Expression and Characterization of Recombinant Soluble Peptide: I-A Complexes Associated with Murine Experimental Autoimmune Diseases[1]

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Structural and functional studies of murine MHC class II I-A molecules have been limited by the low yield and instability of soluble, recombinant heterodimers. In the murine autoimmune diseases experimental autoimmune encephalomyelitis and collagen-induced arthritis, MHC class II molecules I-A^u and I-A^q present peptides derived from myelin basic protein and type II collagen, respectively, to autoreactive T cells. To date, systems for the expression of these two I-A molecules in soluble form for use in structure-function relationship studies have not been reported. In the present study, we have expressed functional I-A^u and I-A^q molecules using a baculovirus insect cell system. The chain pairing and stability of the molecules were increased by covalently linking the antigenic peptides to β-chains and adding carboxyl-terminal leucine zippers. Peptide:I-A^q complex quantitatively formed an SDS-stable dimer, whereas peptide:I-A^u formed undetectable amounts. However, the two complexes did not show any significant difference in their response to thermal denaturation as assessed by circular dichroism analyses. The autoantigen peptide:I-A complexes were highly active in stimulating cognate T cells to secrete IL-2 and inducing Ag-specific apoptosis of the T cells. Interestingly, the T cells were stimulated by these soluble molecules in the apparent absence of experimentally induced cross-linking of TCRs, indicating that they may have therapeutic potential in autoimmune disease models. ***The Journal of Immunology,*** **1998, 160: 5915–5921.**

T cells recognize peptide fragments bound to the groove of MHC class I or class II molecules and are therefore MHC restricted (1). In murine models of autoimmunity, I-A molecules of different haplotypes are almost invariably the restricting elements. High resolution structures of human HLA-DR and murine I-E molecules have been reported (2, 3), but both I-A and its human equivalent, HLA-DQ, molecules have proven difficult to produce for use in such studies. These problems are most likely related to the relatively weak association of α- and β-chains of I-A or HLA-DQ, which may be exacerbated when the transmembrane regions are truncated in attempts to express soluble molecules. To overcome these difficulties for I-A^d, approaches in which the antigenic peptide is covalently tethered (4) or the $\alpha\beta$ association is stabilized by either leucine zippers (5) or by a synthetic peptide linker (6) have been employed.

The current study describes the production of two I-A molecules associated with murine autoimmune diseases. I-A^u presents the immunodominant N-terminal 11 mer (or nonapeptide) of myelin basic protein (MBP)[3] to T cells during experimental autoimmune encephalomyelitis in H-2^u mice (7) and I-A^q presents residues 260 to 270 of type II collagen (CII) during collagen-induced arthritis in H-2^q mice (8). We have made use of a system that ensures effective pairing of the α- and β-chains of MHC class II molecules to express functionally active MHC class II molecules with antigenic peptides covalently linked to the β-chains. Biochemical and functional characterization of these peptide:I-A complexes have been conducted and indicate that these proteins have possible applications in the therapy of autoimmune disease models and in high resolution structural studies.

Materials and Methods

Cell lines

The murine 1934.4 T cell hybridoma (9) is specific for the N-terminal 11-mer (or nonamer) of MBP bound to I-A^u and was a generous gift from Dr. D. Wraith (University of Bristol, Bristol, U.K.). An I-A^u MBP1-11-restricted T cell line expressing the 1934.4 TCR (our manuscript in preparation) was made by transfecting TCR genes into a TCR-negative cell line 58α-β- (Ref. 10; kindly provided by Dr. S. Hedrick with permission from Dr. B. Malissen, Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique, Marseille-Luminy, France) using α and β shuttle vectors (11). The α and β shuttle vectors were kindly provided by Dr. M. Davis (Stanford University School of Medicine, Palo Alto, CA). TCR expression was analyzed by indirect immunofluorescence using the anti-Vβ8 mAb F23.1 (12), a generous gift from Drs. J. Kappler and P. Marrack (University of Colorado Health Sciences Center, Denver, CO). The murine qCII85.33 and qCII92.33 T cell hybridomas recognize bovine CII 260-270 presented in the context of I-A^q (13) and were kindly provided by Dr. E. Rosloniec (University of Tennessee, Memphis, TN). These cells were maintained in RPMI complete medium (RPMI 1640 from Cellgro, Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FCS, penicillin/streptomycin (100 U/ml), 2 mM glutamine, and 5.5×10^{-5} M β-mercaptoethanol). CTLL-2 (14), an IL-2-dependent

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[3] Abbreviations used in this paper: MBP, myelin basic protein; CII, type II collagen; CD, circular dichroism; TUNEL, TdT-mediated dUTP nick-end labeling; Tm, midpoint temperature; FPLC, fast protein liquid chromatography.

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mouse T cell line, was kindly provided by Dr. E. Vitetta (University of Texas Southwestern Medical Center, Dallas, TX).

Antibodies

The anti-polyhistidine mAb (IgG2a) HIS-1 was obtained from Sigma (St. Louis, MO). The following mouse B cell hybridomas were purchased from the American Type Culture Collection (ATCC, Rockville, MD): CRL-1729, which secretes 9E10, an anti-myc Ab (15) (IgG1); TIB-93, which secretes 10.2.16, an anti-I-A^k Ab (16) (IgG2b) cross-reactive with I-A^u; HB-183, which secretes Y-3P, an anti-I-A Ab (17) (IgG2a) specific for conformational epitopes of the α-chain of I-A^u and I-A^q; and HB-32, which secretes 14-4-4S, an anti-I-E^k Ab (IgG2a).

Construction of baculovirus vectors for the expression of I-A^u and I-A^q

Generation of I-A^u with covalently linked antigenic peptides. cDNA clones encoding I-A^u were generous gifts of Dr. D. Wraith. Truncated versions (at codon 217 for α-chain and 222 for β-chain) of MHC class II I-A^u α and β genes were generated by PCR. A strategy similar to that described by Kozono et al. (4) was used. A *Kpn*I restriction site was inserted between codons 3 and 4 of the β-chain gene using site-directed mutagenesis. A flexible linker including a thrombin cleavage site (GGGGSLVPRGSGG) was inserted using a synthetic DNA duplex that deleted the 3′ *Kpn*I site while retaining the 5′ *Kpn*I site. This site was used to insert the codons encoding the antigenic peptide, the [4Y] derivative of the rat MBP1-11 (ASQYRPSQRHG). For MBP1-11, a glycine was inserted before the MBP sequence as this extension mimics the role of the acetyl group in removing the charged N terminus of the peptide, which is necessary for T cell recognition (D. Wraith, personal communication). The α and β genes were tailored with *Bam*HI and *Bgl*II sites and ligated into the corresponding sites of the baculovirus expression vector pAcUW51 (PharMingen, San Diego, CA), which contains polyhedrin and p10 promoters upstream of the two cloning sites for dual expression of the α and β polypeptides.

Insertion of the leucine zipper motifs at the 3′ ends of the α and β genes. Sequences encoding the leucine zipper peptides, ACID-p1 and BASE-p1 (18) (a generous gift of E. L. Reinherz, Harvard Medical School, Cambridge, MA) were extended at the 3′ end with a 15-amino acid peptide linker containing an *Xho*I site (for the acidic zipper) or a *Sal*I site (for the basic zipper) and a thrombin site (SSL/AE/DLVPRGSTTAPS). The 3′ end of the acidic zipper was extended with a polyhistidine tag (his6 tag) followed by a stop codon and a *Bgl*II site. A *Bst*EII site was inserted at the 3′ end of the basic zipper. The modified leucine zippers were then fused to the α^u and β^u chain genes (acidic zipper to the α-chain and the basic zipper to the β-chain-peptide) using splicing by overlap extension (19). The PCR product encoding the α-chain-acidic zipper was restricted with *Bgl*II and cloned into the corresponding cloning sites of pAcUW51. The PCR product encoding the β^u chain-basic zipper was restricted with *Bst*EII and the fragment containing the carboxyl-terminal 140 codons of the truncated β^u chain fused with the basic zipper was inserted into the pAcUW51β^u-peptide construct containing the N terminal 73 codons of the β-chain, linked peptide, and the myc-tag (15) followed by a stop codon. The peptide (bovine CII260-270-IAGFKGEQGPK)-linked form of I-A^q-zipper was constructed using a similar strategy to that for the I-A^u constructs, but the α^q-zipper and β^q-peptide-zipper were cloned into the *Bgl*II and *Bam*HI sites of the pBlueBac4.5 vector (Invitrogen, Carlsbad, CA). All the expression constructs were sequenced and determined to be free of mutations.

Production and purification of soluble I-A^u and I-A^q molecules

pAcUW51α^u/β^u-peptide, pAcUW51α^u/β^u-peptide-zipper, pBlueBacα^q-zipper, and pBlueBacβ^q-peptide-zipper were each cotransfected into Sf9 insect cells with wild-type baculoviral DNA (linear AcMNPV DNA or Bac-N-Blue DNA, Invitrogen). Sf9 cells were grown in TNM-FH medium (JRH Biosciences, Lenexa, KS or Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS. Recombinant viruses were identified and plaque purified by visual screening (occ$^-$ plaques) (20) or by the X-galactosidase overlay method (21). The expression of the recombinant proteins was checked in immunoblots using the anti-myc (9E10) and anti-polyhistidine mAbs. After three rounds of plaque purification, 200-ml viral stocks were prepared in Sf9 cells. The stocks were used for the production of MHC class II molecules by infecting High Five cells (Invitrogen) at a multiplicity of infection of 10 at a density of 1×10^6 cells/ml in Excell-405 insect cell serum-free medium (JRH Biosciences). High Five cells were used in preference to Sf9 cells because preliminary studies indicated that the expression levels were higher in these cells (not shown). Infected cells were grown at room temperature in Spinner flasks (100–500 ml) for 4 days, the culture supernatant collected, and the MHC class II molecules affinity purified using Y-3P Ab covalently coupled to protein A- or G-Sepharose beads (Pharmacia, Uppsala, Sweden). Before Ab-affinity purification, the supernatants were precleared using protein A- or G-Sepharose beads. Proteins were eluted from Ab affinity columns using 100 mM Na_2CO_3, 500 mM NaCl, pH 11.5, immediately neutralized to pH 7.0, and dialyzed against PBS. After purification, I-A^u and I-A^q molecules were analyzed using 15% SDS-PAGE under reducing conditions with and without heating to 95 to 100°C. Proteins were detected by Coomassie blue staining or by immunoblotting using anti-polyhistidine, 9E10 or 10.2.16 Abs. Proteins were also analyzed by size exclusion chromatography using a Superdex-200 column (Pharmacia) and the Biologic Workstation (Bio-Rad, Hercules, CA).

Circular dichroism (CD) analyses

CD analyses were performed as previously described (22) using an AVIV 62 DS spectropolarimeter (Aviv Associates, Lakewood, NJ), except that the recombinant proteins were in PBS pH 7.2. For thermal denaturation, the samples were progressively heated from 25 to 90°C with an equilibration time of 30 s at each temperature. Recording time was 15 s at each temperature and the CD signal was followed at 203 and 205 nm. Reversibility of the thermal unfolding was demonstrated by analyzing at 25°C the far-UV CD spectra of the peptide:I-A molecules after a 25 to 90°C heating/cooling cycle.

T cell assays

T cell stimulation assays were conducted in triplicate in flat-bottom 96-well plates (Costar, Cambridge, MA). Peptide:I-A^u and I-A^q molecules were adsorbed on the plate for 2 h at 37°C at various concentrations (1–500 ng per well). The wells were washed twice with PBS. T cells (10^5/well) were incubated with the peptide:MHC class II molecules for 24 h. IL-2 levels in the supernatants were determined by quantitating [^{3}H]thymidine incorporation into IL-2-dependent CTLL-2 cells.

For the apoptosis assay, the T cells were washed with 1% BSA in PBS, resuspended in PBS-BSA containing 50 μg/ml propidium iodide, and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) as described (23).

Similar experiments were conducted using soluble molecules. For this, after blocking the wells for 3 h at 37°C using 1 or 2% BSA in PBS or 10% FCS in RPMI, the T cells were added together with various concentrations of soluble peptide:I-A molecules and incubated at 37°C. After 24 h, IL-2 secretion and apoptosis induction were analyzed. To ascertain the specificity, T cell stimulation was also conducted in the presence of an anti-I-A mAb, Y-3P. An anti-I-E mAb, 14-4-4S, was used as a control.

TdT-mediated dUTP nick-end labeling (TUNEL) assay

The 1934.4 T hybridoma cells were incubated with 10 μg/ml of soluble peptide:I-A^u complexes for 1 and 5 h and then washed with PBS and fixed with 0.5% paraformaldehyde. Following a 1-h incubation, cells were permeabilized with Triton X-100 (0.2% v/v) and then incubated with TUNEL reagent (Boehringer Mannheim, Indianapolis, IN) for 1 h as recommended by the manufacturer. Cells were washed twice (0.1% Triton X-100/1% BSA/PBS) and then analyzed by flow cytometry as described above.

Results

Expression and purification of the recombinant peptide:I-A complexes

The peptide MBP1-11[4Y] in which the lysine at position 4 is substituted by tyrosine is known to bind with much higher affinity to I-A^u (24, 25), and this position 4 substitution does not appear to affect T cell recognition (26). In the current study this higher affinity analogue was therefore linked to β^u and coexpressed with α^u (Fig. 1*A*), in an analogous way to that described by Kozono et al. (4). However, this resulted in the production of $\alpha^u\beta^u$ heterodimers in low yields that did not activate T cells (data not shown). The inactivity of the protein appeared to be due to dissociation of α- and β-chains following purification. Similar results have been noted for I-A^d (5). We therefore built constructs in which the α^u and β^u (with linked MBP1-11[4Y]) were tagged with leucine zippers in an attempt to stabilize $\alpha^u\beta^u$ association (Fig. 1*B*). A similar approach was reported for the stabilization of I-A^d heterodimers (5), but in that case the β^d chain was not linked to peptide. This

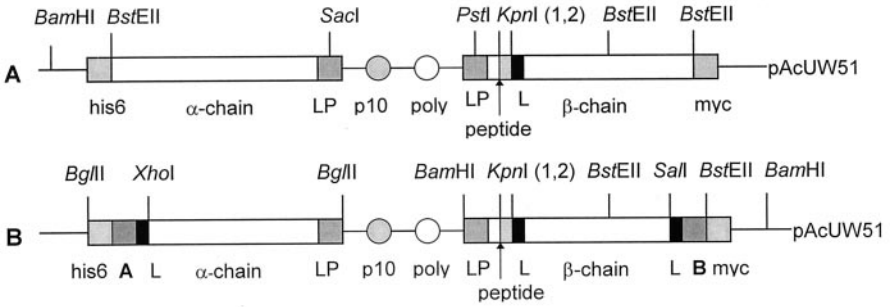

FIGURE 1. Plasmid constructs for the expression of I-A^u in insect cells. *A*, pAcUW51α^u/β^u-peptide; *B*, pAcUW51α^u/β^u-peptide-zipper. The locations of the restriction sites used for cloning are indicated. p10: p10 promoter; poly: polyhedrin promoter; LP: leader peptide; peptide: antigenic peptide; L: synthetic linker containing a thrombin cleavage site; α-chain: truncated I-A^u α-chain; β-chain: truncated I-A^u β-chain; A: acidic zipper; B: basic zipper; his6: polyhistidine tag; myc: myc-tag.

resulted in I-A^d heterodimers that could subsequently be loaded with antigenic peptide (5). However, the empty I-A^d molecules showed limited stability (5), and for this reason in the current study zipper-tagged molecules were expressed with covalently linked peptides.

The recombinant MBP1-11[4Y]:I-A^u was affinity purified using Y-3P, a conformational-dependent Ab (17) coupled to protein A- or G-Sepharose. Although the recombinant molecules were expressed with his6 tags, Y-3P Sepharose was used in preference to Ni^{+2}-NTA agarose, since only $\alpha\beta$ heterodimers in the correct conformation can be purified on Y-3P affinity columns. The yield of MBP1-11[4Y]:I-A^u was approximately 1 mg per liter of culture (10^9 High Five cells). A similar strategy was employed for CII260-270:I-A^q, and the yields were comparable.

SDS stability and CD analyses of the purified I-A molecules

MHC class II molecules are frequently SDS stable, in a form designated compact dimers (27) in which α and β polypeptides remain associated in the presence of SDS at room temperature. This form occurs following antigenic peptide binding, and is taken to represent a mature form of the molecule (27). However, peptide binding is necessary but not sufficient to induce compact dimer formation, and the structural requirements for a peptide to generate the SDS stable form of a class II molecule are as yet poorly defined (28–30). We were therefore interested to determine whether the recombinant purified peptide:I-A complexes formed this SDS-stable form (Fig. 2). Soluble CII260-270:I-A^q forms compact dimers quantitatively, whereas MBP1-11:I-A^u yields undetectable amounts. This marked difference in compact dimer formation led us to carry out CD analyses to assess the thermostability of the I-A complexes. The spectra show that the proteins are correctly folded (Fig. 3), but perhaps unexpectedly, for both proteins the denaturation midpoint temperature (Tm) is 73°C. Thus, for these two complexes, compact dimer formation does not correlate with the thermal stability of the folded state assessed by CD.

Stimulation of cognate T cells

Both peptide:I-A complexes were very efficient in stimulating cognate T cells. MBP1-11:I-A^u induced dose-dependent activation of MBP-specific T cells (1934.4 T cell hybridomas or 1934.4 TCR transfectants) while CII260-270:I-A^q activated collagen-specific T cell hybridomas qCII85.33 and qCII92.33, as determined by IL-2 secretion (Fig. 4). The T cell activation was highly specific as there was no detectable cross-recognition of the complexes (Fig. 4). These molecules also induced apoptosis of T cells in a ligand-specific manner (data not shown). Interestingly, the T cells were also activated when the peptide-MHC complexes were presented in solution and not coated on the plate: both IL-2 secretion and apoptosis could be detected for T cell hybridomas and transfectants (Fig. 5). Similar results were also seen for the qCII92.33 hybridoma in response to soluble CII260-270:I-A^q (data not shown). The TUNEL method was used to confirm that the cell death was apoptotic and not necrotic (Fig. 5*A*). Relative to the soluble I-A complexes, the plate-coated molecules were, however, approximately 20 times more potent on a per weight basis (not shown). Activation with the autoantigen molecules in solution was also specific for both peptide:I-A complexes. It could be blocked by the anti-I-A mAb Y-3P, which recognizes correctly conformed I-Aα polypeptide (Fig. 5). However, for reasons that are not clear Y-3P blockade of 1934.4 hybridoma cell death was not complete (Fig. 5*C*), despite the use of a higher Y-3P/I-A molar ratio than was used for other T cells (Fig. 5, *B* and *D*). As expected, coincubation of peptide:I-A complexes with anti-I-E Ab did not have any effect in these functional assays.

The data suggest that the T cells (transfectants or hybridomas) in the present study could be activated even in the absence of TCR

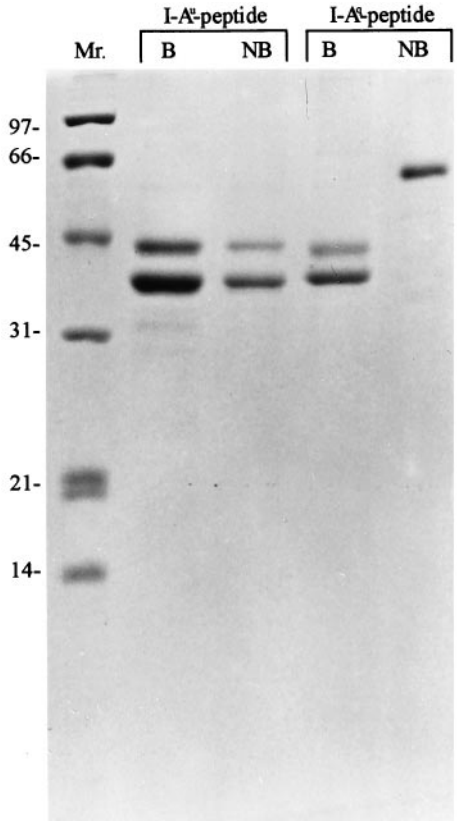

FIGURE 2. SDS-PAGE (15%) analysis of recombinant peptide:I-A molecules, stained with Coomassie brilliant blue. Mr.: m.w. standards; B: the samples (in 2% SDS sample buffer) were heated for 5 min at 95 to 100°C before loading; NB: the samples were kept at room temperature in SDS (2%) sample buffer for 5 min before loading.

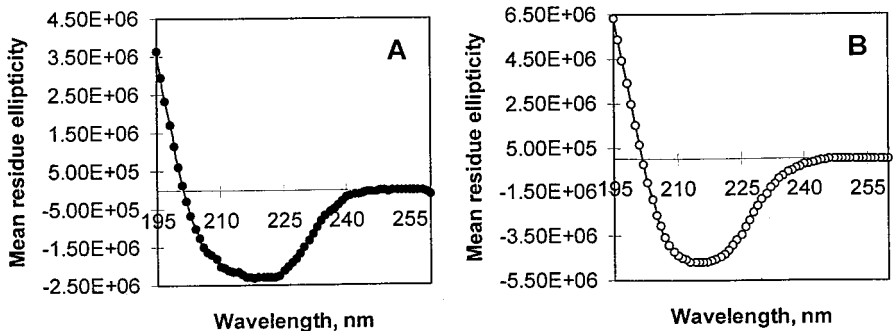

FIGURE 3. Far-UV CD spectra of peptide: I-A molecules. *A*, MBP1-11:I-A^u; *B*, CII260-270:I-A^q. *y*-axis units are mdeg cm^2/dmol.

cross-linking. Analysis of both peptide:I-A complexes by fast protein liquid chromatography (FPLC) showed no evidence of aggregation, although some dissociation into single α and β polypeptides was observed with MBP1-11:I-A^u. However, blockade of T cell activation by Y-3P excludes any involvement of the individual chains in T cell stimulation. To further investigate the possibility that I-A aggregates may be involved in T cell stimulation, a time course of IL-2 secretion and apoptosis induction was performed with soluble MBP1-11:I-A^u and 1934.4 hybridoma cells (Fig. 6). IL-2 secretion and apoptosis are detectable within 2 h of peptide: I-A addition, suggesting that aggregation during the 24-h incubation used in Figure 5 is not responsible for the observed effects. As would be expected, the kinetics of IL-2 production are different from those of apoptosis, with IL-2 levels plateauing before cell death. However, it cannot be excluded that the effects observed in Figures 5 and 6 are due to the presence of levels of preformed aggregated, Y-3P-reactive I-A molecules that are not detectable by FPLC.

Discussion

This study describes the expression and characterization of two peptide:I-A complexes associated with murine autoimmunity. The complexes can be produced in milligram quantities in insect cells and are very efficient at activating ligand-specific T cells. Functional expression has been achieved using a combination of strategies involving covalent linkage of antigenic peptide to the β-chain and stabilization of $\alpha\beta$-chain association with leucine zippers at the C-termini.

The two complexes show a marked difference in compact dimer formation, with CII260-270:I-A^q forming compact dimers quantitatively, whereas MBP1-11:I-A^u yields undetectable amounts. Interestingly, CD analyses indicate that compact dimer formation does not correlate with thermal stability of the folded state, as both complexes exhibit indistinguishable denaturation temperatures. Thus, lack of compact dimer formation does not appear to result in thermal instability of the folded state of the proteins. Furthermore, the Tm for both peptide:I-A complexes is similar to that reported for peptide-loaded I-E^k but higher than that for I-A^d covalently linked to antigenic peptide (31). The difference in Tm for peptide: I-A^d vs peptide:I-A^u/I-A^q may be due to the absence of leucine zippers in the peptide:I-A^d complexes (4).

The structural basis of compact dimer formation is unclear and does not appear to be affected by peptide binding affinities (29). However, for HLA-DR, the interaction of the first anchor residue (P1) in a hydrophobic pocket has been shown to play a role in mediating SDS stability (29). In the current study, the difference in compact dimer formation may therefore be a reflection of the particular anchor residues present in the sequences of MBP1-11[4Y] and CII260-270, as it has been suggested that the polymorphic residue at position 52 of the I-Aα-chain may affect the size and nature of the P1 pocket (32). Both I-A^k and I-A^u have arginine at this position, which is consistent with the preference for acidic residues at P1 for high affinity binding to I-A^k (32). In contrast, I-A^q has threonine at α52, which may result in a preference for larger, bulky residues such as isoleucine at P1 (present in CII260-270 and known to be important for MHC binding (13)), and this in turn may facilitate compact dimer formation. This suggests that P1 of MBP1-11[4Y] may not be optimal for the induction of SDS stability. In this context, I-A^u molecules expressed on the surface of PL-8 cells (I-A^u LPS blasts fused with the I-A-negative B lymphoma M12.C3 (33)) form compact dimers almost quantitatively (A. Q., unpublished observation) indicating that this I-A molecule does have the capacity to form an SDS-stable conformation. This is presumably due to the presence of peptides in PL-8 cells that have the necessary characteristics to stabilize $\alpha^u\beta^u$ polypeptide association. The alternative explanation that I-A^u is dependent upon the presence of invariant chain or H-2M for compact dimer formation, as has been reported for other class II haplotypes (34–36), is excluded by an observation that I-A^u-transfected, invariant chain, and H-2M-negative BW5147 cells (Ref. 37; a generous gift of Dr. H. McConnell, Stanford

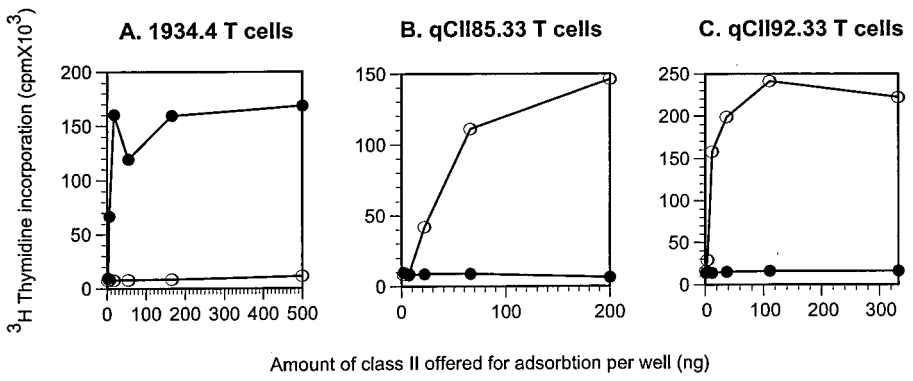

FIGURE 4. T cell stimulation assays with plate-bound peptide:I-A molecules. T cells were incubated with peptide:I-A molecules adsorbed onto flat-bottom wells of a 96-well plate. At 24 h later, culture supernatants were analyzed for IL-2 levels by measuring [^{3}H]thymidine incorporation into the IL-2-dependent cell line, CTLL-2. ●–MBP1-11:I-A^u, ○–CII260-270:I-A^q. *A*, 1934.4 TCR transfectants (I-A^u restricted, MBP1-11 specific); *B* and *C*, I-A^q-restricted, CII260-270-specific T cell hybridomas (qCII85.33 and qCII92.33).

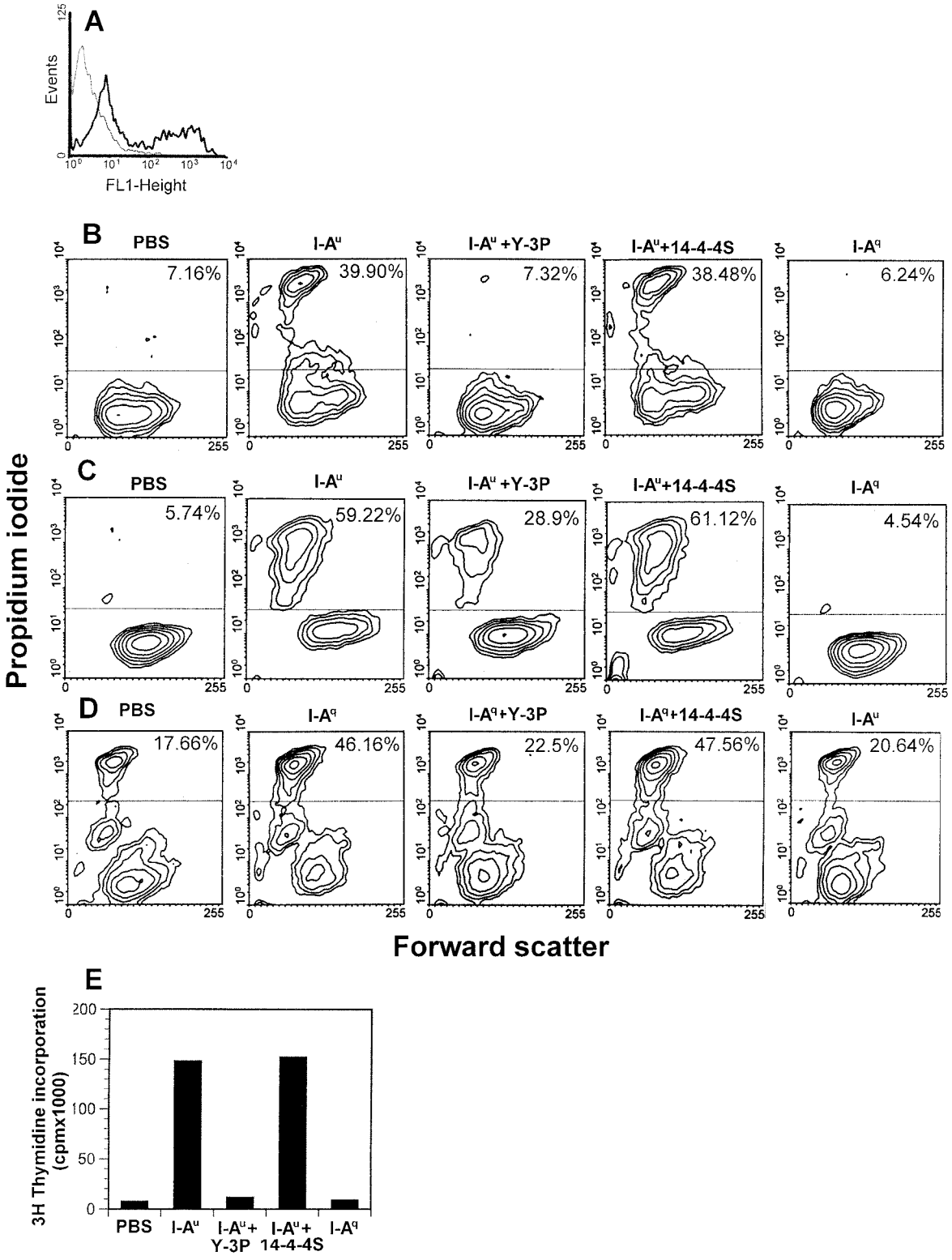

FIGURE 5. T cell assays using soluble molecules. T cells (10^5/well) were stimulated with peptide:I-A complexes in a round-bottom 96-well plate preblocked with 1 or 2% BSA or 10% FCS at 37°C. Following the incubation times indicated in the methods section, T cells were washed with PBS-BSA, and used in the TUNEL assay (*A*) or suspended in PBS-BSA containing 50 μg/ml of propidium iodide (*B–D*). Following staining, cells were analyzed by flow cytometry (*A–D*). For *A*, the TUNEL assay was conducted after 1 h (thin line) and 5 h (thick line) of incubation with peptide:I-A^u complexes. To ascertain the specificity of the assay, in *B* to *D* the peptide:I-A molecules were also preincubated with 5 μg (*B*, *D*) or 15 μg (*C*) of Y-3P or 14-4-4S mAb. *B* and *C* show the effects induced by 10 μg/ml or 1 μg/ml, respectively, of MBP1-11:I-A^u on 1934.4 TCR transfectants (*B*) or 1934.4 T cell hybridomas (*C*). *D* shows the effects induced by 10 μg/ml of CII260-270:I-A^q on qCII85.33 hybridoma cells. The number in the *upper right corner* represents the percentage of apoptotic T cells (low forward scatter and high propidium iodide fluorescence) relative to the total number of cells. *D*, IL-2 secretion (determined by measuring [^{3}H]thymidine incorporation into CTLL-2 cells) by 1934.4 TCR transfectants stimulated as described in *B*. Both 1934.4 and qCII85.33 hybridoma cells secreted IL-2 at similar levels in response to the treatments shown in *C* and *D*, respectively (not shown).

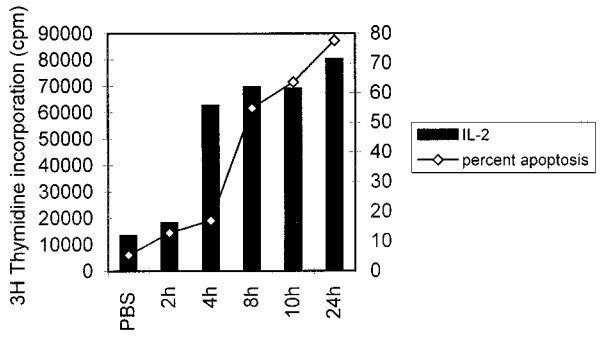

FIGURE 6. Time course of IL-2 production and apoptosis induction for 1934.4 T cell hybridoma cells in response to 10 μg/ml of MBP1-11:I-A^u. PBS: cells treated with PBS for 24 h. IL-2 levels and apoptosis were quantitated as for Figure 5.

University, Stanford, CA) express significant amounts of compact dimers (A. Q., unpublished observation).

The lack of compact dimer formation did not, however, affect the functionality of the MBP-1-11:I-A^u complex as has also been seen in other systems where SDS stability is not a prerequisite for T cell activation (36); this complex efficiently activates 1934.4 T cells (Fig. 4*A*). Similar results were obtained for CII260-270:I-A^q, which specifically activates qCII85.33 and qCII92.33 cells (Fig. 4, *B* and *C*). Furthermore, both recombinant I-A^u and I-A^q complexes are stable for at least 1 mo of storage at 4°C, again indicating that differences in compact dimer formation do not appear to result in differential stabilities.

Our results indicate that the peptide:I-A complexes are also able to mediate IL-2 secretion and apoptosis of cognate T cells in the absence of TCR cross-linking. Although plate-bound complexes appear to be more efficient on a molar basis at apoptosis induction (data not shown), the soluble complexes (added to previously blocked plates, with undetectable amounts of aggregates by FPLC) were also effective at inducing T cells to undergo apoptosis and secrete IL-2 (Fig. 5). In contrast, we do not observe apoptosis of 1934.4 T cell transfectants or 1934.4 hybridoma cells when they are stimulated by I-A^u-expressing PL-8 cells (33) pulsed with antigenic peptide, suggesting that other molecules on the surface of this B lymphoblast-derived cell line protect against apoptosis (A. Q., unpublished observations). The soluble peptide:I-A complexes will be of use in investigating the molecular basis of this phenomenon.

The stimulatory effects of the peptide:I-A complexes on cognate T cells were mediated solely by heterodimers, as any single α- or β-chains that might be formed by dissociation do not bind to the conformational-dependent Ab Y-3P. In our studies, this Ab completely blocks apoptosis and IL-2 secretion for three of four T cell hybridomas/transfectants tested (Fig. 5 and data not shown). These data are in contrast to other reports in which the assays were either designed to induce cross-linking by using plate-bound peptide: MHC (6) or aggregation of soluble complexes was reported to be necessary for efficacy (38). Furthermore, in other studies, the HLA-DR or I-A heterodimers used in soluble form to induce apoptosis were reported to be aggregated (39) or dimeric (40). The possibility that in our study the monomeric peptide:MHC complexes aggregate in solution after addition to the assay wells is unlikely, as the molecules are effective after only 2 h of coincubation with T cells and are diluted into a medium containing 10% FCS. However, although believed to be unlikely, the possibility that amounts of aggregated, Y-3P-reactive I-A molecules that are undetectable by FPLC are responsible for the effects observed in Figures 5 and 6 cannot be excluded. Our data also indicate that soluble I-Aβ-chains alone, complexed with peptide, do not induce apoptosis, as the cell death induction is blocked by Y-3P. This is in contrast to the study in which HLA-DR α- or β-chains plus antigenic peptide were shown to be effective (41). The indications in the current study that soluble, nonaggregated peptide:I-A complexes can induce IL-2 secretion are reminiscent of the report that a single peptide:MHC complex on an APC can induce T cell activation (42). This supports serial triggering models of T cell activation (43).

In summary, we have described the functional expression of two peptide:I-A complexes of relevance to murine autoimmune disease models. The complexes can be produced in milligram quantities and are highly active in specifically stimulating T cells to produce IL-2 and to undergo apoptosis. These complexes should be useful in both analyzing the affinities of autoreactive TCRs and high resolution structural studies. Finally, they may have utility as immunotherapeutic reagents in the modulation of autoreactive T cell activation.

Note added in proof. A study describing the X-ray crystallographic analysis of peptide:I-A^d complexes with covalently linked peptides and leucine zippers has recently been reported (44).

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