Major Histocompatibility Complex Class II–restricted Presentation of an Internally Synthesized Antigen Displays Cell-type Variability and Segregates from the Exogenous Class II and Endogenous Class I Presentation Pathways

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Summary

Although reported examples of endogenous antigen (Ag) presentation by major histocompatibility complex (MHC) class II molecules have increased, the mechanisms governing this process remain poorly defined. In this communication, we describe an experimental system designed to examine the mechanisms governing class II presentation of internal Ag. Our target peptide is processed from a transmembrane protein constitutively expressed by a variety of nucleated cells (MHC class I, H-2L^d), is naturally displayed by MHC class II molecules in vivo, and is recognized by a class II–restricted, CD4$^+$ T cell hybridoma. Our results indicate that presentation of the L^d target Ag is independent of its plasma membrane expression, may not involve endosomal proteolysis, and thus may be distinct from the classically defined class II presentation pathway. In addition, the observations that L^d presentation does not require a functional TAP-1 complex, is not blocked by invariant chain, and cannot utilize cytoplasmic forms of H-2L^d, suggest that a classical class I pathway is not involved in this presentation event. Finally, our data suggest that different cofactors participate in MHC class II presentation of exogenous and endogenous Ag, and that disparate Ag presenting cells, such as B, T, and pancreatic islet cells, may differentially express these two class II pathways of Ag presentation.

T lymphocytes recognize Ag as peptide fragments associated with MHC molecules on the surface of APC (1, 2). MHC class II molecules have the specialized capacity to present peptides generated from extracellular Ag which enter the cell by endocytosis (3, 4). The resulting class II–peptide complexes, products of the endosomal–lysosomal processing pathway, are recognized by CD4$^+$ T cells. In contrast, class I MHC molecules primarily present peptides derived from proteins with access to the cytosol and its resident proteolytic machinery. Resulting class I–peptide complexes are important in directing the immune response of CD8$^+$ cytolytic T lymphocytes against virally infected cells.

It has become increasingly clear, however, that class II can efficiently bind and present peptides derived from proteins synthesized within the APC (5–13). This suggests that, in the absence of receptor-mediated uptake of extracellular protein, internally synthesized proteins may be the principal source of class II peptides. Like typical extracellular Ag, intracellular Ag could potentially access the endocytic processing pathway by internalization from the plasma membrane, by secretion and pinocytosis, or by receptor-mediated endocytosis. Internal Ag might also access the endosomal/lysosomal pathway by direct sorting from the *trans*-Golgi network (TGN)[1], by heat shock protein (HSP)–mediated uptake from the cytosol (14), or by autophagy (15). Although the extent to which class II displays peptides derived from internally synthesized proteins is becoming increasingly apparent, the mechanisms governing their presentation remain poorly defined. Our understanding of class II presentation is largely derived from studies involving exogenous protein Ag. Interestingly, these studies have implicated the likely importance of several protein cofactors in class II Ag presentation including

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; GAM, goat anti–mouse Ig; gIi, genomic invariant chain; hMYO, horse myoglobin; HSP, heat shock protein; Ii, invariant chain; MXH, mycophenolic acid, xanthine, and hypoxanthine; MYOT, tryptic digest of MYO; OVAT, tryptic digest of OVA; TGN, *trans*-Golgi network.

the invariant chain (Ii) (16), HSPs (17), and an as yet undefined protein(s) encoded by genes within the MHC (18). It is not known if the same protein cofactors will regulate the presentation of internal versus external Ag or if these cofactors will vary with cell type.

To examine the regulation of class II presentation of an intracellular Ag, we developed an experimental system in which a peptide processed from an MHC class I molecule (L^d) is displayed by the murine class II molecule I-A^d (19). The I-A^d/L^d peptide complex is recognized by a T cell hybridoma derived from the L^d-negative, BALB/c-H-2^{dm2} mouse. This system affords us the opportunity to examine presentation of a typical ubiquitous Ag which, under physiologic levels of expression, gains access to class II independently of an ongoing viral infection. Using recombinant DNA technology and cellular transfection techniques, we altered (*a*) subcellular localization of the class I target Ag; (*b*) lineage of the cell expressing both the target Ag and class II restriction element; and (*c*) expression of Ii within the APC, all parameters of potential relevance for Ag presentation and subject to variation in vivo. Collectively, our data suggest a pathway of endogenous class II Ag presentation, distinct from both the classically described class I and II presentation pathways, which may function selectively in the APC of different lineages.

Materials and Methods

Animals. BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). H-2 L^d-negative BALB/c-H-2^{dm2} (dm2) mice (20) were bred at the University of Chicago in a barrier facility from stock provided by Dr. Roger Melvold (Northwestern University, Chicago, IL).

Tissue Culture Reagents. Cell lines were maintained at 37°C and 5% CO_2 in supplemented RPMI as described (21) plus selection drugs G418 (0.2 mg/ml), MXH (6 μg/ml mycophenolic acid, 0.3 mg/ml xanthine, and 15 μg/ml hypoxanthine) (Sigma Chemical Co., St. Louis, MO), and histidinol (5 mM) as indicated. All media and supplements were purchased from GIBCO BRL (Grand Island, NY) unless otherwise noted. The synthetic peptide corresponding to amino acids 61-85 of L^d was synthesized at the University of Chicago Cancer Center. Chicken OVA and horse myoglobin (hMYO) were purchased from Sigma Chemical Co. and tryptic digests of each (OVAT and MYOT, respectively) were prepared as described by Shimonkevitz et al. (22). Purified soluble L^d/Q10 was provided by L. Boyd and D. Margulies (National Institutes of Health [NIH]).

mAbs. mAb-producing hybridomas reactive with I-A^d (MKD6 [23], M5/114 [24], K24-199 [25], 40B [26]); I-E (14-4-4S [27]); I-A^k (10.2-16 [28]); H-2K^k (16-1-2N [25], 16.1.11N [25]); and with H-2L^d (30.5.7 [29], 28.14.8 [29]) were acquired from American Type Culture Collection (Rockville, MD). 64.3.7 (anti-L^d [30]) was provided by D. Sachs (NIH), whereas F23.1 (anti-TCR Vβ8 [31]) culture supernatant was kindly provided by J. Bluestone (University of Chicago). Ii-reactive mAbs (P4H5 [32], IN-1 [33]) were gifts from J. Miller (University of Chicago). The mAbs 2.43 (anti-CD8 [34]) and GK1.5 (anti-CD4 [35]) were provided by F. Fitch (University of Chicago). Antibodies were used as culture supernatants except for in vivo T cell depletion experiments where an ascites preparation of 2.43 was used.

Flow Cytometry. Cells were evaluated for MHC molecule cell surface expression using mAb as culture supernatants and a secondary staining reagent (FITC-labeled goat anti-mouse Ig [FITC-GAM]; Cappel Laboratories, Cochranville, PA) as described (21), and analyzed on a FACScan® cytofluorimeter (Becton Dickinson & Co., Mountain View, CA). Background control fluorescence was measured using an irrelevant mAb plus FITC-GAM or medium alone and FITC-GAM (as indicated in each figure, see Results).

Gene Constructs. The L^d genomic clone *pL^d.4* was kindly provided by T. Hansen (Washington University, St. Louis, MO) and *pL^d/Q10* (36) was provided by D. Margulies. An L^d cDNA (37) was cloned into the Lk444 (38) vector utilizing the β-actin promoter (*pLd444*), and a leaderless L^d construct (*pL^d_{cyt}*) was generated from *pL^d444* utilizing PCR mutagenesis to remove the sequence encoding the leader peptide from L^d and then adding an initiation sequence (39) 5′ to the α_1 domain of H-2L^d. The altered L^d gene was sequenced and subsequently subcloned into the Lk444 β-actin expression vector.

Transfectants. The class II-negative cell line DAP.3 was transfected with I-A^d α and β chain genes and the selection marker Ecogpt by the calcium phosphate method (40) and selected in MXH. Supertransfection of I-A^d positive cells (DAP-AD) was subsequently performed using a second selection marker (pSV2neo) and either *pL^d.4*, *pLd444*, *pL^d/Q10*, or the *pL^d_{cyt}* construct. Cells resistant to G418 were then subcloned or sorted for cell surface expression of H-2L^d. DAP-LD and DAP-LD/Q10 were derived by transfection of DAP.3 with *pL^d.4* or *pL^d/Q10*, respectively. Those cells transfected with *pL^d/Q10* were subcloned by limiting dilution and screened for L^d/Q10 expression by immunoprecipitation and intracytoplasmic staining.

Ltk^- cells transfected with I-A^d only (LAD) or I-A^d plus genomic Ii (LADgIi) (41), the thymic tumor cell line EL4 transfected by electroporation with I-A^d and *ECOgpt* (ELAD) (42), and a βTC-3 (H-2^d) insulinoma cell line transfected with I-A^d (β-AD) (43) were generously provided by J. Miller. LAD and LADgIi were subsequently transfected with genomic L^d and the selection marker *pSV2his* (44) by the calcium phosphate method yielding LAD-LD (MGT1) and LADgIi-LD (MGT2), respectively. Sublines were selected in MXH, G418, and histidinol. Transfectants matched for class II and L^d expression were obtained by mAb and magnetic bead cell sorting. The EL4 cell line (ELAD) was subsequently supertransfected with genomic L^d (*pL^d.4*) and *pSV2neo* by electroporation, and the resulting cells (ELAD-LD) selected in G418 and MXH.

The embryonic cell line EE2H3 (H-2^k) originally derived by Silverman et al. (45) and provided by J. Bluestone was transfected with I-A^d, genomic L^d, and *pSV2neo* by the calcium phosphate method. EE2H3-ADLD transfectants (GEE.4) were selected in G418 and sorted for I-A^d expression.

T Cell Lines and Hybridomas. The $CD4^+$ T cell clones 93.1 (I-A^d restricted, L^d peptide 61-85 specific) and 50.13 (H-2L^d restricted) were obtained from dm2 mice immunized with full thickness BALB/c skin grafts (19). T cell clones were fused with the hypoxanthine guanine phosphoribosyl transferase (HGPRT) negative thymoma line α^- β^- BW5147 by fusion with polyethylene glycol (PEG 1500; Boehringer Mannheim, Mannheim, Germany) (46). Hybridomas were selected with HAT (GIBCO BRL) and plated at limiting dilution. Hybridomas from the 93.1 clone (GTH) were screened on irradiated (2,000 rad) BALB/c splenocytes and DAP-ADLD transfectants for their ability to produce lymphokines, whereas those from 50.13 (EM50.13) were screened on A20 and DAP-LD.

A $CD4^+$, L^d-reactive bulk T cell population was generated by

immunization of CD8-depleted dm2 mice with irradiated BALB/c splenocytes. In vivo T cell depletion was performed as described (19) and CD8-depleted dm2 mice were then immunized intraperitoneally with 3×10^7 irradiated BALB/c splenocytes. 11 d after immunization, spleen cells from the dm2 mice were subjected to in vitro depletion using anti-CD8 mAb (2.43), anti-Ia mAbs (MKD6, K24-199, M5/114, 14.4.4S), and Low-Tox-M rabbit complement (Cedarlane Laboratories Ltd., Hornby, ON, Canada). Appropriate cellular depletion was confirmed by flow cytometry. For stimulation of the dm2 anti-BALB/c bulk T cell population, 2.5×10^5 T cells were placed in microwells with 5×10^3 mitomycin-treated L cells or 5×10^5 irradiated splenocyte APC. After 48 h of incubation at 37°C, cultures were pulsed with 1 μCi/well of [^{3}H]thymidine and T cell proliferation was measured as a function of [^{3}H]thymidine incorporation. Cultures using L cells as APC were performed in the presence of the prostaglandin inhibitor indomethacin (5 μg/ml).

T Cell Hybridoma Assays. APC used at the concentrations indicated (see figures) and hybridomas used at $5–7.5 \times 10^4$ cells/well were cocultured for 16–20 h and supernatants tested for lymphokine content by culture with the growth factor–dependent T cell line CTLL as described (19). Results appear as mean cpm of triplicate cultures unless otherwise noted.

Cytokine Induction of MHC Expression. APC were treated for the times indicated with 10 U/ml of IFN-γ (Genzyme Corp., Cambridge, MA) and 100 U/ml of TNF (gift of Dr. Hans Schreiber, University of Chicago). Before initiation of the Ag presentation assay, APC were washed thoroughly and an aliquot of APC stained to evaluate cytokine-induced changes in surface MHC expression.

Coculture Assays. Irradiated dm2 or BALB/c splenocytes were incubated with L^d/Q10 containing supernatant as described (see Fig. 3 legend). Cell–cell coculture experiments were performed in 25 cm^2 culture flasks using equal numbers of DAP-AD and DAP-LD/Q10, as indicated (see Fig. 3 legend). L. Boyd and D. Margulies kindly provided us with purified L^d/Q10 and performed quantitative analysis of L^d/Q10 contained in supernatants from DAP-ADLD/Q10 and DAP-LD/Q10 cultures using an ELISA assay.

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE. Metabolic labeling and immunoprecipitation of proteins from transfected cells was performed as previously described (47). Immunoprecipitated material was analyzed by SDS-10% PAGE. All SDS gels were treated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and autoradiographs prepared at −70°C.

Results

The T Cell Hybridoma GTH Maintains the Reactivity Pattern of the Parent T Cell Clone. We generated a T cell hybridoma from the clone 93.1 and α^-,β^- BW5147 (19). The use of a hybridoma diminishes the contribution of accessory molecules to T cell stimulation and provides a more direct method of assessing peptide–MHC complex expression. Fig. 1 demonstrates stimulation of one such T cell hybridoma (GTH) by L cells (DAP.3: H-2^k, class II$^-$) transfected with both the target Ag (L^d) and the I-A^d restriction element (DAP-ADLD). This response was specifically blocked by mAbs directed against I-A^d. GTH also produced lymphokines in response to a synthetic peptide of L^d (amino acids 61–85) added to either live or fixed I-A^d–bearing APC that lack L^d. These results establish that GTH is restricted

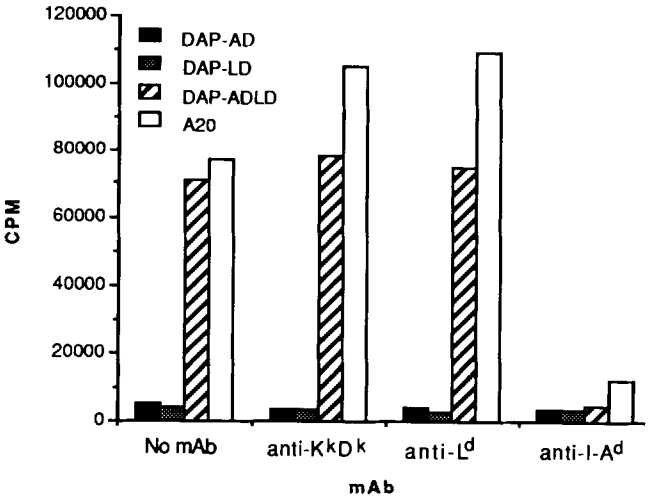

Figure 1. Expression of both I-A^d and L^d is required for stimulation of GTH. 5×10^4 of indicated APC were incubated with 5×10^4 GTH T cell hybridomas for 18 h in an Ag presentation assay as previously described. Shown are cpm of [^{3}H]thymidine incorporated by growth factor–dependent CTLL. The ability of anti-I-A^d (MKD6, M5/114) mAbs and the inability of anti-L^d (30.5.7) and anti-K^kD^k (16.1.2 N) mAbs to block the stimulation of GTH are shown above.

by I-A^d and specific for a fragment of L^d, mirroring the reactivity of the parent dm2-derived T cell clone.

Cell Surface Expression of H-2L^d Is Not Required for Processing and Presentation by I-A^d. The most obvious route for an endogenously synthesized transmembrane Ag to gain access to the endocytic processing compartment is by internalization from the plasma membrane. To determine whether cell surface expression of L^d is required for processing and presentation of the L^d peptide, a gene encoding a soluble form of the target Ag (L^d/Q10) was transfected into DAP.3 with or without the I-A^d restriction element. L^d/Q10 (comprised of the NH_2-terminal α_1 and α_2 domains of L^d and the α_3 COOH-terminal domain of Q10 [36]) associates with β_2 microglobin, maintains normal α_1 and α_2 antibody epitopes, lacks a transmembrane domain, and is thus not expressed at the cell surface, but is secreted into the extracellular medium. Fig. 2 *A* shows metabolically labeled L^d/Q10 precipitated from whole L cell transfectant lysates (lane *3*) or from the extracellular medium (lane *9*). As expected, L^d/Q10 was not detected at the cell surface by immunofluorescence staining (Fig. 3 *D*), nor was an L^d-restricted alloreactive T cell hybridoma (EM50.13) stimulated by L^d/Q10 transfectants (data not shown). Despite a lack of L^d surface expression, DAP-ADLD/Q10 transfectants processed and presented the target L^d peptide to GTH as effectively as DAP-ADLD (Fig. 2 *B*).

To further test the possible involvement of a cell surface intermediate in L^d presentation, we sought to alter processing of L^d and L^d/Q10 with anti-L^d antibodies. If transient surface or extracellular expression of L^d is critical for generation of the GTH-specific peptide, then addition of anti-L^d antibody might be expected to modulate L^d Ag presentation. Despite using a broad panel of anti-L^d antibodies of

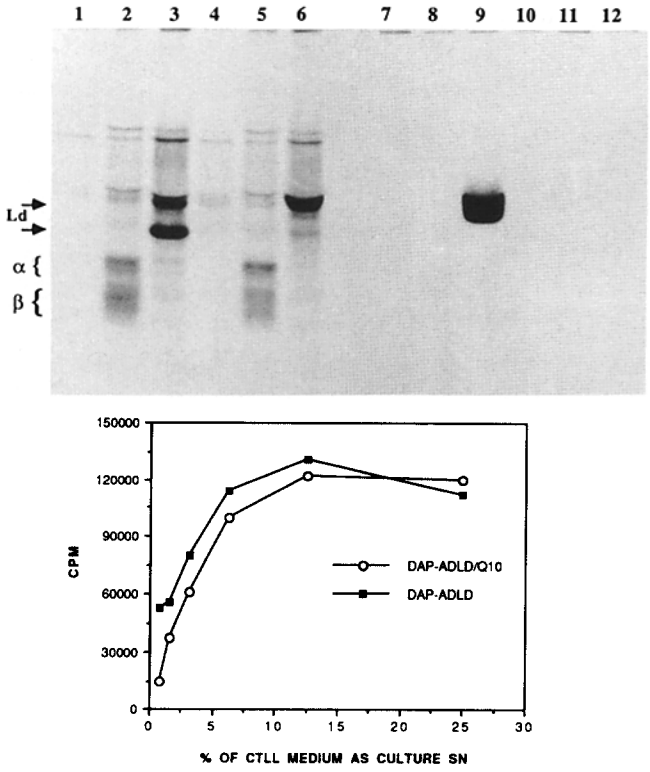

Figure 2. Cell surface expression of L^d is not required for effective processing and presentation of stimulatory determinant(s) to GTH. (*A*) L^d/Q10 is expressed at high levels intracellularly and in the extracellular medium of DAP-ADLD/Q10 transfectants. DAP-AD cells supertransfected with either wild-type genomic L^d (lanes *4–6* and *10–12*) or L^d/Q10 (lanes *1–3* and *7–9*) were labeled for 12 h with [^{3}H]leucine followed by a 2.5-h chase period. Immunoprecipitates from cell lysates (lanes *1–6*) or from the culture medium (lanes *7–12*) were analyzed by SDS-10% PAGE under reducing conditions. L^d molecules (*arrows*) were immunoprecipitated with the mAb 30.5.7 (lanes *3*, *6*, *9*, and *12*). A lower molecular weight form of L^d present in the cell-associated lysates from both L^d/Q10 (lane *3*) and genomic L^d (lane *6*) transfectants represents an immature form of the target Ag (48). Class II α and β chains (*small* and *large brackets*, respectively) were isolated with the mAb M5/114 (lanes *2*, *5*, *8*, and *11*). Control precipitates using the irrelevant mAb 10.2-16 (anti-I-A^k) are shown in lanes *1*, *4*, *7*, and *10* for comparison of background bands. (*B*) DAP-ADLD and DAP-ADLD/Q10 APC were plated at 5×10^4 cells per well with an equal number of I-A^d–restricted, L^d peptide-specific T cell hybridomas (GTH) and incubated at 37°C for 18 h. Supernatants were assayed for lymphokine content. Shown are mean cpm of duplicate cultures resulting from [^{3}H]thymidine incorporation by CTLL in the presence of various concentrations of supernatant from the original APC assay.

varying domain specificity and increasing antibody incubation times, no specific modulation of L^d Ag presentation was detected for either DAP-ADLD or DAP-ADLD/Q10 (data not shown).

Presentation of the Secreted L^d/Q10 Does Not Occur by Internalization from the Medium. To test the possibility that secreted L^d/Q10 enters the class II Ag processing compartment by reuptake from the extracellular medium, DAP-AD cells were incubated for 4 d, or irradiated dm2 splenocytes (spln) were incubated overnight, with supernatant (SN) containing L^d/Q10. Neither DAP-AD nor dm2 splenocytes stimulated GTH when pulsed with L^d/Q10 (Fig. 3, *A* and *B*). The L^d/Q10-containing supernatant used in these experiments was freshly obtained from confluent flasks containing DAP-ADLD/Q10 transfectants, and cells from these same flasks were effective stimulators of GTH (Fig. 3, *A* and *B*, shaded bars). Moreover, purified L^d/Q10 added to DAP-AD at a concentration 1,000-fold higher than that maximally accumulated in the medium of L^d/Q10-secreting cells did not stimulate GTH (Fig. 3 *E*). To test whether suboptimal levels of peptide–MHC complexes were expressed by DAP-AD in the presence of purified soluble L^d/Q10, we performed a dose–response assay using synthetic L^d 61-85 peptide. We reasoned that if a significant but subthreshold level of processed L^d peptide was displayed in the presence of high-dose L^d/Q10, then less L^d 61-85 peptide should be required to stimulate GTH. This should result in a shift to the left in the L^d 61-85 peptide dose–response curve. No such shift in the peptide dose–response curve was evident in the presence of exogenously added soluble L^d/Q10 (Fig. 3 *E*, ▲ vs □). These results strongly suggest that the epitope recognized by GTH is not readily generated after endocytosis of target Ag from the culture medium.

Although exposure of APC to extracellular L^d/Q10 did not result in detectable levels of GTH-specific peptide, we considered the possibility that cell–cell contact might facilitate L^d/Q10 uptake. To address this possibility, cells transfected with L^d/Q10 alone (DAP-LD/Q10) were cocultured with DAP-AD. APC from these mixed cultures did not stimulate GTH, indicating that presentation of the determinant recognized by GTH does not occur by uptake of L^d/Q10 from the medium or from adjacent cells (Fig. 3 *B*, open bar).

To test whether the secreted L^d Ag could serve as substrate for the generation of peptide epitopes other than that recognized by GTH, we generated a polyclonal dm2 anti-BALB/c $CD4^+$ T cell population. As shown in Fig. 3 *C*, $CD4^+$ T cells obtained from immunized dm2 mice were able to recognize and proliferate in response to I-A^d–bearing APC cocultured with either DAP-LD/Q10 cells or supernatant containing L^d/Q10. This suggests that a recognizable epitope(s) is generated from L^d/Q10 after endocytosis, and that this epitope is likely distinct from that generated from internally synthesized L^d/Q10.

In summary, our results using the L^d/Q10 construct indicate that cell surface expression of the target Ag is not required, nor is addition of the target Ag to the extracellular medium sufficient, for generation of peptide–class II complexes recognized by GTH. This suggests that the L^d peptide is generated during intracellular biosynthesis and transport of the newly synthesized target Ag. Thus, internally derived L^d either accesses the class II endosomal processing compartment with far greater efficiency than exogenous Ag, or it employs proteolytic machinery different from that used to process extracellular Ag taken up by endocytosis.

A Cell Line Deficient in Presentation of Exogenous Nominal Ag Efficiently Presents the Endogenously Derived L^d Peptide. To explore the possibility that L^d and exogenous Ag utilize distinct presentation pathways, we examined a cell deficient in exogenous Ag presentation for its ability to process and present

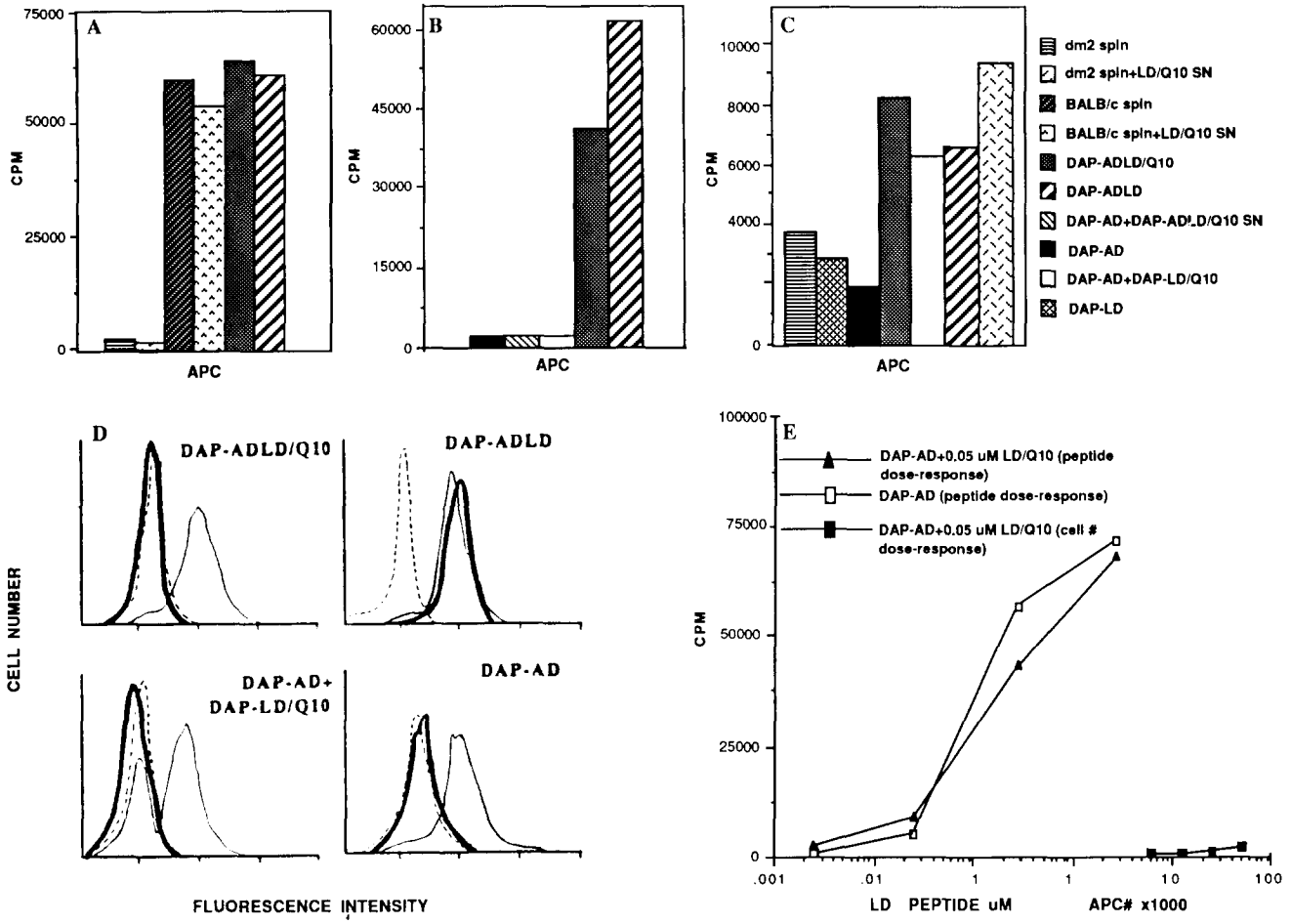

Figure 3. Ag presentation to GTH by cells secreting L^d does not occur by reuptake from the culture medium. (*A*) 5 × 10^5 irradiated dm2 and BALB/c splenocytes (*spln*) were placed in culture with 7.5 × 10^4 GTH T cells in the presence or absence of L^d/Q10-containing supernatant (LD/Q10 SN). DAP-ADLD and DAP-ADLD/Q10 were also examined for their ability to stimulate GTH providing positive controls. (*B*) The Ag presentation assay was performed as described in the legend to Fig. 1 with the following modifications. DAP-AD were preincubated for 4 d in the presence of L^d/Q10-containing supernatant (DAP-LD/Q10 SN) or cultured for 4 d in the presence of L^d/Q10-secreting cells (DAP-LD/Q10) before initiation of the Ag presentation assay and incubation with GTH. 5 × 10^4 APC and responder T cells were placed in triplicate wells of a 96-well plate. Shown are cpm incorporated by CTLL in a secondary culture. (*C*) I-A^d-bearing APC cultured in the presence of L^d/Q10 containing supernatant (LD/Q10 SN) or L^d/Q10-secreting cells (DAP-LD/Q10) stimulate the proliferation of a dm2 anti-BALB/c bulk $CD4^+$ T cell population. dm2 mice were depleted of $CD8^+$ T cells, then immunized with L^d-bearing BALB/c splenocytes. 2.5 × 10^5 of the resulting T cells were then placed in microwells with 5 × 10^3 mitomycin-treated L cells or 5 × 10^5 irradiated splenocytes as indicated. After 48 h of incubation, T cell proliferation was measured as a function of [^{3}H]thymidine incorporation. (*D*) Cell surface staining of cells used in coculture experiments. As indicated, single and mixed cell cultures were stained for I-A^d (———) and L^d (▬▬) surface expression using the mAbs MKD6 and 30.5.7, respectively. After addition of the FITC-GAM secondary staining reagent, cells were fixed and histograms generated by FACScan®. Control staining was performed using FITC-GAM alone (----). (*E*) 5 × 10^4 GTH T cells were placed in microwells with varying numbers of DAP-AD APC exposed for 15 h to 0.05 μM purified L^d/Q10 (■). To examine for subthreshold levels of peptide–MHC complex at the APC surface, peptide dose–response curves were generated using untreated APC (□) or APC pretreated with 0.05 μM purified L^d/Q10 (▲).

endogenous L^d, exogenous L^d/Q10, and synthetic L^d 61-85 peptide. The lymphoblast tumor cell line ELAD (EL4 transfected with I-A^d) (42) is unable to present exogenous Ag to OVA-specific hybridomas (Miller, J., unpublished observations, and 49). Fig. 4 *A* demonstrates the inability of ELAD to stimulate the hMYO-specific, I-A^d–restricted T cell hybridoma ACHhMyo/27.7 in the presence of whole hMyo Ag. In the presence of MYOT, however, ELAD stimulated ACHhMyo/27.7 as effectively as DAP-AD (Fig. 4 *B*). We also found ELAD incapable of presenting exogenous nominal Ag to a panel of I-A^d–restricted hybridomas while ELAD stimulated these same T cells when Ag processing was bypassed by providing peptide (data not shown). This indicates that the Ag presentation defect in ELAD is due to an intracellular processing event, not to a defect in class II or in accessory molecule function. We next examined the ability of ELAD to process and present purified L^d/Q10. As shown in Fig. 5 *D*, ELAD could not utilize exogenously added purified L^d/Q10 to generate peptide–MHC complexes sufficient for GTH stimulation. Moreover, no shift was observed when

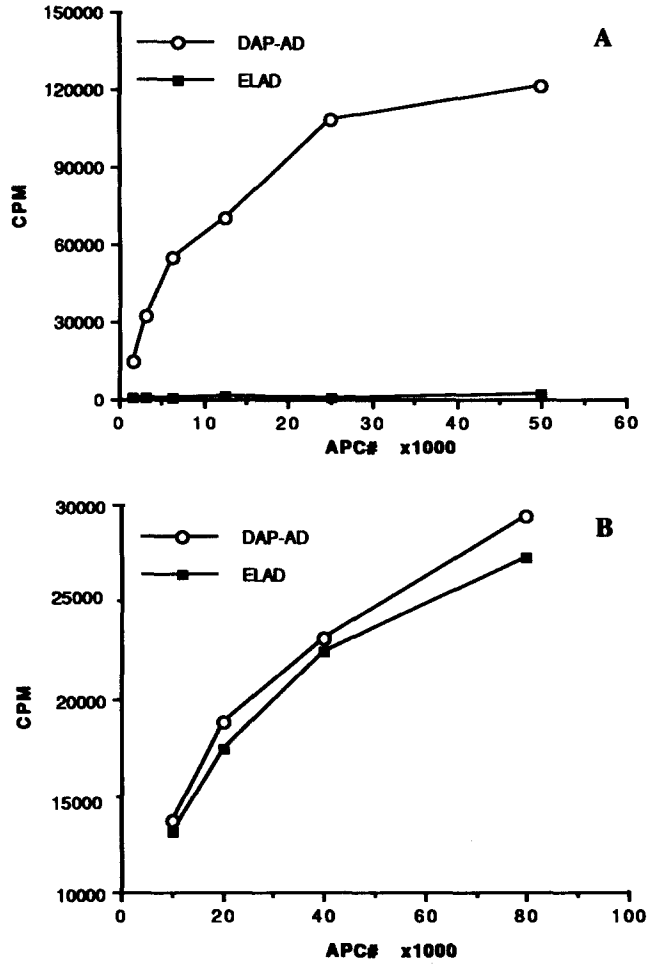

Figure 4. EL4 cells transfected with I-A^d cannot effectively process and present nominal Ag (*A*) but can present peptide (*B*). (*A*) ELAD and DAP-AD cells were incubated with 0.05 mg/ml of hMYO for 11 h, washed, fixed (0.1% paraformaldehyde × 20 min), and plated at varying numbers with 5 × 10^4 I-A^d-restricted, hMYO-specific T cell hybridomas (ACHhMYO/27.7). (*B*) ELAD and DAP-AD APC were fixed as in *A* and then incubated with (10 μg/ml) of MYOT in the presence of ACHhMYO/27.7. The number of APC per well was titrated while T cells were plated at 5 × 10^4 cells/well. T cell lymphokine production was measured by CTLL incorporation of [^{3}H]thymidine.

peptide dose–response curves were generated in the presence or absence of exogenously added soluble L^d/Q10 (Fig. 5 *D*, ▲ vs □). Thus, we conclude that ELAD are deficient in the presentation of nominal Ag including exogenously provided L^d/Q10.

To determine if ELAD can process and present internally derived L^d peptide, ELAD cells were supertransfected with the gene encoding L^d (ELAD-LD) and tested for their ability to stimulate GTH. As shown in Fig. 5 *C*, these cells efficiently stimulated GTH suggesting that events critical for class II presentation of internally synthesized L^d are fundamentally different from those critical for class II presentation of exogenous Ag. A similar conclusion was recently reached by Bikoff (49) using this cell line.

We considered several possibilities to explain this result. First, staining profiles for ELAD-LD and DAP-ADLD using several anti-I-A^d and anti-L^d mAbs (Fig. 5 *A*) demonstrate that the two APC have comparable expression of both the restriction element and the target Ag. Second, it was possible that ELAD effectively presented L^d because of an enhanced avidity of ELAD for the GTH hybridoma. But when Ag processing is bypassed through addition of synthetic L^d 61-85 peptide, ELAD and DAP-AD are nearly equivalent APC for GTH (Fig. 5 *B*). Finally, we considered the possibility that stimulation of GTH occurred because of supersaturating amounts of L^d peptide generated by the internal route of presentation. This excess would allow ELAD-LD to display peptides derived from internal L^d at threshold levels for T cell stimulation compared to subthreshold levels from peptides derived from exogenous Ag. To more accurately quantify the efficiency of presentation, Ag presentation blocking experiments were performed using varying concentrations of anti-I-A^d (M5114) mAb added to ELAD-LD or to control DAP-ADLD APC (Fig. 5 *F*). Parallel blocking studies were performed in L^d-negative DAP-AD and ELAD cells using the L^d 61-85 peptide (Fig. 5 *E*) to control for the adhesion/accessory properties of these APC. Antibody concentrations capable of effecting a 50% reduction in the maximal GTH response were then calculated for each APC. Based on these assays, we conclude that ELAD-LD processing and presentation of internally derived L^d is in fact approximately threefold more efficient than DAP-ADLD (Fig. 5, *E* and *F*). These data argue against the possibility that EL4 transfectants present internal L^d Ag, but not exogenous Ag, because endogenous Ag gain access to the class II presentation pathway more efficiently than exogenous Ag. These results, therefore, support our hypothesis that the L^d peptide is generated by intracellular processing events that may differ from those typically used to process exogenous Ag.

Pancreatic Islet Cells Fail to Present Internally Synthesized L^d. Our experiments using EL4 suggest that presentation of exogenous Ag by MHC class II is not a ubiquitous activity shared by all APC. We therefore examined a large panel of cells for the ability to present the L^d to GTH to evaluate whether presentation of internally synthesized Ag is an activity universally shared by APC. One cell line tested, a pancreatic islet cell line (βTC-3 [H-2^d]) (43) transfected with I-A^d (β-AD), can be induced to express high levels of MHC molecules upon treatment with IFN-γ and TNF (50, 51). The I-A^d islet transfectant was grown for 5 d in the presence or absence of 10 U/ml IFN-γ and 100 U/ml TNF and stained with mAbs reactive with L^d and I-A^d. Fig. 6 *A* shows that in untreated cells (M), the L^d surface staining is only slightly above background levels obtained with an irrelevant control mAb. In contrast, when cells are grown in the presence of cytokine, H-2L^d expression increases 10–20-fold. High levels of L^d surface expression were detected with both peptide-dependent (30-5-7) and -independent (28-14-8) anti-L^d mAb (Fig. 6 *A* and data not shown) suggesting that the MHC class I presentation pathway is functional in the cytokine-treated cells.

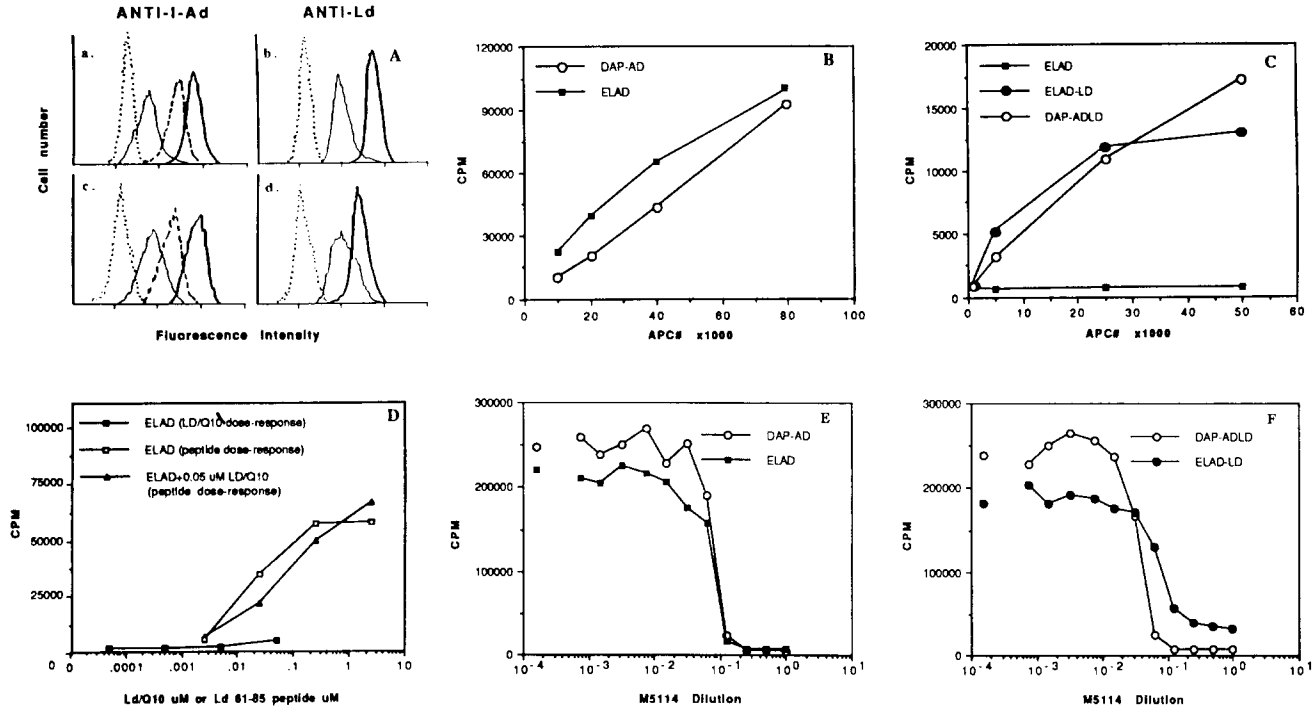

Figure 5. ELAD-LD efficiently presents the endogenously derived L^d target Ag. (*A*) DAP-ADLD and ELAD-LD express similar surface levels of both I-A^d and L^d. DAP-ADLD (*a* and *b*) and ELAD-LD (*c* and *d*) were stained with anti-class II (*a* and *c*) and anti-L^d (*b* and *d*) mAbs. For *a* and *c*, cells were stained with the mAbs MKD6 (———), K24-199 (·····), and 40B (– – –). (*b* and *d*) Staining with the mAbs 30.5.7 (anti-L^d) (———) and 64.3.7 (anti-L^d) (– – –). Background staining was performed using the irrelevant mAb 14.4.4S and FITC-GAM. (*B*) ELAD and DAP-AD APC, fixed to prevent Ag processing, stimulate GTH similarly in the presence of L^d 61-85 peptide. APC, fixed by incubation in 0.1% paraformaldehyde for 20 min, were plated at various cell concentrations with a constant number (5×10^4) of GTH T cells and 0.5 μM of L^d 61-85 peptide. (*C*) ELAD-LD stimulates GTH comparably to DAP-ADLD. ELAD, ELAD-LD, and DAP-ADLD were plated at various cell concentrations with 5×10^4 GTH T cells in an Ag presentation assay. (*D*) 5×10^4 ELAD APC were placed in an Ag presentation assay with increasing concentrations of purified L^d/Q10 (■) and examined for the ability to stimulate GTH. Peptide dose–response curves were generated using ELAD APC, and GTH T cells, and increasing concentrations of L^d 61–85 peptide in the presence (▲) or absence (□) of 0.05 μM L^d/Q10. (*E*) M5114 (anti-I-A^d) mAb was incubated at various concentrations with 5×10^4 APC, 5×10^4 GTH T cells, and a suboptimal dose of L^d 61-85 peptide (0.025 μM). Results are recorded as mean cpm of duplicate cultures. The initial M5114 dilution (10^0) corresponds to 100 μl of mAb culture supernatant in a final vol of 200 μl. Control cpm recorded in absence of M5114 are presented as points adjacent to the y-axis. (*F*) 5×10^4 of the indicated APC and 5×10^4 GTH T cells were incubated in an Ag presentation assay in the presence of decreasing concentrations of M5114. As in Fig. 5 *E*, the initial M5114 concentration was 100 μl culture supernatant in a final vol of 200 μl, and control data points (no mAb) are presented adjacent to the y-axis.

Untreated islet cells (β-AD [M]) did not stimulate GTH (Fig. 6 *B*) but did when provided with exogenously added L^d peptide, indicating that the islet cells express sufficient accessory molecules to effectively interact with the T cell hybridoma. To determine whether increased target Ag expression would permit β-AD to stimulate GTH, we induced L^d expression with IFN-γ and TNF. Time course experiments demonstrated that although islet cell L^d surface expression increased within 24 h of cytokine treatment, presentation of internally synthesized L^d did not occur even after 5 d of treatment (Fig. 6 *B*). Nor was a shift in the L^d 61-85 peptide dose–response curve observed after induction of L^d expression (Fig. 6 *B*). Control experiments demonstrated that TNF and IFN-γ treatment had no effect on β-AD exogenous Ag presentation (Fig. 6, *C* and *D*) and did not antagonize exogenous or endogenous Ag presentation in control APC (Fig. 6, *E–H*). From these experiments, we conclude that expression of endogenous L^d within the default exocytic pathway and at the cell surface is insufficient for presentation of the target peptide to GTH. Rather, these data suggest that presentation of internally synthesized Ag by class II requires protein cofactors in addition to MHC class II, and that these cofactors may be differentially expressed in different cell types.

Expression of a Leaderless Form of the Target Ag Does Not Result in Stimulation of GTH. Transmembrane viral Ag are proteolyzed in the cytosol and presented by class I molecules by a process that presumably employs a pool of viral Ag ineffectively translocated into the endoplasmic reticulum (ER) (52). Since presentation of L^d does not require either internalization from the plasma membrane or endocytic uptake from the extracellular medium, we next sought to determine whether the cytosolic class I pathway is used to generate L^d peptides for I-A^d binding. If this was the case, we predicted three experimental results: (*a*) a cytosolic form of L^d should

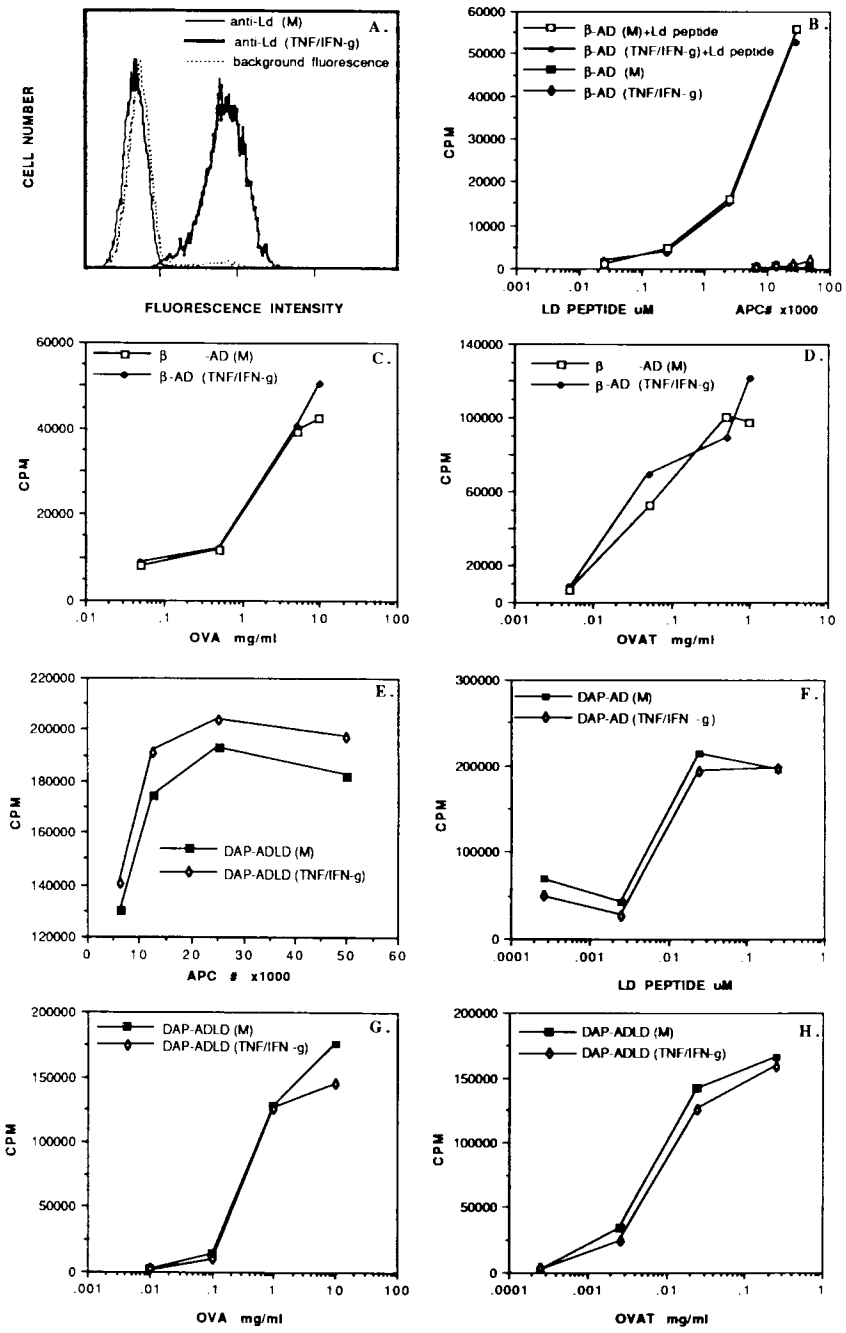

Figure 6. (*A*) β-AD transfectants were incubated for 5 d in the presence (TNF/IFN-γ) or absence (*M*) of TNF (100 U/ml) and IFN-γ (10 U/ml) and then evaluated for MHC expression by flow cytometry. Two-step immunofluorescence staining was performed using the mAb 28.14.8 (anti-L^d) or an irrelevant mAb (*background fluorescence*) followed by FITC-GAM. The resulting histograms are recorded as relative cell number vs mean fluorescence intensity. (*B*) β-AD does not present the endogenously derived determinant recognized by GTH, but effectively presents exogenously added L^d 61-85 peptide. APC were incubated at increasing cell numbers with 5 × 10^4 GTH T cells as previously described. (◇) GTH responses to varying numbers of cytokine-treated β-AD APC; (■) responses to untreated APC. The GTH response to increasing concentrations of synthetic L^d 61-85 peptide was evaluated using 5 × 10^4 cytokine-treated (◆) or untreated control (□) islet cell APC. (*C* and *D*) Untreated (□) or treated (◆; TNF, 100 U/ml and IFN-γ, 10 U/ml for 4 d) islet cell APC were incubated with 5 × 10^4 54.8 T cells and increasing concentrations of OVA (*C*) or OVAT (*D*). (*E–H*) Untreated (■) and treated (◇; TNF, 100 U/ml and IFN-γ, 10 U/ml for 5 d) DAP.3 transfectants were incubated in an Ag presentation assay with 5 × 10^4 GTH (*E* and *F*) or 54.8 (*G* and *H*) T cells under the indicated conditions.

stimulate GTH; (*b*) expression of Ii should block binding of target L^d peptide to I-A^d and, as a result, inhibit stimulation of GTH; and (*c*) a cell line deficient in class I Ag presentation should not present the L^d target peptide and, therefore, should not stimulate GTH.

To address the first hypothesis, we tested the ability of L cells expressing a cytosolic form of L^d to stimulate GTH. A cytosolic form of L^d (L^d_{cyt}) was generated using PCR mutagenesis of a cDNA of L^d to remove the sequence encoding the leader peptide. An initiation sequence was then added 5′ to the L^d α_1 domain. DAP-AD cells were transfected with genes encoding either L^d_{cyt} (DAP-$ADLD_{cyt}$) or wild-type L^d cDNA (DAP-ADLDc) in identical expression vectors. Unlike cells expressing wild-type L^d, cells transfected with L^d_{cyt} were unable to stimulate GTH (Fig. 7 *A*). In addition, peptide dose–response curves for DAP-AD and DAP-$ADLD_{cyt}$ were virtually identical (Fig. 7 *B*) suggesting no appreciable presentation of the cytoplasmic L^d target Ag. The inability of L^d_{cyt} to gain access to the appropriate processing compartment indicates that cytosolic subsets of Ld/Q10 or wild-type L^d that fail to translocate normally into the ER are unlikely to serve as precursors for the processed peptide recognized

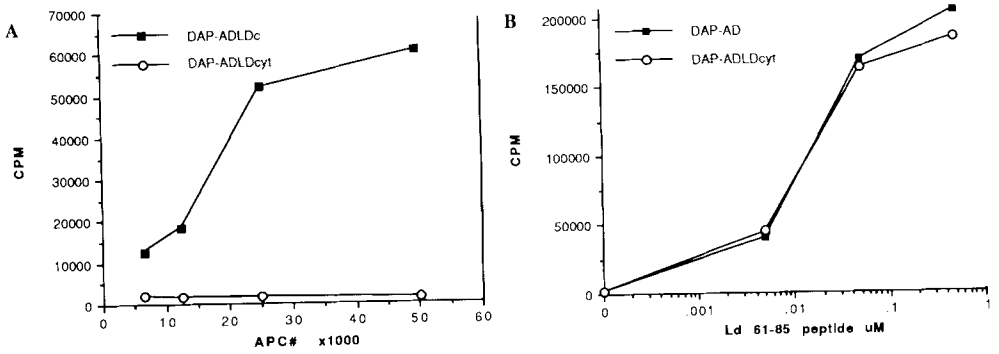

Figure 7. APC transfected with a cytosolic form of the target Ag do not stimulate GTH. (*A*) DAP-AD cells transfected with cDNA encoding a cytoplasmic form of L^d ($DAP\text{-}ADLD_{cyt}$) or wild-type L^d cDNA (*DAP-ADLDc*) were examined for their ability to stimulate GTH. The Ag presentation assay was performed as described in the legend to Fig. 1 using 5×10^4 GTH T cells and increasing numbers of APC per well. (*B*) Subthreshold levels of the L^d peptide–MHC complex are not present on the surface of DAP-$ADLD_{cyt}$ APC. The Ag presentation assay was performed using 5×10^4 GTH T cells and an equal number of APC per well. Peptide was added at the indicated final concentrations at the initiation of the Ag presentation assay.

by GTH. Thus, unlike the results of Townsend et al. (52) in their analysis of class I–restricted presentation of a truncated cytosolic form of influenza hemagglutinin, these results argue that class II–restricted presentation of internally synthesized L^d does not occur via the cytosolic presentation pathway.

Ii Expression Has No Effect on Presentation of L^d Peptide and Stimulation of GTH. To test our second prediction, we examined the effect of Ii on Ag presentation. As a subset of L^d is poorly transported out of the ER (53), it seemed plausible that resident ER proteolytic machinery (54) might process L^d and generate peptides capable of binding newly synthesized class II molecules within the ER. Because experiments performed in vitro have demonstrated that Ii inhibits peptide binding to class II (16, 55, 56), we reasoned that ER presentation should be blocked in APC which express high levels of Ii (Fig. 8 *A*). We therefore examined L^d peptide presentation by Ltk^- APC transfected with I-A^d and L^d with and without genomic Ii (LADgIi and LAD, respectively). As shown in Fig. 8 *C*, the Ii-positive and -negative APC stimulated GTH similarly, indicating that Ag presentation of L^d is independent of Ii expression.

A Cell Line Deficient in Class I–restricted Ag Presentation Presents the Internally Synthesized L^d by MHC Class II. To address whether L^d presentation is dependent on the functional TAP proteins which are important for class I Ag presentation, we examined the ability of a cell line deficient in MHC class I assembly (EE2H3) to stimulate GTH. EE2H3 is an H-2^k embryonic cell line that is deficient in the expression of the putative peptide transporter TAP-1 and has a pheno-

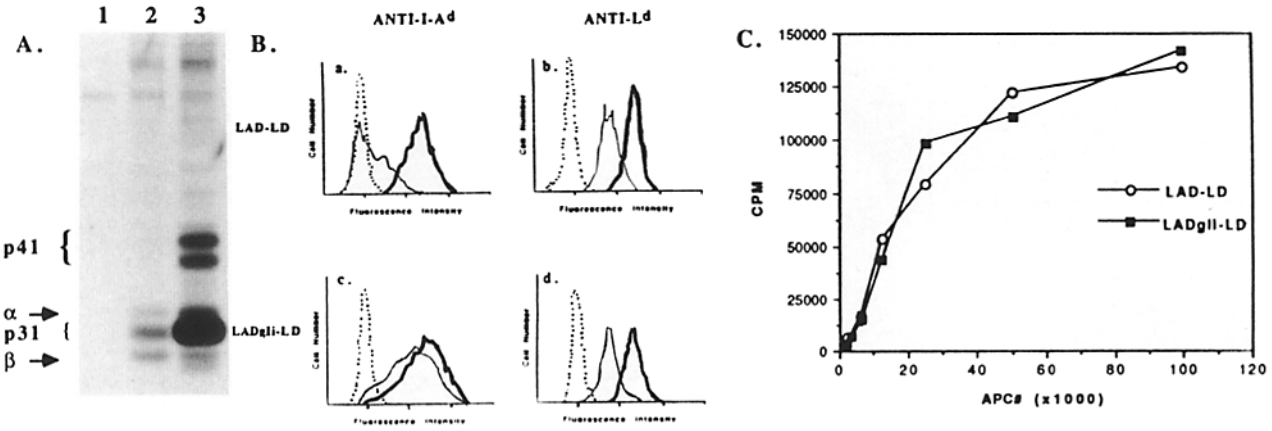

Figure 8. Ii expression does not affect the presentation of endogenous L^d target Ag. (*A*) Immunoprecipitation of LADgIi-LD cell lysates demonstrates saturating levels of Ii in association with class II. Cells were labeled for 30 min with [^{3}H]leucine and Ii precipitated from the lysates with the mAbs IN-1 and P4H5 (lane *3*), whereas I-A^d was precipitated using the mAb MKD6 (lane *2*). The proteins were separated on SDS-10% PAGE under reducing conditions. ({) p31 and p41 Ii forms; (→) α and β chains. (Lane *1*) Demonstrates precipitation with an irrelevant mAb for comparison of nonspecific background bands. (*B*) Flow cytometry of Ii^- LAD-LD (*a* and *b*) and Ii^+ LADgIi-LD (*c* and *d*) transfectants was performed as previously described. For *a* and *c*, cells were stained with the anti-class II mAbs MKD6 (———) (anti-I-A^d, Ii-independent) and 40B (———) (anti-Ia, Ii-dependent) (39, 40). (*b* and *d*) Surface staining using the anti-L^d mAbs 30.5.7 (———) and 64.3.7 (———). (·····) Staining with an irrelevant mAb (14.4.4S, all panels). (*C*) Ii expression does not inhibit presentation of endogenous L^d to GTH. LAD and LADgIi transfectants differing only in Ii expression were subsequently supertransfected with genomic L^d yielding LAD-LD and LADgIi-LD, respectively. These APC were then examined for their ability to stimulate GTH. Hybridomas were plated at 5×10^4 cells/well with varying numbers of APC.

type resembling that of RMA-S (45, 57). EE2H3 was transfected with genes encoding L^d and I-A^d and the resulting transfectants (EE2H3-ADLD) tested for the ability to stimulate GTH. As shown in Fig. 9, EE2H3-ADLD effectively stimulated GTH despite near background levels of L^d surface staining. In conjunction with the results described above, these data suggest that class II–restricted presentation of L^d to GTH may not follow typical class I or II presentation pathways.

Discussion

Class II molecules are specialized to present peptides derived from exogenous Ag but are also capable of presenting peptides derived from proteins synthesized within the APC. Most of the studies addressing the mechanisms of class II Ag presentation have focused on the former (58, 59). These studies, in conjunction with those examining class II trafficking (60–62), the relationship between class II and Ii (16, 42, 55, 56), and the formation of stable class II dimers (63), have led to the general model that class II binds to peptides derived from Ag that enter the APC by endocytosis and are proteolyzed within acidic vesicles of the endosomal/lysosomal pathway. Clearly, internally synthesized secretory or integral membrane proteins theoretically have access to the putative endosomal/lysosomal processing pathway. However, it is becoming increasingly clear that internal Ag may access endosomes/lysosomes by mechanisms other than endocytosis, including chaperone-mediated trafficking from the cytosol, autophagy from the cytosol and ER, and direct trafficking from the TGN. It is less clear whether the route by which an Ag enters the endosomal/lysosomal pathway affects the repertoire of peptides revealed (64), or if different routes employ different processing cofactors. For example, prolonged Ag expression within the ER or Ag interaction with HSPs might influence processing such that the dominant epitopes generated are different from those revealed after endocytosis from the extracellular fluid.

We have established a model system designed to examine the mechanisms governing MHC class II presentation of an endogenous Ag. Unique to our system is the use of a target Ag (MHC class I, L^d) that is constitutively expressed by a variety of nucleated cells, providing us with the opportunity to study the processing requirements for a normally expressed protein in cells of different lineages. T cells recognizing this epitope are stimulated by unmanipulated BALB/c splenocytes indicating that this peptide–class II complex is not only one which APC can be designed to generate, but is also one which is naturally displayed in vivo.

One possible explanation for the presentation of internally derived surface proteins by class II is that these proteins gain access to the endocytic pathway by internalization from the cell membrane. Using a secreted form of L^d, we demonstrated that surface expression is not essential for L^d presentation. Experiments using purified soluble L^d/Q10 demonstrated that GTH was not stimulated even at Ag concentrations 1,000-fold greater than those present in the culture medium of DAP-ADLD/Q10 transfectants. Moreover, dm2 splenocytes, which include cells considered to be professional APC, were unable to present the exogenously added soluble form of L^d. These results, coupled with those obtained in a series of coculture experiments, suggest that the secreted target Ag does not gain access to the class II presentation pathway by uptake from the extracellular milieu. We are confident that 4 d of coculture provided more than adequate time to accumulate MHC–peptide complexes at the cell surface because I-A^d–positive APC transiently transfected with L^d were

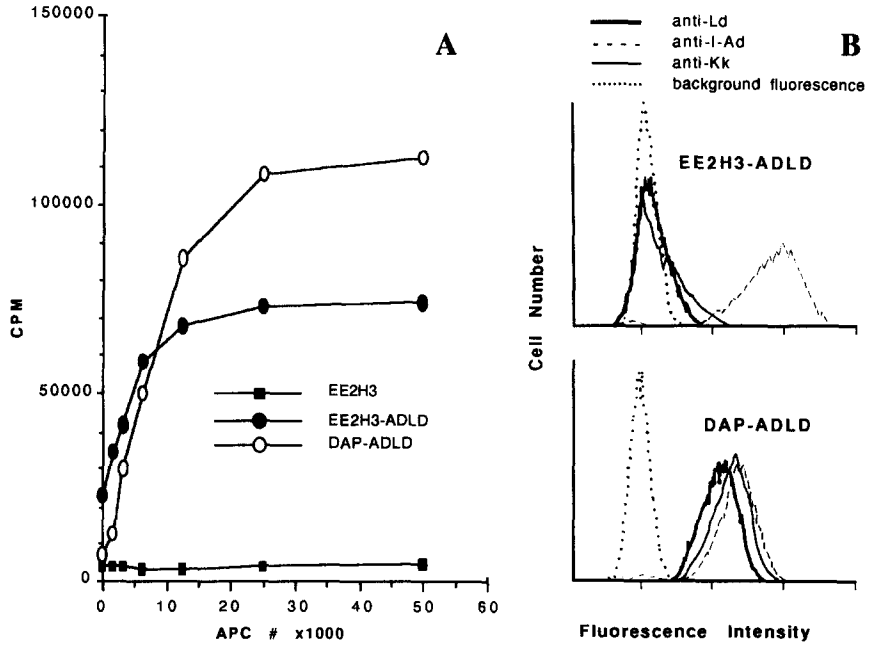

Figure 9. (*A*) EE2H3-ADLD stimulates GTH. EE2H3 cells transfected with I-A^d and L^d were examined for their ability to stimulate GTH. DAP-ADLD and untransfected EE2H3 were examined in parallel as positive and negative controls, respectively. APC were plated at increasing cell numbers with 5×10^4 GTH T cells. (*B*) EE2H3-ADLD (H-2^k) express low levels of class I. EE2H3-ADLD (*top*) and DAP-ADLD (*bottom*) transfectants were stained with MKD6 (anti-I-A^d, ·····), 28.14.8 (anti-L^d, ———), 16.1.11N (anti-K^k, ———) and with an irrelevant antibody 14.4.4S (•••••••) followed by FITC-GAM as previously described.

effective stimulators of GTH within 2 d of transfection (data not shown). The finding that soluble L^d/Q10 added to I-A^d–bearing APC resulted in the stimulation of a bulk population of $CD4^+$ anti-BALB/c T cells demonstrates that peptide determinants other than those recognized by GTH were generated from L^d/Q10. Attempts to modulate L^d processing and presentation with exogenously added anti-L^d antibody proved ineffective using APC expressing either the cell surface form of L^d or the secreted L^d/Q10 (data not shown). This is consistent with our hypothesis that a cell surface or extracellular intermediate is not involved in generating the L^d peptide recognized by GTH. Moreover EL4, a cell line defective in exogenous Ag presentation, effectively processed and presented internally synthesized L^d. Taken together, these data support the hypothesis that processing of L^d resulting in the peptide recognized by GTH takes place during biogenesis and intracellular transport of the target Ag.

Our experiments also address whether the L^d target Ag accesses class I proteolytic machinery within the cytosol. Transfection of APC with a cytosolic form of L^d did not result in stimulation of GTH. This is consistent with class II–restricted endogenous Ag presentation systems reported by Brooks et al. (10) and Weiss and Bogen (7), although several studies have indicated that cytosolic proteins may gain access to class II if they are expressed at very high levels (6, 8). Attempts to augment expression of L^d_{cyt} by increasing gene copy number in a transient transfection assay or by regulating expression with the strong β-actin promoter did not result in stimulation of GTH, nor was L^d_{cyt} presentation revealed by subjecting APC to serum starvation or heat shock (data not shown). These results suggest it is unlikely that improperly translocated L^d plays any role in stimulation of GTH. In support of this conclusion, transfectants of EE2H3 (45, 57), a cell line deficient in class I Ag presentation, effectively stimulated GTH. Finally, using three different cell types, we have found that Ii fails to block class II–restricted presentation of L^d. It is therefore improbable that the classical class I processing pathway generates the L^d peptide recognized by GTH.

One of the most intriguing results from this series of experiments relates to the differences observed between different cell types in exogenous and endogenous Ag presentation. A lymphoblast tumor cell line (EL4) defective in exogenous class II Ag presentation effectively stimulated GTH. In contrast, a pancreatic cell line (βTC-3) capable of exogenous Ag presentation, was unable to present endogenous L^d despite high surface expression of both the restriction element and the L^d target Ag. These results suggest that to generate and present the L^d peptide, it is insufficient to merely express the target Ag and the appropriate MHC class II restriction element. Taken together, our results suggest that different cofactors may regulate endogenous and exogenous class II Ag presentation and that expression of these cofactors may be regulated in a tissue-specific manner. This possibility is consistent with earlier work showing that some APC, differing in cell type or physiologic state, show differences in their ability to present epitopes derived from extracellular Ag (65, 66). Although the biological basis for the observed differences remains to be determined, our observations suggesting that different cell lines process and present Ag differently have potential implications for a number of immune phenomena including thymic selection, where different APC types are believed to be responsible for positive and negative selection, and autoimmune responses, where tissue-specific Ag presentation might play a role in the pathogenesis of localized, T cell–mediated autoimmune disease.

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