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Immunology

Formation of functional peptide complexes of class II major histocompatibility complex proteins from subunits produced in *Escherichia coli*

(T-cell recognition/antigen presentation/protein folding/prokaryotic expression)

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ABSTRACT **Class II major histocompatibility complex molecules play a major role in the immune response by binding peptide fragments of exogenous antigens and displaying them on the surfaces of antigen-presenting cells, where they can be recognized by T cells. To facilitate structural and functional studies of these molecules, we have produced truncated α and β chains of the murine class II molecule I-E^k in *Escherichia coli* (*Ec*-I-E^k) and have developed conditions to fold them in the presence of specific peptides with yields of complex approaching 2%. Reconstitution is specific since only unlabeled peptides known to bind I-E^k compete with biotinylated peptide, as assessed by ELISA. Complexes of the refolded heterodimer (*Ec*-I-E^k) with either of two different peptide antigens remain associated during nonreducing SDS/PAGE. Immobilized *Ec*-I-E^k–peptide complexes stimulate lymphokine production by three T-cell clones in an antigen-specific manner with a dose–response relation comparable to previously described soluble I-E^k molecules produced in CHO cells. These results demonstrate that folding of E$^k_\alpha$ and E$^k_\beta$ polypeptides does not require any other protein to produce the biologically relevant conformation and that carbohydrate modification of this class II molecule is not necessary for $\alpha\beta$ T-cell recognition.**

Major histocompatibility complex (MHC) molecules are heterodimeric glycoproteins that present antigenic fragments of proteins to the antigen-specific receptors of T lymphocytes (T-cell receptor, TCR). In general, class I molecules present endogenously synthesized peptides to cytotoxic T cells, which express the CD8 coreceptor, whereas class II molecules present exogenously derived peptides to CD4-positive helper T cells. There is extensive structural information available from x-ray crystallographic studies for class I MHC (1, 2), and equivalent data is only recently emerging for class II MHC (3). Some of the class I MHC structural studies have been facilitated by the production of soluble class I molecules in insect cells (4) or through *in vitro* folding of proteins produced in *Escherichia coli* (5, 6). Although there are several eukaryotic expression systems for class II proteins (7, 8), until now *in vitro* folding of class II proteins produced in *E. coli* has not been demonstrated.

Class II MHC heterodimers present an inherently difficult folding problem. Unlike class I molecules, where the α1 and α2 domains form an "intramolecular dimer" (9) or "superdomain" (10), the peptide binding site of class II molecules is formed from two domains, α1 and β1, that are encoded on separate subunits (3). Together, the two domains form an eight-stranded β-pleated sheet that lies at the floor of the peptide binding groove (3). The N-terminal regions of the two subunits likely form a single cooperative unit of structure, and Braunstein *et al.* (11) have argued that the two cis-encoded subunits constitute a single unit of evolution. The class II MHC proteins, therefore, contrast with other heterodimeric proteins of the immune system that have been folded *in vitro*, such as F_{ab} fragments and class I MHC proteins, where each domain or superdomain is composed of protein segments from a single subunit (5, 12, 13).

In this paper, we describe the reconstitution of biologically active class II MHC–peptide complexes from denatured subunits. Using α and β subunit proteins synthesized in an insoluble form in separate *E. coli* transformants (*Ec*-I-E^k), we show that peptide binding specificity of the reconstituted class II protein is identical to that of the same isotype and allele synthesized in mammalian cells. Finally, we show that the reconstituted *E. coli*-synthesized class II MHC–peptide complexes stimulate antigen-specific T cells, demonstrating that posttranslational modifications of I-E^k are not required for T-cell recognition.

MATERIALS AND METHODS

Construction of Truncated Genes Encoding the *Ec*-I-E^k Subunits. DNA fragments encoding the soluble portions of the I-E^k α and β chains were isolated by the polymerase chain reaction (PCR), using the plasmid pBJ1-Neo/MHC$\alpha\beta$ (7) as a source of target DNA. The following oligonucleotides were used in the PCR: α forward, 5′-CATATGGCTAGCATCAAAGAGGAACACACCAT; α reverse, 5′-CCGGAATTCTCAGAGGAGGGTTTTCTCTTCAA; β forward, 5′-CATATGGCTAGCTTGGTCAGAGACTCCAGACC; β reverse, 5′-CGCGGATCCTCATGTGGACTGTGCTTTCCACT. Each truncated gene was amplified in a separate reaction by 10 cycles of denaturation (94°C for 1 min), annealing (51°C for 1 min), and extension (72°C for 1.5 min), digested with the appropriate restriction enzymes, and subcloned into the pGEMEX-1 plasmid (Promega). The truncations occurred in the middle of the connecting peptide, as for the soluble I-E^k produced in CHO cells (pi-I-E^k) (7).

Production of *Ec*-I-E^k Subunit Proteins. The *Ec*-I-E^k α and β subunit proteins were produced as inclusion bodies in the BL21(DE3)pLysS strain of *E. coli* by following established protocols (14) and solubilized as described (15). The recombinant proteins represented >90% of the total guanidinium chloride-solubilized proteins. Approximate extinction coefficients were calculated based on the composition of the proteins: for α, ε_{280} = 20,277 liter·mol^{-1}; for β, ε_{280} = 37,395 liter·mol^{-1}.

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Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor; MCC, moth cytochrome *c*; bioMCC, biotinylated moth cytochrome *c* peptide residues 82–103; *Ec*-I-E^k, MHC class II I-E^k heterodimers refolded from subunits produced in *E. coli*; pi-I-E^k, soluble I-E^k produced in CHO cells; OVA, ovalbumin; HEL, hen egg lysozyme; SWMb, sperm whale myoglobin; Hb, hemoglobin.

Peptide Synthesis. Peptides used in this study were as follows: bioMCC-(82–103), biotin-FAGIKKANERADLIAY-LKQATK [moth cytochrome *c* is MCC); biotinylated MCC is bioMCC]; MCC-(88–103), ANERADLIAYLKQATK; MCC-95E-(88–103), ANERADLEAYLKQATK; MCC-95K-(88–103), ANERADLKAYLKQATK; MCC-99E-(88–103), ANERADLIAYLEQATK; MCC-99E/103A-(88–103), ANERADLIAYLEQATA; OVA-(323–339), ISQAVHAAHAEINEAGR (chicken ovalbumin peptide, provided by Anand Gautam, Stanford University); HEL-(46–61), NTDGSTDYGILQINSR (hen egg lysozyme); SWMb-(129–153), GAMNKALELFRKDIAAKYKELGYQG (sperm whale myoglobin); Hbβ^{dmin}-(53–76), AIMGNPKVKAHGKKVITAFNEGLK (minor allele of murine hemoglobin β subunit). All peptides were synthesized at the Stanford University Protein and Nucleic Acid Facility unless otherwise noted. The bioMCC peptide was produced as described (16).

***In Vitro* Folding of *Ec*-I-E^k.** Prior to *in vitro* folding, the buffer containing the guanidinium chloride-solubilized α and β subunits was exchanged for 6 M urea/10 mM acetic acid on PD-10 desalting columns (Pharmacia). Refolding was initiated by the dilution of urea-solubilized proteins into a buffer that generally contained 50 mM sodium phosphate (pH 7.5), 1 mM EDTA, 3 mM reduced glutathione, 0.3 mM oxidized glutathione, 25% (vol/vol) glycerol, and 20 μM bioMCC-(82–103). Buffer components varied as described in figures. Protein subunit concentration was 2 μM and the reactions were incubated at 25°C for 66 h unless otherwise noted. The volume of urea-solubilized α and β chains added to refolding reactions was never >1% of the total volume.

Peptide Competition Experiments. Peptide competition for bioMCC binding during the *in vitro* folding reactions was assayed as were standard folding experiments with the following modifications. The concentration of the biotinylated peptide was reduced to 2 μM, so that it equaled the concentration of protein subunits. Separate reaction mixtures were set up with 1:3 serial dilutions of competitor peptide, starting at a concentration of 60 μM in the folding reaction mixtures. Conditions for peptide binding competition using intact pi-I-E^k were as follows: pi-I-E^k was used at 100 nM, bioMCC was used at 2 μM, and serial dilutions of competitor peptides were prepared, starting at 60 μM. All reactions were incubated for 66 h in citrate/phosphate buffer at pH 5 or 7 (16). Binding of bioMCC to pi-I-E^k was assayed as described (16).

ELISA. G32 is an anti-I-E^k IgG1 mouse monoclonal antibody and will be described elsewhere (P.A.R., M.D., and Kristen Baldwin, unpublished data). ELISAs were performed as described (7, 16) with minor modifications. Each microtiter well (Dynatech) was coated with 50 μl of G32 at 20 μg/ml in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Folding reaction mixtures containing biotinylated peptide complexes of *Ec*-I-E^k were diluted 1:10 into a buffer containing 150 mM Tris·HCl (pH 8.0), 2% (wt/vol) bovine serum albumin, and 10 mM sodium azide. Complexes were captured by the application of 50 μl of this solution to the preblocked microtiter wells, followed by incubation for 2 h at room temperature. A standard curve was prepared by the capture of serially diluted pi-I-E^k–bioMCC complexes of known concentration in the same buffer. The standard curves were not linear but were well fit to the empirically derived equation $y = a + b(1 - e^{-cx})$, where y is the rate of increase in absorbance at 405 nm in OD units ($\times 10^{-3}$)/min, x is the concentration of MHC–peptide complex in the standard, and a, b, and c are adjustable parameters.

***Ec*-I-E^k Purification.** Preparative-scale reactions of *Ec*-I-E^k complexes were carried out in 0.25–1.0 liter of the buffer described above. Peptide concentrations were 20 μM. After 3–7 days, solutions containing *in vitro*-folded proteins were dialyzed twice against at least 40 vol of PBS at 4°C in dialysis tubing with a molecular mass cutoff of 10 kDa or less. Precipitated aggregates were removed by centrifugation, and native *Ec*-I-E^k complexes were purified by immunoaffinity chromatography on 14-4-4 columns and concentrated as described (7). Purified complexes appeared to be free of single-chain molecules by size-exclusion HPLC (data not shown). In addition, the ratio of α to β chains appeared equimolar by SDS/PAGE analysis.

T-Cell Stimulation Assays. The 96-well microtiter plates were coated with preformed peptide–MHC complexes of either soluble pi-I-E^k (7) or *Ec*-I-E^k (this report) as described (17). Supernatants from T cells after stimulation were assayed for interleukin 2 (2B4 and Y01.6) using HT-2 indicator cells (7, 18) or for interleukin 3 (5C.C7) with R6X indicator cells (7, 19) as described.

RESULTS

Optimization of *Ec*-I-E^k *in Vitro* Folding Conditions. We optimized folding conditions for *Ec*-I-E^k by using the modified capture ELISA method. Monoclonal antibody G32,

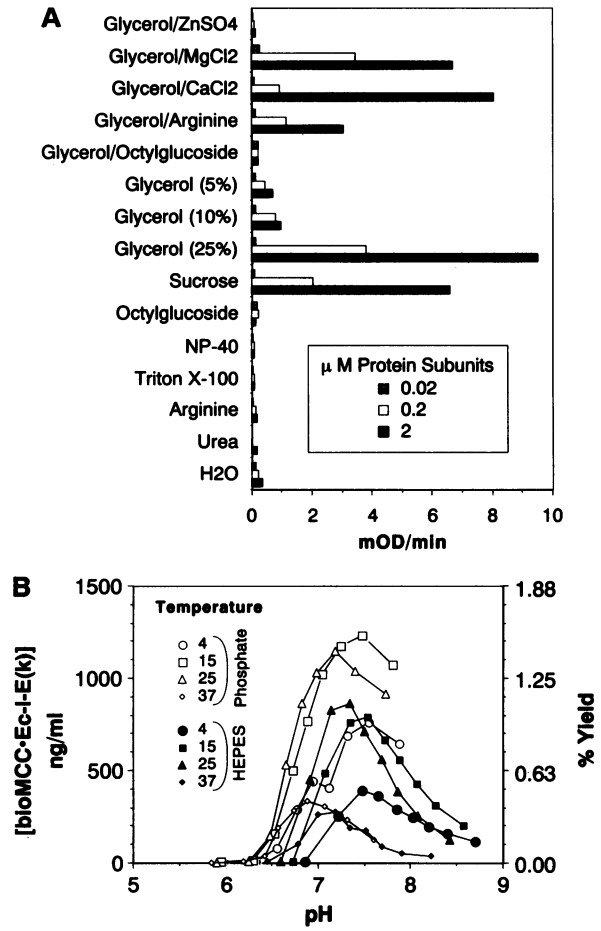

FIG. 1. (*A*) Solvent effects on *in vitro* folding of *Ec*-I-E^k. *In vitro* folding reactions were performed using solvent additives as indicated. Triton X-100, Nonidet P-40 (NP-40), and octylglucoside were included at 1%, urea and arginine were at 0.5 M, metal ions were at 5 mM, and glycerol was at 25%, unless otherwise noted. Folding reactions were carried out at three concentrations of α and β subunits; the bioMCC peptide was included at a concentration 10-fold greater than the subunit concentration. mOD, OD units $\times 10^{-3}$. (*B*) *In vitro* folding yields of *Ec*-I-E^k are a function of pH and temperature. *In vitro* folding reactions were performed with phosphate or Hepes buffers at 50 mM. Solutions at different pH values were prepared by addition of predetermined ratios of 0.5 M stock solutions of the acid and conjugate base forms of the buffers, such that the total buffer concentration was 50 mM. pH values were then measured at the temperatures shown in °C.

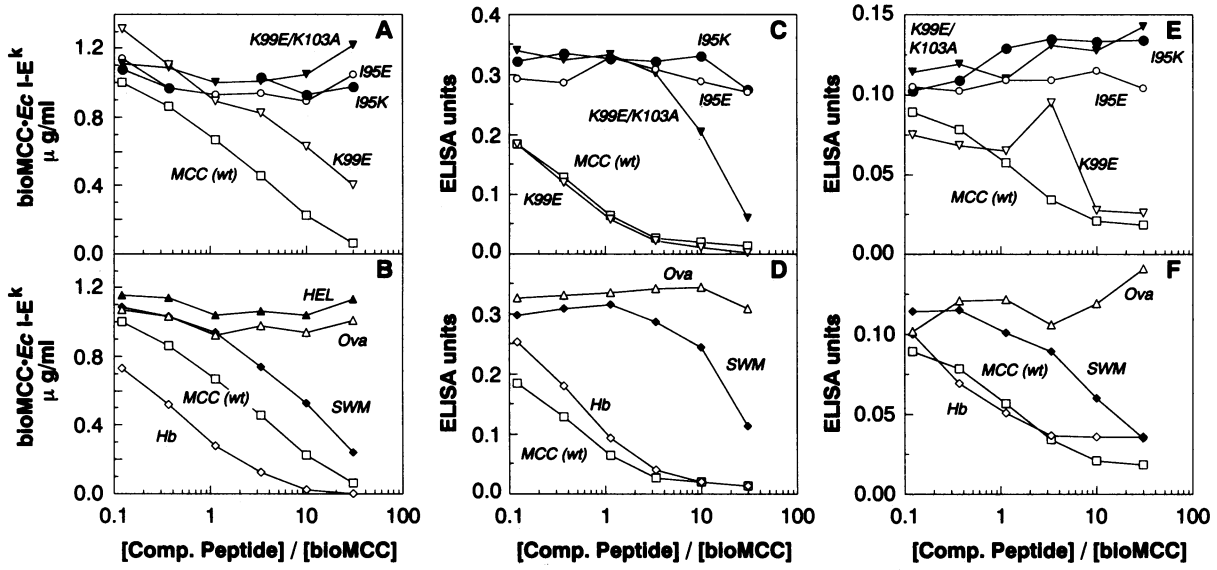

FIG. 2. *Ec*-I-E^k shows the same peptide specificity as pi-I-E^k. (*A* and *B*) Competition (comp.) for binding of bioMCC peptides by *Ec*-I-E^k during *in vitro* folding reactions. (*C–F*) Peptide binding competition using pi-I-E^k. (*C* and *D*) Incubated for 72 h at 37°C at pH 5. (*E* and *F*) Incubated for 72 h at 37°C at pH 7. (*A*, *C*, and *E*) Use variants of the MCC peptide. (*B*, *D*, and *F*) Compare peptides from a variety of proteins.

which replaces the polyclonal antisera, provides an assay that can detect bioMCC–I-E^k complexes at concentrations of 1–10 ng/ml. This modified assay is ≈10-fold more sensitive than the original assay of Reay *et al.* (7).

We screened the effects of three classes of solvent modifiers known to alter protein stability or solubility for their ability to promote *in vitro* folding of *Ec*-I-E^k–peptide complexes: (*i*) mild chaotropes such as urea and arginine, which might destabilize aggregates (12); (*ii*) nonionic detergents, which have also been reported to suppress protein aggregation (20); and (*iii*) nonionic protein stabilizers such as glycerol and sucrose (21). *Ec*-I-E^k–peptide complexes are clearly produced in the presence of glycerol and sucrose, at levels >10-fold above the quantities produced in the absence of solvent additives (Fig. 1*A*). Divalent metal ions such as Ca^{2+} and Mg^{2+} do not affect folding, but Zn^{2+} nearly abolishes reconstitution, probably through interaction with cysteine thiols necessary for disulfide formation. Although *in vitro* folding yields, as a percentage of total protein subunits, are greater at lower protein concentrations, greater absolute total folded protein concentrations are obtained at higher subunit concentrations.

We also examined the effects of temperature and pH on *Ec*-I-E^k–peptide complex formation using Hepes and phosphate buffers in the presence of 25% glycerol (Fig. 1*B*). The optimum pH at all temperatures occurs in between pH 7.4 and 7.6, declining steeply on either side of the peak. This optimum likely reflects a balance between disulfide bond formation, which is favored at pH values above the pK_a of cysteine thiols (usually between pH 8 and 9), and peptide–MHC complex formation, which is optimal near pH 5 (7, 16, 22). We don't yet know the cause of the observed difference in yield when using Hepes versus phosphate buffers. Yields of *Ec*-I-E^k are comparable when *in vitro* folding is performed at 15 and 25°C but are decreased at temperatures of 4 and 37°C. The temperature profile is qualitatively similar to that observed in the refolding of other heterodimeric molecules that show a tendency to aggregate, such as F_{ab} fragments. Based on the results in Fig. 1*B*, subsequent refolding reactions were carried out at 25°C with buffers containing 25% glycerol, 40 mM Na_2HPO_4, and 10 mM NaH_2PO_4. The

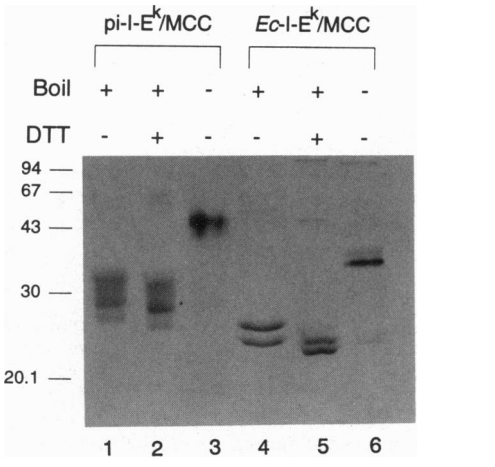

FIG. 3. SDS/PAGE analysis of purified *Ec*-I-E^k complexes. Purified *Ec*-I-E^k (lanes 4–6) complexes were analyzed by SDS/PAGE on 10% gels and compared to preformed complexes using pi-I-E^k (lanes 1–3). The intact heterodimers (lanes 3 and 6) migrate with anomalously low molecular masses. Some samples were boiled (+) and some were not (−). Dithiothreitol (DTT) was added to some samples (+) but not to others (−). Molecular masses in kDa are indicated.

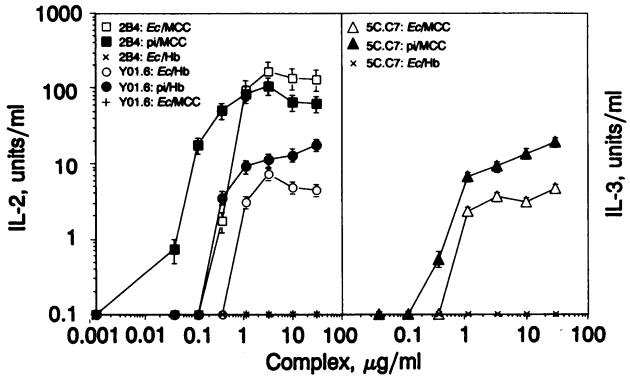

FIG. 4. *Ec*-I-E^k and pi-I-E^k complexes stimulate T cells. The 2B4 T-cell hybridoma and 5C.C7 T-cell clone were stimulated by the MCC complexes (squares and triangles, respectively) but not by the *Ec*-I-E^k–Hb complexes (× and + symbols). The Y01.6 T cell hybridoma was stimulated by the Hb complexes (triangles) but not by the pi-I-E^k–MCC complexes (×).

optimum *in vitro* folding yield in this experiment, based on the total concentration of α and β subunits, was ≈1.5%. In other experiments, yields of biologically active *Ec*-I-E^k complexes were between 1 and 2%.

Competition by Specific Peptides Resembles that of Eukaryotic-Expressed I-E^k. Individual MHC molecules bind diverse sets of peptides that may share a particular sequence motif (for review, see ref. 23). We carried out competition experiments to test for inhibition of the reconstitution of *Ec*-I-E^k–bioMCC by peptides known to bind intact I-E^k (ref. 16 and references therein). We tested variants of the MCC peptide and peptides from unrelated proteins that have a range of affinities for I-E^k (Fig. 2).

Several peptides were used as negative controls and show no inhibition of *Ec*-I-E^k–bioMCC reconstitution or bioMCC binding to pi-I-E^k at either pH. These include the MCC-(88–103) variants 95E and 95K (Fig. 2 *A*, *C*, and *E*) and peptides that bind to I-A^d [OVA-(322–339)] and I-A^k [HEL-(46–61)] but not to I-E^k (Fig. 2 *B*, *D*, and *F*). In contrast, the MCC-(88–103) variant 99E and the peptides murine Hbβ^{dmin}-(53–76) and SWMb-(129–153) reduce the signal in a concentration-dependent manner, consistent with previous results (16). The SWMb-(129–153) peptide is a less-effective competitor than the wild-type MCC-(88–103), consistent with its lower affinity for I-E^k (Fig. 2 *B*, *D*, and *F*). While Hbβ^{dmin}-(53–76) is equally as effective as MCC-(88–103) in competition for binding to intact pi-I-E^k (Fig. 2 *D* and *E*), it appears to be a more effective inhibitor of the *Ec*-I-E^k–bioMCC reconstitution reaction (Fig. 2*B*). Additionally, the MCC 99E variant shows the same dose–response relation as the wild-type peptide in competition for binding to pi-I-E^k (Fig. 2 *C* and *E*) but is a less-effective inhibitor of the bioMCC–*Ec*-I-E^k reconstitution reaction (Fig. 2*A*). We don't yet know the cause of these apparent differences in the two assays, but there may be an effect of peptide length in the *Ec*-I-E^k reconstitution assays that does not affect the pi-I-E^k binding reaction. Finally, high concentrations of the MCC 99E/103A variant peptide inhibit bioMCC binding to pi-I-E^k at pH 5 (Fig. 2*C*) but not at pH 7 (Fig. 2*E*); since the bioMCC–*Ec*-I-E^k reconstitution reaction is carried out at pH 7.4, it is not surprising that this peptide is unable to compete for bioMCC in the reconstitution reaction (Fig. 2*A*). Thus it is clear that *Ec*-I-E^k has at least qualitatively the same peptide binding specificity as pi-I-E^k.

Purification and Physical Characterization of *Ec*-I-E^k–Peptide Complexes. To further characterize this material, we isolated complexes of *Ec*-I-E^k with the MCC-(88–103) and the murine Hb β^{dmin}-(64–76) peptides. Each 500-ml reaction mixture yielded ≈200 μg of protein. The *Ec*-I-E^k–peptide complexes displayed characteristics similar to the pi-I-E^k complexes when analyzed by SDS/PAGE (7) (Fig. 3). Samples not reduced and boiled prior to electrophoresis ran predominantly as noncovalent heterodimers with anomalously high mobilities, whereas reduced and boiled samples ran as resolved monomers (8, 24). The *Ec*-I-E^k peptide complexes ran at ≈35 kDa, compared to the calculated molecular mass of 46 kDa, and were much sharper than the pi-I-E^k band, which ran at 47 kDa, compared to its approximate calculated molecular mass of 65 kDa. These features reflect the absence of large and heterogeneous carbohydrates on side chains and the glycan-phosphatidyl inositol tail on *Ec*-I-E^k.

Antigen-Specific T-Cell Activation by *Ec*-I-E^k–Peptide Complexes. The most stringent test of correctly folded I-E^k peptide complexes is to determine whether these proteins can stimulate T cells. We tested three antigen-specific T cells for stimulation by their cognate peptide antigen–MHC complexes immobilized in wells of 96-well plastic plates (Fig. 4). Both 2B4 (a T-cell hybridoma) and 5C.C7 (a T-cell clone) recognize the MCC-(88–103) peptide bound to I-E^k; the YO1.6 hybridoma recognizes the Hbβ^{dmin}-(53–76) peptide bound to I-E^k and its TCR is unrelated to the 2B4 and 5C.C7 TCRs. Activation of the 2B4 and Y01.6 cells in this manner does not require costimulatory molecules (7). When the TCRs on these cells are cross-linked by interaction with immobilized MHC–peptide complexes, the cells secrete lymphokines that can be quantitated with a bioassay using indicator cell lines that are dependent on the lymphokines for proliferation. Preformed peptide complexes with pi-I-E^k, and I-E^k-bearing antigen-presenting cells pulsed with antigenic peptides served as positive controls; the "wrong" peptide–MHC complexes served as negative controls. In each case, the correct *Ec*-I-E^k–peptide complexes stimulated T cells to produce lymphokines, whereas the wrong peptide–MHC complex failed to stimulate the T cells. By this last very stringent criterion, we conclude that the *in vitro*-folded proteins are functional class II MHC–peptide complexes. Furthermore, this is a direct demonstration that carbohydrate attachments or other post-translational modifications on I-E^k are not required for the activation of these T cells.

The plateau values for the stimulation of 2B4 cells by the *Ec*-I-E^k and pi-I-E^k complexes were very similar, and maximum levels of lymphokine production by 5C.C7 and Y01.6 were greater for the pi-I-E^k complexes than for the *Ec*-I-E^k complexes. In each case, the dose–response curves for the *Ec*-I-E^k complexes were shifted toward higher protein concentrations by 3-fold compared to the pi-I-E^k complexes. Both versions of the immobilized soluble protein produced lower maximal T-cell stimulation compared to controls using peptides presented on antigen-presenting cells (data not shown), consistent with previous results (7). The significance of the difference in dose–response curves between *Ec*-I-E^k and pi-I-E^k complexes is unknown; it may be that not all of the *Ec*-I-E^k protein is correctly folded or that it coats the plates less efficiently or in a different average orientation.

DISCUSSION

In this paper we show that truncated class II MHC α and β chains can be expressed in *E. coli* and that a biologically active peptide complex can be reconstituted *in vitro*. Dornmair and McConnell (25) have reported the reconstitution of native class II–I-A^d peptide complexes, but they did not confirm the peptide specificity of reconstitution by competition experiments, nor did they assess biological activity. In our experiments, we rely on the binding of a labeled peptide as an indicator of reconstitution. However, we have shown that unlabeled peptide effectively competes with the biotinylated peptide in reconstitution reactions on a molar basis. Moreover, only peptides known to bind to I-E^k are effective competitors. The use of negative control peptides that do not bind to I-E^k, such as the MCC-(88–103) position 95 variants and the OVA and HEL peptides, argues strongly against a general inhibition of class II folding by the high concentrations of peptides (60 μM) used in the experiments. The simplest interpretation of these data is that unlabeled peptides are competing for a site formed during *in vitro* folding that is similar to the site in intact pi-I-E^k.

For each antigen-specific T cell we tested, the immobilized *Ec*-I-E^k complexes elicit cytokine responses comparable to the immobilized pi-I-E^k complexes. Although the 2B4 and 5C.C7 cells bear structurally related TCRs, the TCR of the Y01.6 hybridoma uses distinct α and β chain variable germline segments. The stimulation of the three T cells tested validates the approach of using MHC–peptide complexes reconstituted from nonglycosylated proteins produced in *E. coli* to study the physical characteristics of peptide–MHC–TCR ternary complexes.

Any demonstration of unassisted *in vitro* folding supports the hypothesis of Anfinsen (26), which states that all of the

information necessary for the proper folding of proteins is contained in their primary sequences. We have shown that truncated MHC class II α and β chains can spontaneously fold and assemble in the presence of peptides but in the absence of other accessory molecules. The actual role of the peptide in this process remains a matter of controversy (27). While both MHC class I and class II heterodimers are clearly stabilized by peptide ligands (8, 28), at least two eukaryotic class II expression systems yield apparently empty heterodimers that are nonetheless recognized by conformation-sensitive antibodies (7, 8, 16). In addition, eukaryotic class I expression systems can also yield apparently empty heterodimers at the cell surface, although these molecules are unstable at physiological temperatures (28). On the other hand, Wiley and colleagues (5, 13) have shown that peptides are necessary for *in vitro* folding and assembly of class I subunits. We do not yet know whether peptide is absolutely necessary in the system described here, but the results cited above suggest that empty class II molecules are more robust than class I and the peptide may not be required. One interesting possibility, however, is that the addition of peptides to MHC *in vitro* folding reactions mimics the action of one or more chaperone molecules that are involved in the *in vivo* folding of MHC proteins in the eukaryotic systems that yield empty MHC molecules.

Despite the fact that their peptide binding region derives from two separate polypeptides, the class II MHC proteins show much less combinatorial diversity than the other major antigen binding molecules of the immune system (11). From structural data, it can be seen that interactions between the immunoglobulin domains of antibodies are much less extensive than are those of the outer domains of class II α and β chains (3); thus, the peptide binding region of these molecules is much more reminiscent of a single protein domain that folds and unfolds in a cooperative manner. The stringent folding requirements of this superdomain may provide the selective pressure that keeps the class II α and β genes tightly linked on the same chromosome, allowing them to be selected as a single evolutionary unit (11). This is in contrast to the genes encoding antibody or TCR polypeptides, in which none of the various pairs are tightly linked to each other.

Folding and assembly of class II MHC molecules may follow exceedingly stringent rules, but we have now demonstrated that the process can occur spontaneously in the absence of accessory proteins. The ability to produce quantities of biologically active class II MHC proteins in *E. coli* now provides—quickly and cheaply—the amounts of homogeneous material needed for x-ray structural studies (3), two-dimensional NMR analysis (29), and affinity measurements (17). It should also permit much more rapid and extensive mutagenesis for structure–function studies.

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