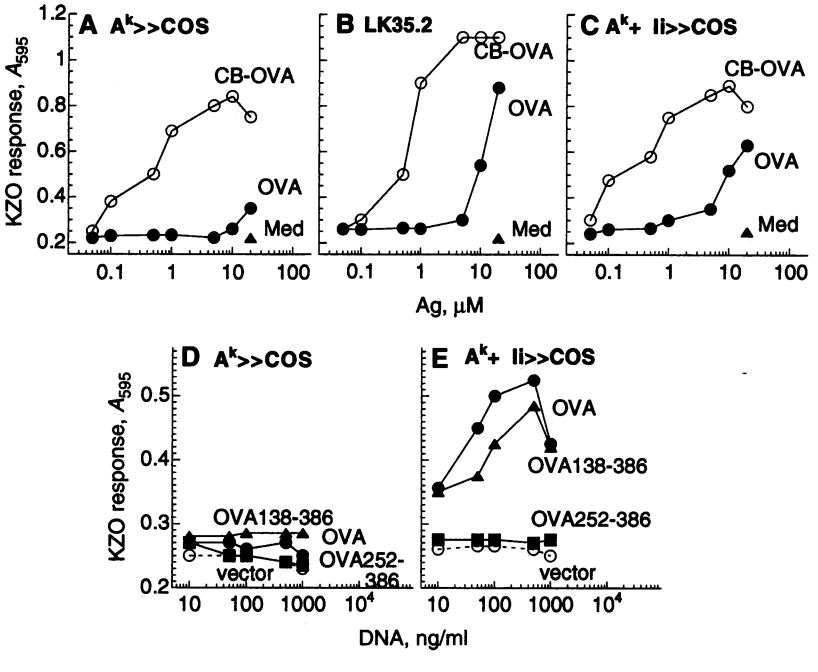

FIG. 1. Generation of OVA peptide–A^k complexes with exogenous or endogenous antigens in transfected COS cells. COS cells were transfected with $A^k\alpha$ and $A^k\beta$ alone (*A* and *D*) or with $A^k\alpha$, $A^k\beta$, and Ii (*C* and *E*) cDNA constructs. Each construct was at 500 ng/ml. (*A–C*) Exogenous presentation with medium alone (Med), native OVA (OVA), or CB-OVA was tested with transfected COS cells (*A* and *C*) or with LK35.2 lymphoma (H-2^{kxd}) cells (*B*). For endogenous presentation, COS cells were transfected with various concentrations of the indicated OVA expression constructs (see Fig. 2), along with A^k ($A^k\alpha$ + $A^k\beta$) alone (*D*) or A^k + Ii ($A^k\alpha$ + $A^k\beta$ + Ii) (*E*) cDNA constructs. Generation of peptide–MHC complexes was measured by stimulation of OVA–A^k-specific KZO T cells.

contrast to A^k COS cells, only A^k + Ii COS cells generated the OVA–A^k complex from full-length OVA and from OVA-(138–386) constructs (Fig. 1 *D* and *E*). The OVA-(252–386) construct was inactive due to deletion of 10 N-terminal residues within the epitope recognized by KZO T cells (Fig. 2). Note that unlike full-length OVA, which is glycosylated and secreted by COS cells (28), the OVA-(138–386) fragment does not contain the N-terminal ER translocation signal of OVA and, therefore, remains unglycosylated within the cytoplasm (see Fig. 4 below and data not shown). The OVA–A^k complexes were generated by endogenous loading of the A^k MHC molecules rather than by secretion and subsequent endocytosis of OVA as an exogenous antigen. Coculture of cells expressing OVA or the OVA-(138–386) fragment mixed with cells expressing A^k and Ii was unable to stimulate T cells (data not shown). Furthermore, only full-length OVA, but not OVA-(138–386) or any other Ii–OVA fusion proteins (see below), was detected in the culture medium by ELISA (data not shown). The concentration of secreted OVA was, however, only 200 pM, far below the minimal concentration (>1 μM) required for exogenous presentation (Fig. 1 *A* and *C*). These findings are consistent with previous results (8, 10–12) in constitutive Ii-expressing B cells that showed that endogenous antigens that are translocated into the ER enter the MHC class II presentation pathway more efficiently than their cytoplasmic forms. For the endogenous OVA–A^k complex, the secreted ER form of OVA was far more active than the cytoplasmic OVA-(138–386) fragment, but T-cell responses to cells expressing either of these polypeptides were generated only in presence of the Ii. Similar results have also been recently reported for presentation of HEL in fibroblasts cotransfected with the Ii (35). Interestingly, another study with APCs from transgenic mice expressing the antigen but lacking Ii indicated that the requirements for the Ii in presentation of endogenous antigen were often variable (22). The disparity in the requirement for Ii in allowing endogenous peptide–MHC class II expression among these model systems may reflect the manner in which different antigens are processed in different types of APCs (fibroblasts, macrophages, B cells, etc.) or in the amount of antigen expressed in the cells that can also affect the efficiency of peptide–MHC class II expression (36, 37).

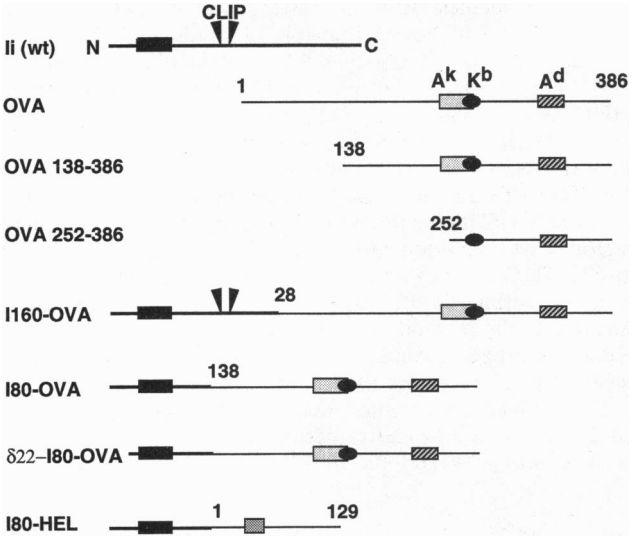

FIG. 2. Schematic representation of constructs for expression of Ii, OVA, and the Ii–OVA and Ii–HEL fusion proteins. The wild-type (wt) murine p31 Ii cDNA encodes a type II transmembrane protein containing a cytoplasmic region, a transmembrane region, and the ER luminal region that includes the class II-associated Ii peptide (CLIP) (2, 3, 33, 34). δ22–Ii encodes an Ii lacking N-terminal residues 1–22. Constructs encoding OVA and its two truncated fragments, OVA-(138–386) and OVA-(252–386), have been described (28). Peptides of OVA or HEL that are recognized by the T-cell hybrids KZO [OVA-(243–274)], DO.11.10 [OVA-(323–339)], B3Z [OVA-(257–264)], and KZH [HEL-(34–45)] are indicated by boxes. Ii–antigen fusion proteins show the residues derived from Ii as thick lines and OVA or HEL as thin lines, respectively. The I160–OVA construct encodes N-terminal Ii residues 1–160 and OVA residues 28–386. The I80–OVA and δ22–I80-OVA constructs contain, respectively, N-terminal Ii residues 1–80 and 23–80 followed by OVA residues 138–386. The I80–HEL construct encodes N-terminal Ii residues 1–80 followed by HEL residues 1–129.

Efficient Presentation of Endogenous Antigens from Ii–Antigen Fusion Proteins in the Absence of Wild-Type Ii. The requirement for Ii in obtaining processed OVA–A^k complexes from both exogenous and endogenous sources suggested that signals within the Ii could be used to target endogenously synthesized proteins to the antigen-processing compartment. Furthermore, both in normal and in mutant APCs, Ii-derived peptides are among those bound to MHC class II molecules (2–4). We prepared cDNA constructs by ligating Ii and OVA or HEL cDNA fragments to yield the I160–OVA, I80–OVA, δ22I80–OVA, and I80–HEL polypeptides (Fig. 2). Similar to native OVA and the OVA-(138–386) polypeptides, each Ii–OVA fusion protein was efficiently expressed in transfected cells as determined by Western blot analysis with polyclonal anti-OVA antibodies (data not shown). Most significantly, whereas the KZO T-cell response to OVA or OVA-(138–386) fragments required coexpression of Ii (Fig. 1*E*), expression of the Ii–OVA fusion constructs alone was sufficient to generate the OVA peptide–A^k complex (Fig. 3*A*). The highest level of OVA–A^k expression was obtained with the I80–OVA construct and was 5- to 50-fold greater than that obtained with either the I160–OVA or the δ22I80–OVA constructs. In contrast to the dramatic differences in the generation of peptide–MHC class II complexes from OVA or the Ii–OVA fusion proteins, the OVA–K^b MHC class I complex was efficiently generated from the OVA or with the Ii–OVA fusion proteins (Fig. 3*B*). Note that the comparable activity of these

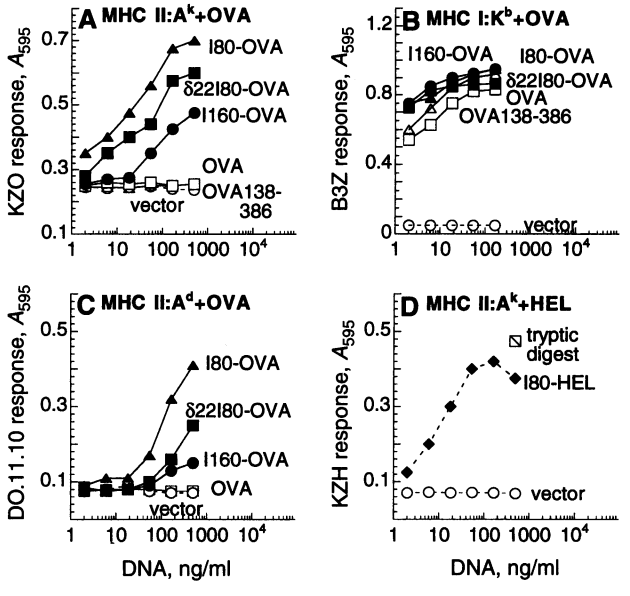

FIG. 3. Generation of endogenous peptide–MHC class II and MHC class I complexes. COS cells were cotransfected with cDNAs encoding $A^k\alpha$ and $A^k\beta$ (*A* and *D*) or $A^d\alpha$ and $A^d\beta$ (*C*) and the indicated constructs encoding OVA fragments or Ii–OVA fusion proteins (*A* and *C*) or the Ii–HEL fusion construct (*D*). Stable K^b-COS cells were transfected with the indicated cDNAs (5, 28) (*B*). After 48 h KZO (*A*), B3Z (*B*), DO.11.10 (*C*), or KZH (*D*) T cells were added, and their activation was measured by the induced *lacZ* activity (*A, C,* and *D*) or interleukin 2 response (*B*).

Ii–OVA fusion proteins in generating the OVA–K^b complex also indicates that they were available in similar amounts. No KZO responses were obtained with cells that had been transfected separately with A^k + Ii and I80–OVA cDNAs alone and then cocultured for 2 days (data not shown). Thus as with endogenous presentation of OVA polypeptides in Ii-expressing cells, the generation of OVA–A^k complexes required endogenous expression of both A^k and the Ii–OVA fusion proteins in the same cell.

To establish whether expression of peptide–MHC class II complexes from Ii–antigen fusion proteins was a general phenomenon, we tested two other model systems. (*i*) We used the OVA–A^d-specific T-cell DO.11.10 (24). COS cells were cotransfected with $A\alpha^d$, $A\beta^d$, and Ii–OVA fusion constructs (Fig. 3*C*). Again, DO.11.10 T cells were stimulated by cells expressing A^d and the Ii–OVA fusion constructs, with similar hierarchy in responsiveness to cells expressing I80–OVA, I160–OVA, and δ22I80–OVA. (*ii*) The analogous I80–HEL cDNA construct was prepared (Fig. 2*A*) and was cotransfected with $A\alpha^k$ and $A\beta^k$ constructs into COS cells. The stimulation of KZH T cells showed that as for OVA, HEL–A^k complexes were also generated from the I80–HEL fusion protein (Fig. 3*D*). Note that the peptide recognized with A^k by KZH [HEL-(34–45)] and KZO [OVA-(243–274)] and with A^d by DO.11.10 [OVA-(323–336)] differs in its proximity to the Ii residues (Fig. 2). Thus, the endogenous generation of peptide–MHC complexes did not depend upon the location of the antigenic peptide relative to the Ii residues in the fusion proteins. We conclude that peptide–MHC class II complexes can be efficiently generated from endogenously synthesized Ii–antigen fusion proteins.

Association of Ii Fusion Proteins with MHC Class II. A substantial body of evidence indicates that native Ii, due to its association with MHC class II molecules in the ER (38, 39) and the presence of an endosomal targeting signal at the N terminus (14, 15), targets MHC to the antigen-processing endosomal compartment (19, 40), in which Ii dissociates to permit MHC to bind processed peptides (41). To test possible interactions between the A^k molecules and the Ii–OVA fusion proteins, immunoprecipitation analysis was carried out with lysates from metabolically labeled COS cells expressing these constructs. To identify associated proteins, the immunoprecipitates of anti-A^k, anti-Ii, or anti-OVA antibodies were electrophoresed in parallel (Fig. 4). Immunoprecipitation with anti-A^k monoclonal antibodies showed that both the $A^k\alpha$ (≈36 kDa) and $A^k\beta$ (≈28 kDa) chains were present in all transfectants (Fig. 4). In cells expressing A^k and the wild-type Ii, anti-A^k antibodies coprecipitated the Ii, as indicated by the presence of an additional 33-kDa band that was also precipitated with the anti-Ii antibody. As expected the anti-Ii antibody (In1) that recognizes an N-terminal epitope of Ii failed to precipitate δ22Ii protein that was present as the 31-kDa band in the anti-A^k immunoprecipitates. Significantly, compared to vector alone, in cells expressing A^k and the Ii–OVA fusion proteins, a fraction of each Ii–OVA fusion (I80-OVA, ≈42 kDa; I160–OVA, ≈65 kDa; δ22I80–OVA, ≈40 kDa) protein coprecipitated with anti-A^k antibody, indicating that they were also capable of interacting with A^k MHC. This association required the Ii residues because neither native OVA (≈ 45 kDa) nor the OVA-(138–386) (≈25 kDa) fragment coprecipitated with A^k, despite their high levels of expression, as judged by immunoprecipitating the same lysates with anti-OVA antibodies. Precipitation with anti-OVA antibodies also allowed coprecipitation of the $A^k\alpha$ and $A^k\beta$ chains with each fusion protein. Furthermore, all the A^k-bound Ii–OVA proteins were glycosylated because their size was 3–6 kDa larger than expected for the core polypeptides and because of their sensitivity to digestion with endoglycosidase H (data not shown), indicating that they were translocated to the ER/Golgi compartment. The differences in the mobilities of the I80–OVA and δ22I80–OVA bands precipitated with anti-A^k and anti-OVA are likely due to differential posttranslational modification of A^k-associated vs. free fusion proteins. These data and other pulse–chase experiments (data not shown) showing that Ii–OVA fusion proteins were coprecipitated with anti-A^k antibody even after a 4-h period suggest that A^k MHC molecules remained bound to the Ii–OVA fusion proteins during their transit through the Golgi. Thus, the Ii residues within the fusion proteins allowed interactions with MHC class II molecules and suggest that this association could account for the efficient generation of peptide–MHC class II complexes.

The fact that Ii–OVA fusion proteins were associated with the A^k MHC molecules also suggests that residues 23–80 of the Ii contain the signal(s) necessary for association with MHC class II molecules. It is interesting that a recent deletion analysis of the Ii showed that loss of residues 81–127, which includes the class II-associated Ii peptide (CLIP) region Ii-(85–100) (2, 3, 33, 34), resulted in loss of association with the MHC class II molecules (42). In more recent studies, C-terminal deletions of Ii residues beyond residues 96 or 82 also caused loss of association with MHC class II molecules (43, 44). These results and our findings suggest that association with the MHC molecules may depend upon more than one Ii region. One contained within Ii residues 23–80 that is present in δ22I80–OVA and another that was lost upon deletion of residues within 81–127 that may together determine this function of the Ii. Analysis of mutations in these regions of the Ii and the antigen should allow a more precise definition of the residues involved in the association function.

The antigen-presentation activity was also strongly influenced by the endosomal targeting signals of Ii because with both A^k and A^d MHC, the I80–OVA construct was far more

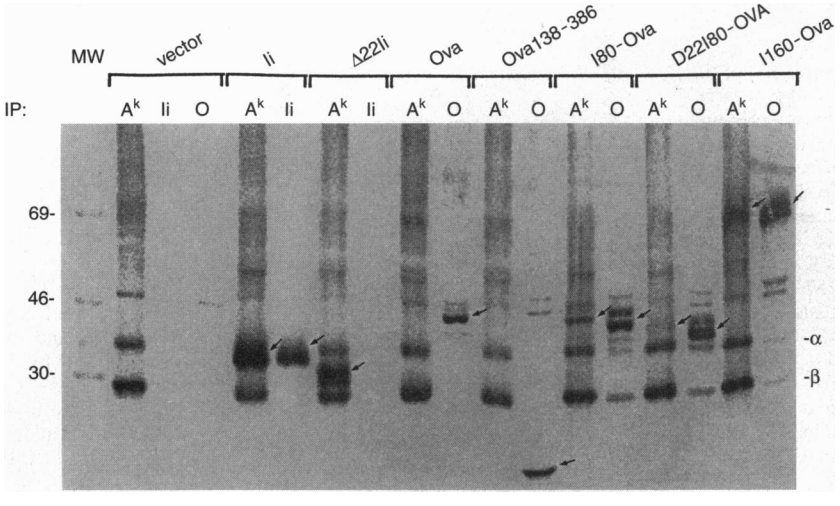

FIG. 4. Ii and Ii–OVA fusion proteins associate intracellularly with A^k MHC class II molecules. COS cells were transiently transfected with $A^k\alpha$, $A^k\beta$, and the cDNA constructs shown at the top (see Fig. 2) and, after 2 days, were metabolically labeled with [^{35}S]methionine. Cells were lysed and divided into aliquots that were precipitated (IP) with monoclonal antibody 10.2.16 (anti-A^k), In-1 (anti-Ii), or rabbit anti-OVA IgG fraction indicated as A^k, Ii, or O, respectively. Immunoprecipitates were electrophoresed in parallel on a 15% SDS/PAGE gel and visualized by phosphoimaging. The image shows the labeled proteins contained in each precipitate and specific bands corresponding to the Ii or Ii–antigen fusion proteins are indicated by arrows. The positions of Aα and Aβ subunits labeled as α and β, respectively, are to the right. Lane MW contains proteins of the indicated molecular masses shown to the left.

(5- to 50-fold) active in generating peptide–MHC complexes than δ22I80–OVA or I160–OVA constructs (Fig. 3). The 22 N-terminal residues, absent in δ22I80–OVA, include the endosomal targeting signal of the human Ii (14, 15). Thus, it was possible that absence of this targeting signal in δ22I80–OVA fusion protein lowered its presentation activity by compromising its ability to reach the endosomal processing compartment (45–47). We confirmed that in contrast to the wild-type Ii, δ22Ii did not allow presentation of either endogenous OVA cDNA or exogenous native OVA (data not shown), despite the fact that comparable amounts of wild-type Ii and δ22Ii were coprecipitated with anti-A^k antibody (Fig. 4). Thus, the 22 N-terminal residues of Ii were essential for exogenous and endogenous presentation and could explain how lack of these residues lowered the presentation efficiency of Ii–OVA fusion constructs. Note, however, that in contrast to virtually complete loss of function of Ii lacking residues 1–22, the δ22I80–OVA was active (Fig. 3 *A* and *C*), indicating that the MHC class II presentation pathways followed by wild-type Ii and the Ii–OVA fusion proteins may be different. This was also consistent with the complete inability of I80–HEL fusion protein to substitute for native Ii in allowing presentation of either exogenous or endogenous OVA (data not shown). The reason why inclusion of 80 additional Ii residues in I160–OVA caused a severe decrease in the presentation activity relative to I80–OVA is presently unclear. Because the I160–OVA construct includes not only the 81–160 luminal Ii residues but also additional OVA-(28–137) residues (Fig. 2), possible competition for MHC binding sites between the class II-associated Ii peptide CLIP (34), and other potential MHC binding peptides from OVA cannot be ruled out. Alternatively, this fusion protein, although stably associated with the MHC class II molecules (Fig. 4), may have influenced some other property of the MHC molecules such as conversion from the "floppy" to the "compact" forms that occurs during normal maturation of the MHC class II molecules (48, 49). Further analysis of these parameters and the intracellular localization of the Ii–antigen fusion proteins should determine the extent to which their expression coincides with or differs from the normal pathways of MHC class II and wild-type Ii.

In conclusion, we have shown that signals within the Ii can be used to target endogenously synthesized proteins efficiently into the MHC class II presentation pathway. We provide direct evidence that these signals include those required for physical interaction with the MHC class II molecules and show that efficient presentation requires the presence of N-terminal cytoplasmic residues but not the luminal residues. The ability to introduce intracellularly synthesized Ii–antigen fusion proteins into the MHC class II presentation pathway overcomes an important limitation of expression cloning strategies for identifying antigens recognized by $CD4^+$ T cells. Available strategies for identifying these antigens depend upon antigen presentation assays with exogenously added antigens. These strategies require biochemical purification of donor antigens or MHC-bound antigenic peptides and are, therefore, limited by availability of material. In contrast, this expression cloning strategy requires only mRNA for construction of appropriate cDNA libraries. This could prove particularly useful in the identification of unknown antigens involved in eliciting $CD4^+$ T-cell responses in protection from pathogens or in autoimmune disorders.

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