Isolation of a functional antigen–Ia complex

(antigen processing/peptide presentation/Ia purification/artificial lipid membranes)

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ABSTRACT The helper T-cell recognition of globular protein antigens requires that the antigen be processed and presented by an *I*-region associated (Ia)-expressing antigen-presenting cell (APC). Processing involves the uptake of antigen into an intracellular, proteolytic, acidic compartment; release of peptide fragments containing the T-cell antigenic determinant; association of these peptides with Ia; and presentation of these complexes on the cell surface for recognition by the specific T cells. The molecular mechanisms by which processed antigenic peptides associate with Ia within the APC are poorly understood. To date, functional antigen–Ia complexes have not been isolated from cells that have processed native antigens, although the resolution of the structure of a major histocompatibility complex (MHC) class I protein indicates that peptide is bound in a groove between two α -helical regions of the molecule and synthetic peptides have been demonstrated to bind purified MHC both in detergent solution and incorporated into planar membranes, where the MHC–peptide complexes function to activate specific T cells. Here we demonstrate that Ia purified from APCs that have processed the native globular protein antigen cytochrome *c*, when incorporated into lipid membranes, stimulates cytochrome *c*-specific T cells in the absence of exogenous antigenic peptide. The T-cell response to Ia purified from cytochrome *c*-pulsed APCs shows the same MHC restriction and antigen fine specificity as the response to antigen-pulsed APCs. Indeed, T-cell recognition of pigeon cytochrome *c* (Pc) shows a well documented high-affinity heteroclitic cross-reaction to insect cytochromes *c*—namely, those of *Drosophila melanogaster* (DMc) and tobacco hornworm moth (THMc). The same heteroclitic response is observed when purified Ia from unpulsed cells, incorporated into lipid membranes, is used to present antigenic peptides of Pc and of THMc. Significantly, Ia purified from APCs that have processed DMc is ≈ 50 -fold more active in stimulating specific T cells compared to Ia purified from APCs that have processed Pc. The peptide–Ia complex isolated here may provide the necessary material for analysis of the physiochemical properties of the processed form of the antigen that is produced by the APC and associates with Ia.

T lymphocytes do not recognize the native structure of protein antigens but require that these be processed by antigen-presenting cells (APCs) to a peptide form (1). Recent evidence indicates that peptides that function as T-cell antigens bind to major histocompatibility complex (MHC) molecules. This includes the observations that synthetic peptides representing T-cell antigenic determinants bind to the MHC class II (2–4) and class I (5) molecules in detergent solution and when the MHC molecules are incorporated into lipid membranes (6–8). In addition, the resolution of the structure of the MHC class I molecule shows a cleft formed by two α -helical regions, which contains electron-dense material (9, 10). One possibility proposed for this heterogeneous, non-

resoluble electron density is that it represents processed antigen that remains bound to the class I molecule throughout purification. Based on structural similarity, the class II molecule is suggested to have a similar peptide binding site (11). While significant progress has been made in understanding which synthetic peptides function as T-cell antigens and bind to MHC molecules, the nature of the processed peptide–*I*-region associated (Ia) complexes assembled by the APCs has yet to be elucidated. Here we describe a functional peptide–Ia complex isolated from APCs that have processed a soluble globular protein antigen.

MATERIALS AND METHODS

Animals. CBA/J female mice, 5–6 weeks old, were obtained from The Jackson Laboratory.

Antigens and Peptides. Pigeon cytochrome *c* (Pc) was isolated and purified according to Brautigan *et al.* (12). *Drosophila melanogaster* cytochrome *c* (DMc) was purified as a recombinant protein from the strain of yeast GM-3C-2 transformed with the plasmid YEpDMc 01, kindly provided by E. Margoliash (Northwestern University). Ovalbumin (OVA) was purchased from Sigma. The cyanogen bromide cleavage fragment of Pc (Pc 81–104) was prepared as described (13). The C-terminal fragment of tobacco hornworm moth cytochrome *c* (THMc) (THMc 81–103) was synthesized by stepwise solid-phase synthesis using the *N*-fluorenylmethoxycarbonyl-*t*-butyl amino acid strategy (14, 15) on a Vega 1000 coupler with 4-methylbenzhydrylamine resin as a solid support.

Cell Lines and Preparation of B Cells. TPc 9.1 is a mouse T-cell hybridoma specific for Pc presented in the context of the I-E^k molecule (16). AODH-3.4 is a mouse T-cell hybrid specific for OVA presented in the context of I-A^k (17). The CTLL-2 line is an interleukin 2 (IL-2)-dependent cell line described by Gillis *et al.* (18). B cells were isolated from the spleens of CBA/J mice by removal of erythrocytes by centrifugation on Ficoll/Hypaque (Pharmacia) gradients and by removal of T cells by incubation with monoclonal antibodies directed toward L3T4, Thy-1, and Lyt-2, and complement, as described (16). When indicated, B cells were fixed by treatment with 0.15% paraformaldehyde (16). CH27 is a B-cell lymphoma kindly provided by G. Haughton (University of North Carolina) (19). The hybridoma cell line 14.4.4s produces an I-E^k-specific monoclonal antibody (20). Hybridoma 10.2.16 secretes a monoclonal antibody specific for I-A^k (21).

Purification of I-E^k from CH27 Cells. Purification of I-E^k from CH27 B-lymphoma cells was performed with the anti-

Abbreviations: APC, antigen-presenting cell; DMc, *Drosophila melanogaster* cytochrome *c*; OVA, ovalbumin; Pc, pigeon cytochrome *c*; Pc 81–104, C-terminal peptide of Pc residues 81–104; THMc, tobacco hornworm moth cytochrome *c*; THMc 81–103, C-terminal peptide fragment of cytochrome *c* residues 81–103; MHC, major histocompatibility complex; Ia, *I*-region associated; IL-2, interleukin 2.

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I-E^k monoclonal antibody 14.4.4s (20) coupled to CNBr-activated Sepharose by a modification of the procedure of Turkewitz *et al.* (22). Briefly, CH27 cells were washed with phosphate-buffered saline (PBS) and a small fraction of these cells was surface labeled by lactoperoxidase-catalyzed iodination (23). The cells were lysed with pH 8 lysis buffer containing 0.5% Nonidet P-40 and protease inhibitors. The lysate was centrifuged at $3000 \times g$ and $100,000 \times g$ for 15 min and 50 min, respectively, and the supernatant was passed over an immunoaffinity column, eluted with a pH 11 buffer containing 30 mM β -octyl glucoside, and the eluate was neutralized with 1 M Hepes buffer. All purification steps were carried out at 4°C. The I-E^k was dialyzed against PBS containing 30 mM β -octyl glucoside and was concentrated by using Centricon microconcentrators. The concentration of I-E^k was estimated by Pierce's BCA-protein assay. On average, $\approx 75 \mu\text{g}$ of I-E^k was obtained from 10^9 CH27 cells. A fraction of this material was analyzed by SDS/PAGE on a 10% polyacrylamide gel.

To obtain I-E^k from antigen-pulsed cells, CH27 cells (2.5×10^9) were incubated for 12 hr in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and other additives (16) with Pc (20 μM) or DMc (5 μM) at 37°C in 5% CO₂/95% air. The cells were harvested and washed with PBS, and I-E^k was purified as described above.

Preparation of I-E^k-Containing Lipid-Coated Glass Beads. Phosphatidylcholine and cholesterol in a 7:2 molar ratio were dried under N₂ and resuspended in PBS containing 30 mM octyl β -glucoside. The I-E^k and lipids were mixed at a ratio of 1:8 (wt/wt) (final protein concentration, 65 $\mu\text{g}/\text{ml}$) and extensively dialyzed against PBS and then against DMEM over a period of 72 hr at 4°C. A 250- μl aliquot of the resultant liposomes was mixed with 9 mg of acid-washed, 75- to 150- μm glass beads (Sigma) and rocked for 2 hr at room temperature. The I-E^k-containing lipid-coated beads were washed thoroughly with DMEM and resuspended in a vol of 400 μl . The amount of I-E^k on the beads was estimated by specific activity of the I-E^k (1×10^{12} molecules per 25 μl of bead suspension).

Antigen Presentation by B Cells and I-E^k-Containing Lipid-Coated Beads. I-E^k beads (25 μl) or B cells (2×10^5) were incubated with Tpc 9.1 cells (5×10^4) and graded doses of the peptides Pc 81–104 or THMc 81–103. Culture supernatants were assayed for their IL-2 content 24 hr later by their ability to maintain the proliferation of an IL-2-dependent T-cell line (CTLL) measured by incorporation of [³H]thymidine (16).

RESULTS

Purified I-E^k Functions to Present Cytochrome *c* Peptides in a Heteroclitic Manner. The I-E^k-restricted T-cell hybrid Tpc 9.1 responds to native Pc presented by live APCs or to the C-terminal peptide fragment of Pc-containing residues 81–104 (Pc 81–104) presented either by live APCs or by APCs that have been blocked in their processing functions by fixation with paraformaldehyde (16). In addition, Tpc 9.1 cells show a characteristic cross-reactivity with native THMc (24, 25) and DMc, and the corresponding antigenic fragments of these insect proteins composed of residues 81–103 (DMc 81–103 and THMc 81–103, respectively). This cross-reactivity is heteroclitic in that maximal T-cell activation to THMc 81–103 is achieved with 10- to 50-fold less peptide compared to Pc 81–104. To determine whether the heteroclitic response is maintained when the antigenic peptides are presented by Ia alone, I-E^k was isolated, incorporated into lipid membranes, and tested for the ability to present cytochrome *c* antigenic peptides to Tpc 9.1 cells.

I-E^k was purified from the CH27 B-lymphoma cell line by immunoaffinity chromatography using an I-E^k-specific monoclonal antibody coupled to CNBr-Sepharose. A fraction of the CH27 cells was radiolabeled by lactoperoxidase-

catalyzed cell-surface iodination to allow for monitoring of I-E^k during purification. The autoradiogram and the Coomassie blue staining of affinity-purified I-E^k analyzed by SDS/PAGE is shown in Fig. 1. Only the β subunit of the I-E^k appears in autoradiography, while both the α and the β subunits are visualized by Coomassie blue staining, consistent with reports of others (22). Thus, the material appears to be highly pure, with few contaminating proteins of higher molecular weight or peptides of lower molecular weight. Purified I-E^k was incorporated into liposomes and the liposomes were adsorbed onto glass beads (I-E^k beads). The ratio of I-E^k to lipid was 1:8 (wt/wt), where the I-E^k concentration was measured by direct protein determination and the specific activity was calculated. The I-E^k beads activate Tpc 9.1 cells to secrete IL-2 when provided with either Pc 81–104 or THMc 81–103 (Fig. 2B) and show the same heteroclitic pattern of response observed when B cells are used as APCs (Fig. 2A). Indeed, maximal T-cell activation required 16 μM THMc 81–103, while at 64 μM Pc 81–104 the T-cell response had not reached a maximum and was comparable to the half-maximal T-cell response to THMc 81–103. Other protein/lipid ratios ranging from 1:4 to 1:16 were also effective in producing functional I-E^k beads. As expected, I-E^k beads were unable to present the native protein antigen Pc at concentrations up to 64 μM (data not shown). Thus, the heteroclitic response of Pc-specific T cells to the antigenic peptide of THMc is solely a function of this peptide's association with the I-E^k molecule and the T-cell receptor recognizing the I-E^k-peptide complex. However, when comparing the efficiency of presentation, the I-E^k beads are less efficient than live or paraformaldehyde-fixed B cells (Fig. 2C). B cells (2×10^5) contain an estimated 2×10^{10} I-E^k molecules, significantly less than the 1×10^{12} I-E^k molecules contained in 25 μl of I-E^k beads. Increasing the number of I-E^k beads by 2- to 10-fold did not enhance T-cell responses at lower concentrations of antigenic peptides. There are several possible explanations for the relatively poor presenting capacity of the I-E^k beads, including variations in the physical properties of the I-E^k molecules in the lipid-coated beads versus the B-cell plasma membranes, or the presence of additional cell interaction molecules on B cells, which facilitate antigen presentation by B cells.

I-E^k Purified from APCs That Have Processed Antigen Functions in T-Cell Activation. Given that I-E^k and antigenic peptide are sufficient for heteroclitic T-cell activation, it was of interest to determine whether the I-E^k isolated from cells

FIG. 1. Affinity-purified I-E^k from CH27 cells. SDS/PAGE of the purified I-E^k.

FIG. 2. I-E^k beads function as APCs for the heteroclitic response to cytochrome *c* peptides. TPC 9.1 cells (5×10^4) were cocultured with either live B cells (2×10^5) (A), I-E^k beads (25 μl) (B), or paraformaldehyde-fixed B cells (2×10^5) (C) in the presence of graded concentrations of either THMc 81-103 ($\blacktriangle$) or Pc 81-104 ($\circ$). Culture supernatants were assayed for the presence of IL-2 24 hr later by the ability to maintain the growth of CTLL cells measured by the incorporation of [³H]thymidine. Lipid beads that contain no I-E^k failed to stimulate IL-2 secretion at THMc 81-103 peptide concentrations up to 64 μM ($\square$) (B). I-E^k beads to which no peptide was added yielded 500 cpm.

that had processed native Pc or DMc would be stimulatory. CH27 cells that are capable of processing and presenting both Pc and DMc to TPC 9.1 cells were incubated with either Pc (20 μM) or DMc (5 μM) for 12 hr at 37°C, washed free of antigen, and used as a source of I-E^k. I-E^k prepared from Pc-pulsed cells and incorporated into lipid-coated beads (Pc-I-E^k beads) activates TPC 9.1 cells in an I-E^k concentration-dependent fashion in the absence of any exogenous peptide antigen (Fig. 3B). The stimulation is low but significantly above that seen for I-E^k beads alone or for lipid-coated beads (Fig. 3B). I-E^k purified from DMc-pulsed cells and incorporated into lipid-coated beads (DMc-I-E^k beads) also stimulates TPC 9.1 cells and is significantly more active than I-E^k from Pc-pulsed cells. Consistent with the stimulation of TPC 9.1 by isolated I-E^k, CH27 cells are also more efficient at presenting DMc than Pc on a per cell basis (Fig. 3A). Thus, the I-E^k purified from antigen-pulsed cells is a functional I-E^k-antigen complex. Moreover, the T-cell response to these I-E^k beads maintains the fine specificity of the heteroclitic response observed with live APCs.

The T-cell response to both Pc- and DMc-I-E^k beads is augmented by the addition of THMc 81-103 (Fig. 3C). As

FIG. 3. (A) CH27 cells (2.5×10^9) were incubated 12 hr with Pc (20 μM) ($\circ$) or DMc (5 μM) ($\bullet$) and washed thoroughly, and a fraction was assayed for the ability to activate TPC 9.1 cells to secrete IL-2 in the absence of any exogenous peptide antigen. (B) Pc- and DMc-pulsed CH27 cells were washed in PBS, and the I-E^k from these cells was purified by immunoaffinity chromatography as described. The purified I-E^k was incorporated into liposomes at a 1:8 (wt/wt) protein/lipid ratio and adsorbed onto glass beads. Graded numbers of Pc-I-E^k beads ($\circ$), DMc-I-E^k beads ($\bullet$), or beads coated with lipid alone (Δ) were cocultured with TPC 9.1 cells (5×10^4) in the absence of any exogenous antigenic peptide. Culture supernatants were assayed for their IL-2 content 24 hr later. (C) Pc-I-E^k beads ($\circ$) (25 μl), DMc-I-E^k beads ($\bullet$) (25 μl), or beads coated with lipid alone (Δ) (25 μl) were incubated with TPC 9.1 cells in the presence of graded concentrations of THMc 81-103. Culture supernatants were tested for IL-2 content 24 hr later.

expected, higher concentrations of THMc 81-103 are required to reach maximal T-cell responses to Pc-I-E^k beads than DMc-I-E^k beads. The T-cell response to DMc-I-E^k beads is MHC-restricted as it can be blocked by the addition of antibody to I-E^k but not to I-A^k (Table 1). The T-cell response to the DMc-I-E^k beads is specific in that the beads do not activate an OVA-specific T-cell hybrid AODH-3.4 (Table 1).

DISCUSSION

The results presented here demonstrate the isolation of a functional I-E^k-peptide complex from APCs that have processed antigen. The fine specificity of the T-cell response to two different cross-reactive cytochromes *c* is maintained when I-E^k is isolated from antigen-pulsed APCs. This finding

Table 1. The specificity and MHC restriction of the T-cell response to DMC-I-E^k beads

Antibody added to culture	T-cell response (IL-2, cpm)	
	TPc 9.1	AODH-3.4
None	18,227	686
Anti-I-E ^k	410	NT
Anti-I-A ^k	17,454	NT

T-cell hybrids (5×10^4), either TPc 9.1 (I-E^k restricted, Pc specific) or AODH-3.4 (I-A^k restricted, OVA specific), were cocultured with DMC-I-E^k beads alone or in the presence of graded concentrations of monoclonal antibodies specific for I-E^k (14.4.4s) (20) or I-A^k (10.2.16) (21). The culture supernatants were collected 24 hr later and assayed for the presence of IL-2 as described in Fig. 2. The results shown are for I-E^k-specific antibody at 0.25 μ g/ml, at which maximal inhibition was observed, and for I-A^k-specific antibody at 4 μ g/ml. In control experiments, AODH-3.4 T cells secrete IL-2 in response to OVA (120 μ g/ml) when presented by CH27 cells as APC. This response was inhibited by anti-I-A^k at 4 μ g/ml. NT, not tested.

is significant as it provides an experimental link between two key observations concerning the T cell's MHC-restricted recognition of antigen. The first of these observations is the electron density observed in the proposed peptide binding groove of the crystallized HLA-A2 molecule (9, 10). The isolation of a functional I-E^k-peptide complex clearly demonstrates that the antigenic fragment produced by processing *in vivo* associates strongly with the class II molecule and remains bound during purification. Secondly, synthetic peptides containing T-cell antigenic determinants not only bind to MHC molecules in detergent solution (2–4) but also activate specific T cells when added to MHC, which is incorporated into lipid bilayers (6–8). Here we show that the incubation of APCs with native protein antigens yields Ia, which is active in stimulating antigen-specific T cells. This purified Ia-peptide complex, when presented on lipid-coated beads, maintains the MHC restriction and fine specificity of T-cell recognition observed with live APCs.

At present, the antigen-processing pathway within the APCs is poorly understood. Therefore, in the experiments presented here it is not known where in the cell the antigenic regions of the native cytochrome become associated with Ia. A recent report by Townsend *et al.* (26) studying the effect of exogenous peptide on a mutant cell line defective in class I protein assembly suggests that peptide may bind class I heavy chain in the endoplasmic reticulum and induce the correct folding of the chain and its association with β_2 -microglobulin, resulting in its transport to the cell surface. In this regard, the yield of I-E^k from CH27 cells that had processed DMC was consistently 2- to 3-fold greater than that from unpulsed cells (M.S., unpublished observation). Thus, it is possible that the processed products of the cytochrome c, which enters the cell through fluid-phase pinocytosis, bind to Ia chains at some early point in the assembly of the functional molecule. The binding of the high-affinity DMC peptides may stimulate Ia assembly or result in a more stable Ia molecule.

An important unresolved issue in the studies of T-cell antigen recognition is the nature of the processed antigen produced by the APCs and associated with the Ia molecule. It is not known whether T-cell stimulatory complexes contain

peptides that resemble the minimal length synthetic peptides, which suffice to activate T cells. Future studies evaluating the peptide-Ia complexes isolated from APCs that have processed radiolabeled DMC will need to address this issue.

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