

# Expression of a Class II Major Histocompatibility Complex (MHC) Heterodimer in a Lipid-linked Form with Enhanced Peptide/Soluble MHC Complex Formation at Low pH

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## Summary

A murine class II major histocompatibility complex (MHC) heterodimer, E<sup>k</sup>, expressed as a glycan-phosphatidyl inositol-anchored chimera on Chinese Hamster Ovary cells, can present peptides, but not processed antigen to T cells. This chimeric MHC requires a 100-times higher peptide concentration to achieve a two- to four-times lower level of T cell stimulation. Cleavage with phosphatidylinositol-specific phospholipase C and purification result in large quantities of heterodimer in a water-soluble form. Plates coated with this material and then incubated with peptide can efficiently stimulate the appropriate T cell hybridomas. This stimulation is significantly enhanced when peptides are preincubated with the plate-bound MHC molecules in a pH range (5.0–5.5) similar to that of late endosomes. More than half of the soluble E<sup>k</sup> molecules can form a specific complex with cytochrome *c* peptides in this pH range. This suggests that class II MHC molecules undergo distinct conformational changes in endosomal compartments that render them more capable of forming functional complexes with peptide antigens, irrespective of other cell components.

T lymphocytes, through their specific antigen receptors, recognize fragments of foreign antigens complexed to molecules of the MHC. Class I MHC molecules appear to present peptides from endogenously synthesized proteins, whereas class II molecules present peptides derived from proteins that have been endocytosed and degraded (1, 2). Numerous examples have been documented demonstrating binding of specific synthetic peptides to isolated class II MHC molecules, resulting in the formation of functional complexes that can be recognized by T cells (3–8). One discrepancy emerging from these studies is that peptide uptake and MHC presentation *in vivo* are more efficient than can be accounted for by the relatively slow rates of peptide/MHC complex formation observed *in vitro* (9). Another problem is that the amount of MHC capable of binding exogenous peptide seems very limited (1–20%) (8), presumably due to the presence of endogenous peptides (10). One way to account for these anomalies is to postulate a role for accessory molecules, which might enhance peptide uptake (11). Alternatively, Jensen (12) has shown that class II MHC-bearing cells that have been fixed with paraformaldehyde can stimulate T cell hybridomas more efficiently after incubation with peptides at weakly acidic pHs (4.5–5.5; approximating those of the late endosomal and lysosomal compartments) (13). As class II MHC molecules

are known to traverse the endosomal compartments (14–17), and at least in some cells seem to be recycled an average of 60 times (18, 19), it is possible that some or all of the discrepancies observed between *in vivo* antigen presentation and the binding of antigen to purified MHC *in vitro* might be explained by passage through an acidic environment. Since the experiments cited involved fixed cells (12), other explanations for this effect are also possible, including bystander molecules and lipid interference or cooperativity.

In our studies, we have been interested in expressing mouse class II MHC molecules in a soluble form in order to study their interactions with peptides and with cognate TCR molecules under detergent-free conditions. We report here a strategy for doing so using the signal sequences for lipid-linked expression derived from human placental alkaline phosphatase (HPAP),<sup>1</sup> which we have defined and successfully used to express a TCR heterodimer (19). E<sup>k</sup> molecules expressed in this glycan-phosphatidyl inositol (GPI)-anchored

<sup>1</sup> Abbreviations used in this paper: CPB, citric acid phosphate buffer; GPI, glycan-phosphatidyl inositol; HPAP, human placental alkaline phosphatase; MCC, moth cytochrome *c*; n, native; PCC, pigeon cytochrome *c*; PI-PLC, phosphatidylinositol-specific phospholipase C.

form are efficiently removed from the cell surface with a specific enzyme, phosphatidylinositol-specific phospholipase C (PI-PLC) (20). Cells bearing lipid-linked (pi) E<sup>k</sup> can present peptide antigens to most T cell hybridomas with an apparent fine specificity that is indistinguishable from wild-type E<sup>k</sup> molecules. In contrast, they are unable to present a whole antigen efficiently, indicating that either the efficiency of cellular uptake of exogenous antigens is affected, or that the chimeric MHC molecule does not traverse the same compartments as the normal molecule. Of particular relevance to the questions discussed above, we have developed a new assay for antigen presentation to T cell hybrids, using tissue culture plates coated with purified PI-PLC-solubilized E<sup>k</sup> and a standard IL-2 assay. Using this assay, we have shown that functional antigen/MHC complexes are formed more efficiently at endosomal pHs (5.0–5.5) than at neutral pH. This indicates that at least part of the discrepancy between the in vivo antigen presentation results and binding of peptide to purified class II MHC molecules lies in the latter being more efficient at these lower pHs. We have also used this finding to generate specific peptide-MHC complexes at high efficiency (> 50%), which will allow a wide range of physical studies to be possible.

## Materials and Methods

**Constructs.** DNA encoding HPAP residues 476–513 (21) was cloned into plasmid Bluescript SK<sup>-</sup> (Stratagene), as described (19). Full-length E<sup>k</sup>  $\alpha$  and  $\beta$  chain cDNAs (22) were cloned into the same vector 5' of the HPAP sequences. E<sup>k</sup>/HPAP in-frame chimeric constructs were then produced using site-directed mutagenesis (23). Correct fusions were confirmed by DNA sequencing using the Sequenase Sequencing Protocol (U.S. Biochemical Corp., Indianapolis, IN). The fused inserts for the  $\alpha$  and  $\beta$  chains were cloned into the eukaryotic expression vector pBJ-Neo in the fashion described for TCR  $\alpha$  and  $\beta$  chains (19). The resulting construct is called pBJ1-Neo/MHC $\alpha\beta$ .

**Oligonucleotides.** The oligonucleotides used to create the chimeras were as follows:  $\alpha$ , 5'-GCCGGCGGGGGCGCCAGG-AGGAGGGTTTTCTC;  $\beta$ 1, 5'-GCCGGCGGGGGCGCCAGTGTGGACTGTGCTTC;  $\beta$ 2, 5'-GCCGGCGGGGGCGCCAGCTGTGCAGATGTGGAC. Synthesis was carried out on a DNA synthesizer (380B; Applied Biosystems, Inc.) (Oligonucleotide Service Center, Dept. of Cell Biology, Stanford).

**Transfections.**  $5 \times 10^6$  CHO cells were trypsinized, washed, and resuspended in 0.5 ml  $1 \times$  HeBS (24) containing 5  $\mu$ g pBJ1-Neo/MHC- $\alpha/\beta$  linearized at the unique vector *Aat* II site. Transfection was by electroporation using a Gene Pulser (Bio-Rad Laboratories) set to 500  $\mu$ F and charged at 230 V for pulse delivery. Cells were iced 10 min before and after electroporation, and then plated as a pool. 36 h after transfection, G418 was brought to 0.5 mg/ml (active drug) and resistant cells were carried as a heterogeneous culture.

**FACS<sup>®</sup> Analysis/Cloning.** For FACS<sup>®</sup> analysis (Becton Dickinson & Co., Mountain View, CA) adherent cells were brought into suspension by treatment with 5.3 mM EDTA in PBS and washed. Cells were stained with the following mAb reagents in the order: Y17 (25) (gift of P. Jones), goat anti-mouse FITC (Sigma Chemical Co., St. Louis, MO), biotin-conjugated 14-4-4 (26) (gift of I. MacNeil) with 1% normal mouse serum, and avidin-conjugated allophycocyanin (biomeda) or PE (biomeda). FACS<sup>®</sup> analyses were

performed essentially as described (27). For FACS<sup>®</sup> cloning G418-resistant cells were stained as above and single cells from the approximately brightest staining 5% were sorted into microtiter wells, expanded, and rescreened by FACS<sup>®</sup> analysis.

**Plate Activation and Lymphokine Release Assays.** IL-2 assays were carried out essentially as previously described in RPMI 1640 supplemented with 10% FCS, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamate, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (28). Briefly,  $10^5$  APC, antigen, and antibody (as described in each figure), and  $10^5$  2B4.11A cells or Jurkat 2B4 (Patten, P.A., E.P. Rock, T. Sonoda, B. Fazekas de St. Groth, and M.M. Davis, manuscript submitted for publication) were added to microtiter wells in a final volume of 0.2 ml for 16–24 h at 37°C. 2B4.11A was derived from 2B4 by recloning and then screening for a high IL-2 producer. For plate activations with soluble MHC, 0.05 ml of 10  $\mu$ g/ml soluble E<sup>k</sup> in PBS was incubated at 4°C overnight to coat microtiter wells (Immunolon 4 plates; Dynatech Laboratories), after which antigen was added at various pHs in McIlvaine's citric acid-phosphate buffer system (CPB) (29), and allowed to incubate for 24 h at 37°C. The wells were then washed twice with 1% FCS/RPMI 1640, and T cells were added in RPMI supplemented with 10% FCS, and other ingredients, as described above. Supernatants from activation wells were analyzed for IL-2 by culturing serial dilutions of each (0.05 ml) with  $4 \times 10^3$  HT-2 cells (0.05 ml) (30). After 22 h at 37°C, wells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine and incubated for another 4 h at 37°C, after which [<sup>3</sup>H] incorporation was determined. 1 U/ml of IL-2 was defined as the concentration at which half-maximal incorporation occurred. The IL-2 data from a standard and from test supernatants was simultaneously fitted with a four-parameter logistic curve using the ARCUS RADIMM program (adapted from Dr. D.J. Finney by Dr. Tony Kyne) to calculate the IL-2 activities.

**Peptides.** Peptides (kindly provided by C. Turck) used in the T cell activation assays were as follows: PCC 81-104, IFAGIK-KKAERADLIAYLKQATAK; MCC 83-103, biotin-AGIKK(K) ANERADLIAYLKQATK; MCC 88-103, ANERADLIAYLKQATK; MCC 88-102, ANERADLIAYLKQAT; MCC 87-103, biotin-KANERADLIAYLKQATK. MCC 83-103 has an additional lysine residue (K) as indicated, due to a clerical error (M.M. Davis). This does not seem to affect either MHC binding or T cell stimulatory activity, however. Peptide controls (kindly provided by T. Mietzner) for binding studies were as follows: OVA 323-339 ISQAV-HAAHAEINEAGR; HEL 46-61 NTDGSTDYGLQINSR.

**Whole Cytochrome c Assays.** IL-3 assays were carried out as follows.  $5 \times 10^4$  5C.C7 T cells and  $5 \times 10^4$  native (n) E<sup>k</sup> or  $\pi$  E<sup>k</sup> APCs were incubated in dilutions of PCC protein (Sigma Chemical Co.) or MCC 83–103 in 200  $\mu$ l 10% FCS/RPMI 1640 in microtiter plates at 37°C. PCC protein and MCC peptide were purified on C-18 reverse phase HPLC using an acetonitrile gradient in 0.1% TFA. After 24 h, the supernatant was assayed for IL-3 activity using the indicator cell line R6X (31).  $5 \times 10^3$  cells were incubated in 100  $\mu$ l in a microtiter plate containing supernatant dilutions. After 42 h, the cells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine for an additional 6 h. The cells were harvested onto glass microfiber filters and [<sup>3</sup>H]incorporation was determined. IL-3 units were calculated as described for IL-2.

**Affinity Purification of Soluble E<sup>k</sup> Molecules.** Soluble E<sup>k</sup> heterodimers were purified from pi E<sup>k</sup> cells as previously described for GPI-linked TCR (19) with the following differences. The supernatant was brought to 5 mM EDTA, 1 mM PMSE, 1  $\mu$ g/ml pepstatin A, 1  $\mu$ g/ml leupeptin, pH 8 (NaOH), and 5 mM NaN<sub>3</sub>, filtered, and then passed over a 14-4-4 affinity column (CNBr Sepharose; Pharmacia Fine Chemicals, Piscataway, NJ). The column

was washed with PBS (pH 8) containing the above cocktail of inhibitors and 10% glycerol. The elution buffer was the same as the wash, except that 50 mM diethylamine, pH 10.5/0.15 M NaCl replaced the PBS. The eluate was neutralized with 1 M sodium phosphate, pH 6.5.

**Gel Electrophoresis.** Protein samples were run on a nonreducing 12% SDS-PAGE (32) gel for the visualization of peptide/MHC complexes. Samples were loaded immediately on the nonreducing gel without prior boiling. Protein bands were visualized by silver staining (33).

**Protein Sequencing.** An aliquot of affinity-purified soluble E<sup>k</sup> was precipitated with 10% TCA and then washed with acetone. NH<sub>2</sub>-terminal protein sequencing of this material was carried out in the presence of 1 mg polybrene using a protein sequencer (470A; Applied Biosystems Inc.) with an on-line HPLC (Protein and Nucleic Acid Analytical Facility, Beckman Center, Stanford, CA).

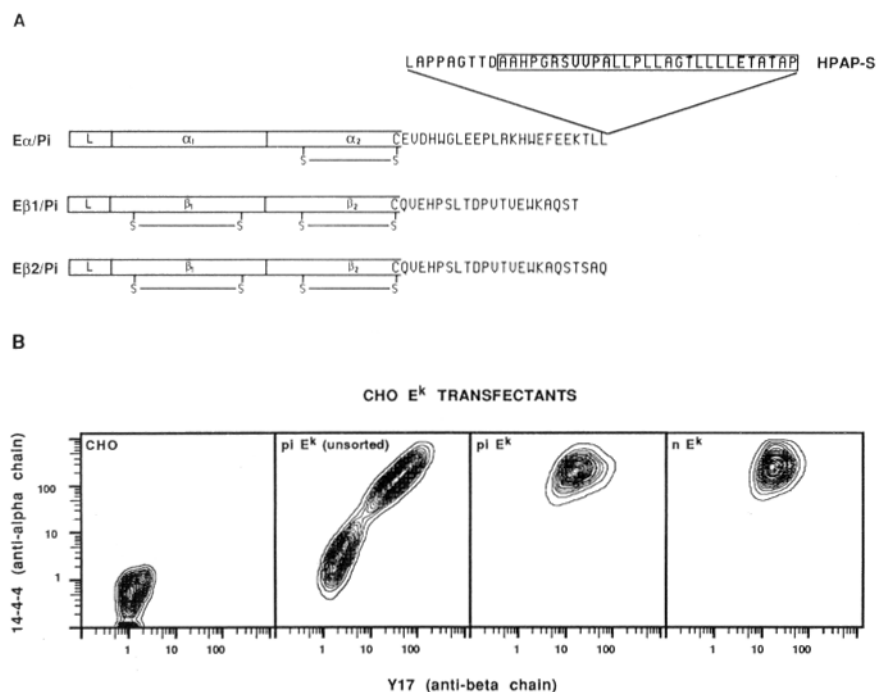
**Peptide Binding by ELISA.** Soluble E<sup>k</sup> at 500 nM was incubated with 15 μM of the biotinylated peptide MCC 83-103 in CPB at varying pH for 60 h at 37°C. Reactions were adjusted to pH 7 by addition of 2 M Na<sub>2</sub>HPO<sub>4</sub> before addition of BSA to a final concentration of 2% (wt/vol). To detect soluble E<sup>k</sup>-associated peptide, microtiter wells (Dynatech Labs) were coated with rabbit antiserum specific to the GPI "tail" by overnight incubation with 50 μl of a 10 μg/ml dilution in PBS at 4°C. After blocking of nonspecific binding by incubation with CPB containing 2% BSA, the binding reactions were "captured" for 90 min at room temperature. MHC-associated peptide was detected using alkaline phosphatase-conjugated avidin (Vector Laboratories, Inc.), before development with the 104 phosphatase substrate (Sigma Chemical Co.). A microplate reader (Molecular Devices) was used to monitor optical density at 405 nm, and this absorbance is directly proportional to the percentage of soluble E<sup>k</sup> molecules associated with biotinylated peptide.

**Peptide Binding for the Visualization of Peptide/MHC Complexes.** Soluble E<sup>k</sup> at 740 nM was incubated with a 10-fold or 100-fold molar excess of MCC peptides (MCC 83-103 or MCC

87-103) or a 100-fold molar excess of control peptides (I-A<sup>d</sup>-restricted OVA 323-339 or I-A<sup>k</sup>-restricted HEL 46-61) in CPB (pH 5.2) for 20 h at 37°C. Before SDS-PAGE, each sample was neutralized by the addition of 2 M Na<sub>2</sub>HPO<sub>4</sub>. As additional controls, MCC 83-103 was incubated with soluble E<sup>k</sup> at a 10-fold excess in CPB (pH 7.0), and MCC 83-103 and MCC 87-103 were incubated together with soluble E<sup>k</sup> at a fivefold excess of each in CPB (pH 5.2).

## Results

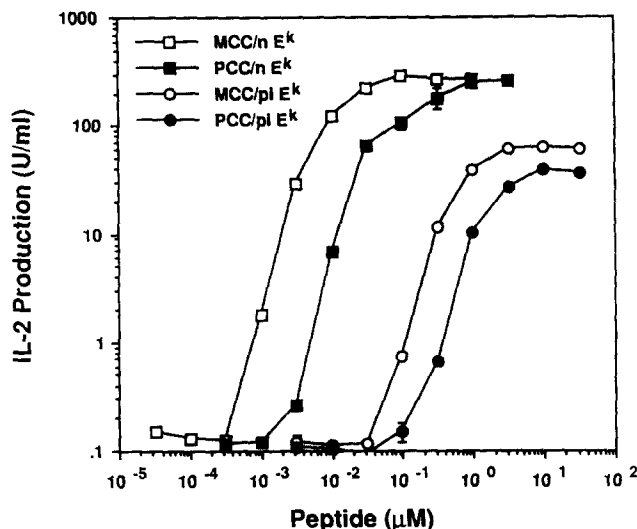
**E<sup>k</sup>/HPAP Chimeric Constructs.** To produce E<sup>k</sup> molecules in a soluble form, we replaced the COOH termini, including the transmembrane regions, of both α and β chains with a signal sequence for GPI linkage, as shown in Fig. 1 A. Previous work had shown that a number of transmembrane proteins can be expressed in this way (34, 35), and in our own work, we have shown that the 38 COOH-terminal amino acids of HPAP are sufficient to act as a signal sequence for GPI linkage of a TCR heterodimer (19). Two different E<sup>k</sup> β chain/HPAP fusions were constructed in order to increase the likelihood that the two chains of E<sup>k</sup> would be in the proper register relative to each other to allow heterodimer formation. One, designated Eβ1/Pi, was made following the same strategy applied in making Eα/Pi, lining up the two sequences from the beginning of the transmembrane region. The second, designated Eβ2/Pi, was constructed using the most COOH-terminal cysteine as the point of reference between the α and β chains. If the chimeric molecules are processed in the same way as HPAP, then the aspartic acid residue in the HPAP sequence will be both the COOH-terminal amino acid residue of the processed chimeric proteins (36) and the point at which these molecules are coupled to the membrane via the GPI moiety (37).



**Figure 1.** Chimeric E<sup>k</sup>/HPAP α and β chain constructs and expression in CHO cells. (A) Schematic diagrams of the E<sup>k</sup>/HPAP single-chain constructs showing the domain structure of each chain and the sequences contributed by each protein. For E<sup>k</sup> sequences, lettering begins with the COOH-terminal half of the intrachain cysteine of the second domain. The 38 COOH-terminal residues of HPAP (HPAP-S) (19) are depicted, and these were fused to each chain. The nine unboxed residues of HPAP-S are those that are predicted to remain in the chimeric chains after formation of the GPI linkage (36). Standard amino acid single letter abbreviations are used. (B) G418-resistant CHO transfectants were stained with anti-α chain (14-4) vs. anti-β chain (Y17) mAbs. As indicated in the figure, the four samples are CHO untransfected (CHO), CHO transfected with Eα/Pi and Eβ1/Pi (pi E<sup>k</sup> [unsorted]), a clone derived from the latter (pi E<sup>k</sup>), and a clone derived from n E<sup>k</sup>-transfected CHO (n E<sup>k</sup>).

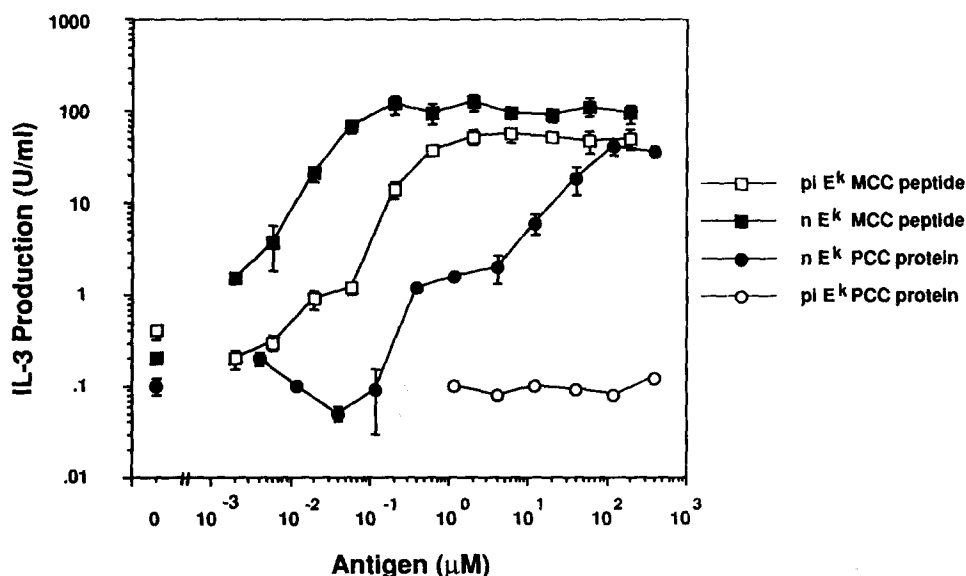
**Chinese Hamster Ovary (CHO)  $E^k$ /HPAP Transfectants.** CHO cells were used as the recipients of  $E^k$ /HPAP chimeric constructs. The cells were transfected with  $E\alpha$ /Pi and either  $E\beta 1$ /Pi or  $E\beta 2$ /Pi inserted together into pBJ1-Neo (as described in Lin et al. [19]), and selected for G418 resistance. The native  $\alpha$  and  $\beta$  chain cDNAs were also inserted into pBJ1-Neo and transfected into CHO cells for use as controls. Cells that survived selection were analyzed by staining with anti- $E\alpha$  and anti- $E\beta$  mAbs, and the FACS<sup>®</sup> profiles are shown in Fig. 1 B (second panel). The initial sample of G418-resistant (pi  $E^k$ ) cells analyzed originated from a pooled transfection and contained two populations of cells, one staining brightly and one weakly. The cells were positive for both chains, with a diagonal pattern of staining indicating that for any given cell, the amount of  $\alpha$  chain staining is comparable with that of the  $\beta$  chain. This suggests that only  $E^k$   $\alpha/\beta$  heterodimers are expressed on the cell surface, unlike the case of TCR GPI chimeras, where monomers are also expressed (19). This interpretation is consistent with the fact that cells transfected with only one GPI-linked  $E^k$  chain are negative for cell surface expression (data not shown). Thus, it appears that only heterodimeric  $E^k$  structures can be stably expressed. Treatment with PI-PLC removes 80–90% of the surface staining, indicating that most or all molecules are GPI linked (data not shown). Subsequent cell cloning resulted in homogeneously high expressing, stable cell lines for both GPI-linked  $E^k$  (Fig. 1 B, third panel) and for clones expressing a similar amount of native  $E^k$  (Fig. 1 B, fourth panel) for use as a control (see below).

**Antigen Presentation by Cells Expressing GPI-linked  $E^k$ .** To evaluate the functional properties of the chimeric molecule, we performed lymphokine release assays using the pi  $E^k$  cells to present antigen. As a control, we used a CHO cell clone transfected with  $E^k$   $\alpha$  and  $\beta$  chains. As a T cell responder, we used the cytochrome *c*-specific, I- $E^k$ -restricted T cell hybridoma 2B4 (38, 39). Fig. 2 shows the dose-response curves



**Figure 2.** Activation of T cells by APC bearing native or GPI-linked  $E^k$ . Antigen dose-response curves using, as antigen presenters, the native  $E^k$ -bearing CHO clone (n  $E^k$ ) or the GPI-linked  $E^k$ -bearing clone (pi  $E^k$ ) shown in Fig. 1. The peptides used are MCC 88–103, and PCC 81–104. The responder cell is 2B4.11A. Error bars represent population SD of triplicate values.

for IL-2 production. Although markedly less effective at stimulating 2B4 at low concentrations of peptide than n  $E^k$  cells, the pi  $E^k$  cells can stimulate 2B4 to make large quantities of IL-2. In addition, the moth cytochrome *c* (MCC) peptide 88–103 is more potent than the pigeon cytochrome *c* (PCC) peptide 81–104, when presented by either cell line, showing that the characteristic fine specificity of cytochrome peptide presentation (40) is preserved with the GPI chimera. The second chimera, made using  $E\alpha$ /Pi and  $E\beta 2$ /Pi, gave essentially identical results (data not shown). Of other  $E^k$ -restricted peptides, hemoglobin  $\beta^{\text{dmin}}$  64–76 (41) and  $\lambda$



**Figure 3.** Failure of pi  $E^k$  cells to present whole antigen. Antigen dose-response curves using, as antigen presenters, the same clones mentioned in Fig. 2. APC were incubated with either PCC protein or MCC peptide 83–103 to stimulate the T cell clone 5C.C7. Shown is one of two independent experiments with similar results. Error bars denote the 95% confidence interval.

repressor 12-26 (42) could also be presented by the pi E<sup>k</sup> cells to specific T cell hybridomas, while hen egg white lysozyme 85-96 (43), could not (data not shown). Thus, three of four peptides can be presented on both GPI-linked E<sup>k</sup> and E<sup>k</sup> molecules, but the effectiveness of stimulation by the GPI-linked molecule is significantly reduced (50-100-fold), and the plateau levels of IL-2 release are somewhat lower (two- to fourfold). Since most pi E<sup>k</sup> molecules can bind peptides specifically (see below), and the ability of cells bearing these molecules to present antigen is removed by PI-PLC treatment (data not shown), the explanation for this phenomenon seems to lie in the more flexible nature of the GPI linkage vs. a transmembrane form of attachment (see Discussion).

We also tested the ability of the pi E<sup>k</sup>-bearing cells to present intact protein. For this assay, we used the IL-3-producing cytochrome *c*-specific, E<sup>k</sup>-restricted cell line 5C.C7 (44), because the detection threshold is ~100-fold lower than the IL-2 assay with 2B4. McCoy et al. (45) have shown that E<sup>k</sup>-bearing CHO cells can process and present whole cytochrome *c*. As shown in Fig. 3, a response to whole PCC is detectable at 0.3 μM, but no response is visible at even 400 μM with the pi E<sup>k</sup> cells. Peptide stimulations with the two cell lines are included as controls. Since it is clear that CHO cells can process cytochrome *c* and transfer the antigenic peptide(s) to native E<sup>k</sup>, it appears that pi E<sup>k</sup> is deficient in this regard.

**The Soluble E<sup>k</sup> Is Heterodimeric.** A high-expressing clone of pi E<sup>k</sup> cells was selected and grown to confluence in a hollow-fiber bioreactor (Cell Pharm, Unisyn Fibertech). Periodic treatment with PI-PLC as described (19) results in 0.2-0.5 mg/harvest of soluble E<sup>k</sup> (see Materials and Methods). To determine whether the α and β chains are noncovalently associated with each other as in the native state, we analyzed a sample of affinity-purified chimeric protein on nonreducing SDS-PAGE. The soluble E<sup>k</sup> migrates with an apparent mass of 65-70 kD (see below), consistent with it being a heterodimer. Additionally, NH<sub>2</sub>-terminal sequencing of the affinity-purified protein revealed three distinct species (Fig. 4), one of which is the α chain, (46) and two of which are β chain sequences (β and β') (47). Whereas the α sequence began as expected, the β sequence began one amino acid previous to the start of the mature protein purified from mouse spleen cells (P. Jones, personal communication). The β' sequence corresponds to the β chain sequence beginning at the third amino acid of the mature E<sup>k</sup>-β polypeptide. The α and β sequences are present in roughly equimolar amounts, and they constitute the majority of the sequencing yield, with the β'

α I K E E H T I I Q A E F  
 β L V R D S R P W F L E Y  
 β' D - R P W F L E

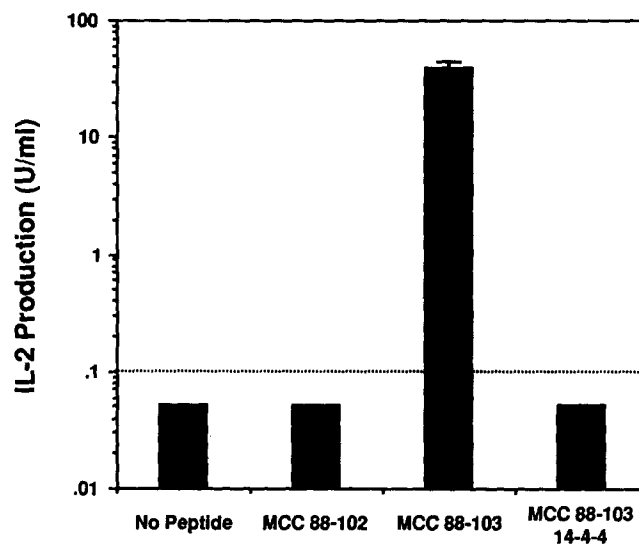
**Figure 4.** NH<sub>2</sub>-terminal sequence of cleaved GPI-linked E<sup>k</sup>. Multiple amino acid residues were detected at each round of NH<sub>2</sub>-

terminal protein sequencing (three in rounds 1 and 3-7; two in the rest). Using available sequence information, the protein sequencing data could be arranged as an α chain and two β chain sequences. The minor β' sequence derives from data obtained in rounds 1-8, but is aligned with the β sequence to show their correspondence (see text). The hyphen indicates absence of sequence information. Standard single letter amino acid abbreviations are used.

sequence accounting for ~10% of the yield. A difference of 10% in the amount of α vs. β chain would not be detectable. As the affinity purification of E<sup>k</sup> used only 14-4-4, an anti-Eα-specific antibody, the presence of the β chain is a further indication that GPI-linked Eα and GPI-linked Eβ are noncovalently associated during expression and purification.

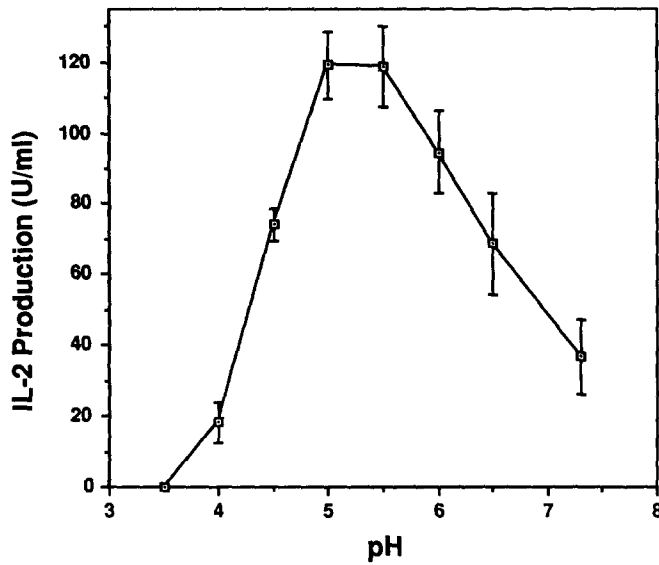
**Activation of T Cells With Soluble E<sup>k</sup>.** To determine whether the purified soluble E<sup>k</sup> obtained above is still functional, the purified protein was substituted for APC in a lymphokine release assay (Fig. 5). Microtiter trays (Immulon 4) were coated with 10 μg/ml soluble E<sup>k</sup>, incubated with peptide, washed, and then T cells were added to the wells (see Materials and Methods). When soluble E<sup>k</sup>-coated plates are incubated with MCC 88-103, this combination alone stimulates 2B4 cells. As shown in Fig. 5, activation was not detected in the absence of this peptide or in the presence of MCC 88-102, an inactive variant (48). Activation was completely blocked with the 14-4-4 antibody. Thus, soluble class II MHC bound to plastic exhibits the same antigen specificity and activation characteristics as cell-bound MHC, with no requirement for a lipid environment. This has previously been shown for detergent solubilized class I MHC by Kane et al. (49).

The simplicity of this assay provides a convenient means of optimizing the formation of functional antigen/MHC complexes. Because of Jensen's previous work with fixed cells (12), we were particularly interested in deriving a pH optimum for peptide/MHC formation by incubating E<sup>k</sup>-coated plates with 100 μM peptide at different pHs for 24 h at 37°C. Free peptide was washed away and the plates were incubated with specific T cell hybridomas. Fig. 6 A shows the results for

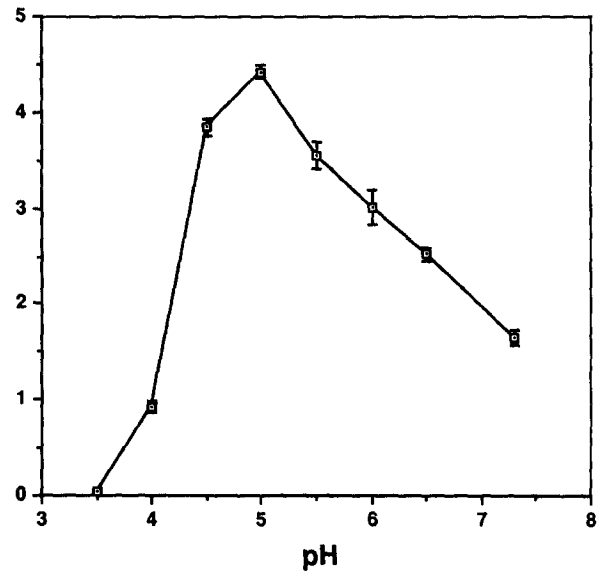


**Figure 5.** Activation of T cells by soluble E<sup>k</sup> bound to plates. Soluble E<sup>k</sup> was used to coat microtiter wells and then incubated with the indicated peptide in 1× PBS and/or blocking antibody. Wells were washed twice and then 2B4.11A T cells were added and IL-2 release assayed as described in Materials and Methods. MCC 88-102 lacks the COOH-terminal lysine residue. The mAb 14-4-4 was used at 2 μg/ml. The dotted line marks the assay limit of sensitivity.

A



B

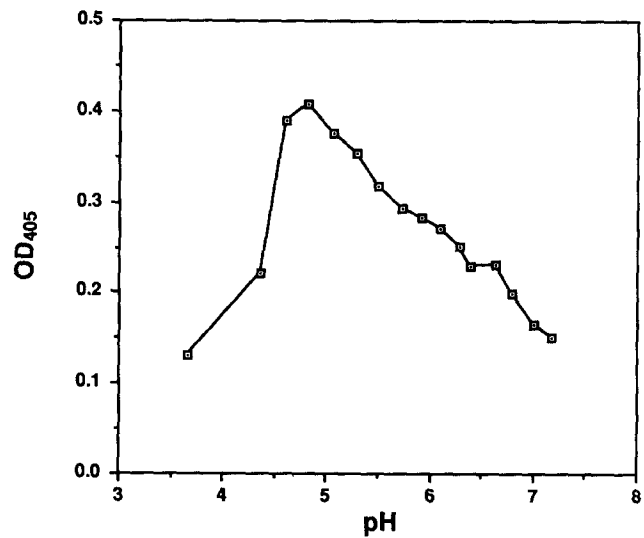


**Figure 6.** Enhanced T cell activation at low pH. Soluble E<sup>k</sup> coated on microtiter wells and incubated with peptide over a range of pHs was used to activate T cell hybridomas. (A) pH titration of MCC 88-103 as assayed by 2B4.11A stimulation. (B) pH titration of hemoglobin  $\beta^{\text{dmin}}$  64-76 as assayed by YO1.6 stimulation. Error bars represent the population SD of triplicate values.

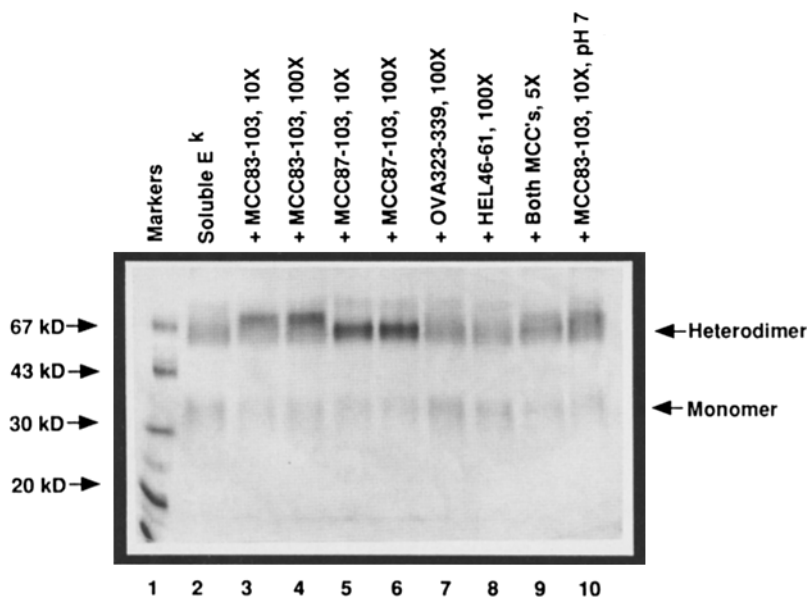
a moth MCC peptide and the 2B4 hybridoma. In this case, there is a clear optimum slightly higher than pH 5.0, leading to an IL-2 release that is four times the value of that obtained at neutral pH (7.3). The same analysis using hemoglobin  $\beta^{\text{dmin}}$  64-76 and the T cell hybridoma (41) YO1.6 gave similar results (Fig. 6 B) with the optimum at pH 5.0. These results are similar to those obtained by Jensen (12), suggesting that much or all of the effects seen in that report, involving peptides restricted to A<sup>d</sup> as well as E<sup>d</sup>, may be attributed to class II MHC molecules and specific peptides alone. Other parameters, such as salt concentration (0.1-2.0 M), or addition of lipids (5 mM lysophosphatidylserine) (50) during the peptide incubation had no effect (data not shown).

**Highly Efficient Formation of Peptide/E<sup>k</sup> Complexes at Low pH.** To investigate whether the low pH optimum seen in the activation assay was associated with enhanced peptide/MHC complex formation, we directly measured the pH dependency of antigen/E<sup>k</sup> complex formation in solution, using a biotinylated cytochrome peptide (MCC 83-103) and a "capture" ELISA using antibodies directed at the GPI tail (Reay, P., et al., manuscript in preparation). As shown in Fig. 7, a pH curve very similar to the T cell activation curve of Fig. 6 A was observed, with an optimum at pH 4.8-5.0. Thus, the enhanced T cell stimulation correlates precisely with increased peptide binding to the soluble E<sup>k</sup> protein. To visualize this complex directly, we also incubated two different lengths of MCC peptide (MCC 83-103 and MCC 87-103) with E<sup>k</sup> for 20 h at pH 5.2, using either a 10-fold or 100-fold molar excess of peptide. When assayed on nonreducing SDS-PAGE gels, Ia heterodimers stay largely intact (51). As shown in Fig. 8, lane 2 soluble E<sup>k</sup> runs as a diffuse hetero-

dimer but resolves into a more distinct band when incubated with either of the cytochrome peptides (lanes 3-6). The diffuse nature of the heterodimer band in the absence of bound MCC peptide may be due to the presence of endogenous peptides, which results in a heterogeneous population of MHC molecules. Either of two irrelevant peptides (OVA 323-339 and HEL 46-61), which are restricted to other class II MHC mol-



**Figure 7.** Enhanced peptide binding at low pH. Soluble E<sup>k</sup> was incubated with the biotinylated peptide MCC 83-103 at the pH indicated for 60 h at 37°C. Samples were neutralized to pH 7, and the amount of biotin associated with E<sup>k</sup> was determined (see Materials and Methods).



**Figure 8.** Visualization of peptide/MHC complexes by SDS-PAGE. Soluble E<sup>k</sup> was preincubated in the absence of peptide (lane 2) or in the presence of a 10- or 100-fold molar excess of MCC 83-103 (lanes 3 and 4), MCC 87-103 (lanes 5 and 6), or a fivefold excess of each (lane 9) at pH 5.2, 37°C for 20 h. Also shown are the results of preincubations with a 100-fold excess of the I-A-restricted peptides OVA 323-339 (lane 7) or HEL 46-61 (lane 8), under the same conditions as above, or with a 10-fold excess of MCC 83-103 (lane 10) at pH 7.0. All samples but the latter were neutralized before SDS-PAGE.

ecules (A<sup>d</sup> and A<sup>k</sup>, respectively), fail to cause an intensification or shift in the E<sup>k</sup> heterodimer band (Fig. 8, lanes 7 and 8), nor can they inhibit this band sharpening when co-incubated with either MCC peptide (data not shown). Also indicative of specific complex formation is the difference in mobility between the two cytochrome peptide/MHC complexes, with the longer peptide showing reduced mobility (70 kD) vs. the shorter (65 kD). Although this difference in apparent mobility is much more than one could normally attribute to the additional five amino acids in the peptides, the longer peptide likely protrudes some distance outside the binding site and thus might disproportionately alter the radius of the complex. When both peptides are incubated with E<sup>k</sup>, both bands are visible (Fig. 8, lane 9). Incubation of one of these same peptides (MCC 83-103) with E<sup>k</sup> at pH 7.0 (lane 10) results in a less distinct heterodimer band, consistent with previous reports that only 1-20% peptide/MHC complexes can be formed at neutral pH (5,3), and our own results summarized in Figs. 6 and 7. The amount of biotinylated cytochrome *c* peptide (MCC 83-103) specifically complexed with soluble E<sup>k</sup> was assessed using fluorescence enhancement of fluorescein-labeled streptavidin (52). Using the enhancement of fluorescence by known concentrations of free biotinylated cytochrome *c* peptide (MCC 83-103) to generate a calibration curve, we estimate that 60% of the soluble E<sup>k</sup> molecules are occupied by MCC 83-103 at pH 5 (data not shown).

## Discussion

The chimeric E<sup>k</sup>/HPAP molecule described here is clearly functional with respect to a number of peptide/T cell combinations and has significant advantages over other approaches for studying class II MHC molecules. Large quantities (1-3 mg/wk) of protein can be produced continuously from one bioreactor in a water-soluble form, without the need for de-

tergents. This is important because even mild detergents can interfere with protein-protein interactions. In particular, the earlier failure to see enhanced peptide binding to detergent-solubilized MHC molecules at low pHs (5) may well be due to detergent effects. Fraser (53) has also reported changes in staphylococcal enterotoxin B binding to class II MHC molecules purified by detergent solubilization vs. surface molecules. The advantage of the methodology described here over existing procedures to prepare soluble class II molecules by papain cleavage (54) is that harvesting conditions are gentler (papain may cleave elsewhere), and the use of cDNA transfectants makes the introduction of modifications easier. Thus far, the papain cleavage procedure has been limited to existing, high-expressing cell lines of human origin, and the solubilized protein has not been tested for its capacity to stimulate T cells (although it can bind specific peptide antigens) (8).

Peptides generated by endogenous processing of cytochrome *c* are available to E<sup>k</sup> expressed in CHO cells, but not (or very poorly) to GPI-linked E<sup>k</sup> expressed in CHO cells, indicating that the chimeric molecules are not treated as native molecules by the recipient cells. The most likely explanation for this is that the GPI anchor does not direct them to the endosomal compartment. A T cell receptor/HPAP chimera reported previously (19) transits from the Golgi apparatus to the surface of CHO cells without any detectable expression in endosomal or pre-endosomal vesicles (R. Klausner, personal communication). One use of the chimera described here is that it may allow the identification and isolation of ubiquitous allo- and autoantigens from cell extracts for which there is no other specific assay.

Although the pi E<sup>k</sup> cells are not as effective at presenting peptides as are cells bearing equal numbers of native molecules, the soluble E<sup>k</sup> is highly stimulatory in the plate assay (Figs. 5 and 6), and most of the soluble E<sup>k</sup> molecules are able to bind peptides specifically (Fig. 8). The kinetics of peptide binding at neutral pH (Reay et al., manuscript in prepa-

ration) are also very consistent with published values for a cytochrome *c* peptide (55). We have also examined the kinetics of the biotin-labeled peptide (MCC 83-103) binding to GPI-linked E<sup>k</sup> and E<sup>k</sup> on cells, and find them indistinguishable (data not shown). Thus, there appears to be no difference in the peptide binding ability of the GPI-linked and native forms of E<sup>k</sup>. Rather, it seems likely that the "floppiness" of the GPI anchor (vs. the more rigid transmembrane-anchored form) might cause fewer or less effective antigen/E<sup>k</sup> complexes to be available for binding to TCRs. The failure of pi E<sup>k</sup> cells to present peptides to some T cell hybrids may be due to differences in glycosylation between the E<sup>k</sup>/HPAP and n E<sup>k</sup> biosynthesis pathways. Perhaps most importantly, the work here confirms and extends that of Jensen (12) to indicate that endosomal pHs can significantly enhance the formation of specific peptide/MHC complexes as judged by both T cell reactivity and formation of specific peptide/heterodimer complexes (Figs. 6, 7, and 8). One difference between our results and those of Jensen (12) is the magnitude of the pH effect on T cell stimulation. He reports 5-15 times differences at analogous peptide concentrations (Fig. 1 of reference 12), whereas we see a three to four times effect. This may reflect the greater sensitivity of a fluid membrane environment (fixed cells in his case), where every MHC molecule is in the correct orientation and groups of molecules can form hyper-stimulatory patches. As shown by Jensen (12) for four different peptides restricted to two

different class II MHC molecules (I-A<sup>d</sup> and I-E<sup>d</sup>), and in the work described here for two other peptides restricted to I-E<sup>k</sup>, pH optima of 4.5-5.5 are observed. Acetylated derivatives of peptides made by Jensen show no significant differences in their pH optima, indicating that this pH dependence is not a function of the peptides. Recent results with detergent-solubilized Ia molecules (Mouritsen, personal communication) binding to radiolabeled peptides also show similar pH optima. It is therefore reasonable to postulate that low pH might cause a distinct conformational change common to class II MHC molecules, which makes it easier to bind peptides. Many proteins that cycle through endocytic compartments exhibit conformational changes related to their function, including the transferrin receptor, influenza hemagglutinin, and Semliki Forest Virus attachment protein (reviewed by Stryer [56]). It is interesting in this regard to note the results of Dornmair et al. (57), who observe a distinct species of detergent solubilized class II MHC heterodimers that can be induced by either heating to 60°C or by incubation at low pH (in their work, between pH 5.0 and 4.0). Also in this context, the classical work of Grey and Unanue, and their colleagues (58, 59), showed that agents that raise intracellular pH, such as chloroquine, inhibit class II (but not class I) MHC presentation. Although the current interpretation is that this is due to interference with protein degradation pathways, another possibility is that the effect is on peptide/MHC association.

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