

# Role of Chain Pairing for the Production of Functional Soluble IA Major Histocompatibility Complex Class II Molecules

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## Summary

Structural studies of cellular receptor molecules involved in immune recognition require the production of large quantities of the extracellular domains of these glycoproteins. The murine major histocompatibility complex (MHC) class II-restricted response has been extensively studied by functional means, but the engineering and purification of the native, empty form of the most-studied murine MHC class II molecule, IA, has been difficult to achieve. IA molecules, which are the murine equivalent of human histocompatibility leukocyte antigen-DQ molecules, have a low efficiency of chain pairing, which results in poor transport to the cell surface and in the appearance of mixed isotype pairs. We have engineered soluble IA molecules whose pairing has been forced by the addition of leucine zipper peptide dimers at their COOH-terminus. The molecules are secreted "empty" into the extracellular medium and can be loaded with single peptide after purification. These IA molecules have been expressed in milligram quantity for crystallization as well as for activation of T cells and measurement of MHC class II-T cell receptor interactions.

The paradigm for studying murine MHC class II-restricted immune response has been systems in which the restricting element was an IA molecule (1). The three-dimensional structure of this particular MHC class II molecule and its human equivalent, HLA-DQ, is still unknown. The barrier to directly addressing this issue has been a lack of availability of large quantities of homogeneous purified IA molecules. In contrast, the engineering of soluble and secreted murine IE (2) and human HLA-DR molecules (3, 4) has enabled structural studies to proceed. This latter approach has not yielded satisfactory amounts of functionally active IA molecules. The addition of a covalently linked peptide to the end of the IA- $\beta$  chain (5) did not successfully overcome the expression problem (L. Teyton, unpublished observation). On the other hand, the promiscuity of both IA and HLA-DQ chains for pairing with chains of other class II molecules has been known for many years (6–8). Their ability to mismatch with IE and HLA-DR chains, for example, is suggestive of a low affinity between homologous chains. As a consequence of this low affinity, the efficiency of assembly and transport is much lower for IA/HLA-DQ molecules than it is for IE/HLA-DR molecules (9, 10). We decided, therefore, to test directly the effect of enhanced IA  $\alpha$  and  $\beta$  chain pairing on the production of soluble secreted IA<sup>d</sup> molecules.

In some proteins, such as the basic leucine zipper transcription factors, dimerization is mediated by coiled coils composed of characteristic seven residues (heptad) repeats (a to g) with hydrophobic residues at position a and d, and polar residues elsewhere (11, 12). These motifs can be transplanted to other proteins, where dimerization through two amphipathic  $\alpha$ -helices can lead to functional proteins (13). We have extended the COOH terminus of each IA chain with such complementary leucine zipper peptides to force  $\alpha$ - $\beta$  association. The resulting effect on dimerization, transport, and secretion was extremely beneficial, and we were able to isolate milligram quantities of highly purified material. In addition, we have optimized conditions to load the purified IA molecules with single peptides. We have now shown that the peptide-loaded molecules are able to bind soluble T cell receptor and to activate T cells. Finally, we have used IEF to demonstrate the formation of a stable complex between peptide and IA<sup>d</sup> molecules.

## Materials and Methods

*cDNA Constructs, Transfection, and Purification.* Full-length IA<sup>d</sup>  $\alpha$  and  $\beta$  chain cDNAs were modified by PCR using 3' primers encoding the linker, the thrombin cleavage site, the leucine zipper peptide, and a Sall cloning site. An EcoRI site was added in the

noncoding sequence of the 5' primer. The extracytoplasmic domain of the human invariant chain was extended at its NH<sub>2</sub>-terminus by a stretch of six histidines and fused to the HLA-A2 signal sequence by modifying the original cDNAs by PCR (14). The PCR fragments were cloned into the polylinker of the metallothionein promoter-driven pRMHa3 vector (15) and sequenced by dye terminator technique on an automated sequencer (model 373A; Applied Biosystems, Foster City, CA). The modified cDNAs were cotransfected at equimolar ratio into *Drosophila melanogaster* SC2 cells along with a neomycin resistance gene (15) at a ratio of 1:60. Stable transfected cell lines were derived by G418 selection in Schneider's medium over a period of 4 wk. Expression was tested 3 d after copper sulfate induction by immunoprecipitation supernatant with MKD6 agarose beads (16), according to the described protocol (15). For large-scale preparation, the same procedure was used, followed by a Mono Q 10/10 ion exchange chromatography and/or a Superdex 200 gel filtration chromatography (Pharmacia, Piscataway, NJ). IA<sup>d</sup>-invariant chain complexes were purified from induced supernatants of triple-transfected cell lines by use of the same strategy. Soluble TCRs were made soluble by a similar approach. In brief, the DNA segment encoding the transmembrane region of both chains of the OVA-2 T cell receptor (F. Carbone, unpublished observations) were removed by PCR mutagenesis using 3' oligonucleotides hybridizing to the 18 base pairs ending the coding sequence of the constant domain and extended by the coding sequences of their respective cytoplasmic tails and a polyhistidine tail. An EcoRI 5' and a SalI 3' cloning site were used for cloning into pRMHa3. Transfections and selection were carried out as described for IA<sup>d</sup>. The initial step of purification was a nickel-agarose affinity chromatography, followed by ion exchange, hydrophobic interaction chromatography, and gel filtration. All purified proteins were kept at 4°C in PBS plus 0.02% NaN<sub>3</sub> at high concentration (>5 mg/ml).

**Biochemical Analysis, SDS-PAGE, and Antibodies.** Purifications were monitored by SDS-PAGE and Coomassie Brilliant Blue R 250 staining. Precast 10–15% gradient acrylamide gels were routinely used for this purpose (Bio-Rad, Hercules, CA). Two-dimensional gel electrophoresis was performed as described (8) with 3.5 to 10.0 nonequilibrium pH gradient electrophoresis (NEPHGE)<sup>1</sup> in the first dimension and a 12% SDS-PAGE in the second dimension. 20 µg of purified protein was used per gel. Proteins were detected by Coomassie blue staining.

Thrombin digestion was performed before gel filtration in morpholinoethanesulfonic acid buffer, pH 6.5, at 37°C for 60 min with 10<sup>-3</sup> U of the enzyme (Calbiochem-Novabiochem Corp., La Jolla, CA) per 10 µg of IA molecules. Endoglycosidase-F digestion was performed in PBS, pH 7.4, for 60 min at 37°C (1 U per 20 µg of IA<sup>d</sup>).

The MHC class II-specific mAbs MKD6 (16) and M5-114 (17) were obtained from the American Type Culture Collection (Rockville, MD). K24-199 (18) and 40B (19) were obtained from Drs. J. Miller (University of Chicago) and M. Pierres (INSERM-CNRS, Marseilles, France), respectively. Abs were purified from ascites by protein A affinity chromatography.

**Peptide Loading and Native IEF.** Purified IA<sup>d</sup> molecules were loaded with peptide immediately after elution at basic pH by adding 20 µM of free peptide and then neutralizing with 2 M glycine. The loaded molecules were subsequently dialyzed in PBS and further purified by gel filtration or, alternatively, were dialyzed against

50 mM Tris-HCl, pH 8.4, and purified by ion exchange chromatography and gel filtration. The three peptides used were OVA<sub>323-339</sub>, hemagglutinin (HA)<sub>126-138</sub>, and HEL<sub>46-61</sub> (non-IA<sup>d</sup>-specific peptide). IA<sup>d</sup>-Ii<sub>56-216</sub> complexes were loaded with single peptides by incubating them with a 20-fold excess of peptide at pH 5.5 for 60 min at 37°C. After neutralization with 4 M Tris base, peptide complexes were purified by ion exchange chromatography and/or gel filtration. The loading experiment shown in Fig. 2 B was analyzed 60 min before the purification step. All peptides were chemically synthesized by T-BOC chemistry on an Applied Biosystems instrument and purified on a C18 reverse phase column. IEF experiments were run on pH 3–10 Servalyte prenet gels (Serva Feinbiochemica GmbH and Co., Heidelberg, Germany) on a flat-bed temperature-controlled Isobox (Hoefer Scientific Instruments, San Francisco, CA) at 2,000 V and stained by the Coomassie/copper sulfate method.

**T Cell Assays.** T cell assays were carried out in triplicate in flat-bottom 96-well plates in which the different IA molecules were adsorbed overnight at 4°C in phosphate buffer at various concentrations ranging from 1 to 0.125 µg. T cells were washed twice in PBS and incubated in DMEM, 0.05% BSA medium for 24 h at 2 × 10<sup>4</sup> cells per well. IL-2 secretion was measured using the NK cell assay (20). In brief, supernatants were harvested after 24 h and serially diluted before adding 5,000 NK IL-2/IL-4 indicator cells in a total volume of 100 µl. After 24 h, NK cell cultures were pulsed with 1 µCi of [<sup>3</sup>H]thymidine. Isotope incorporation was determined the next day. Units are defined as the dilution of supernatant to yield half-maximal stimulation of the indicator cells.

Concentrations of IL-2 were determined by comparison with standard curves generated with recombinant IL-2 (Pharmingen, San Diego, CA). For each assay, the following IA molecules were tested: empty IA<sup>d</sup>, IA<sup>d</sup> loaded with OVA<sub>323-339</sub>, IA<sup>d</sup> loaded with HA<sub>126-138</sub>, IA<sup>d</sup> plus 10 µg of OVA<sub>323-339</sub> peptide, IA<sup>d</sup> plus 10 µg of HA<sub>126-138</sub> peptide, and peptide alone. We used two T cell hybridomas for our functional assays: HA.126, a subclone of 6/22 126.3-2, specific for IA<sup>d</sup>-HA<sub>126-138</sub> complexes (21), and DO11.1.10, a subclone of DO11.10 (22) specific for IA<sup>d</sup>-OVA<sub>323-339</sub> complexes.

**Plasmon Resonance.** A biosensor (BIAcore 2000; Pharmacia, Biosensor, Piscataway, NJ) was used for the measurements, and the data were analyzed with the BIAevaluation 2.1 package. The purified TCR molecules were immobilized to the dextran layer of the sensor chip by classic amine coupling. The different IA<sup>d</sup> complexes and BSA were passed through the flow cell at 20 µl per min in PBS. Association and dissociation phases were fitted using the BIAevaluation 2.1 program.

## Results and Discussion

We have successfully expressed a number of soluble and secreted HLA-DR molecules in SC2 insect cells (L. Teyton, manuscript in preparation) but had failed to produce large quantities of the murine IA molecules by using the same approach. Production levels of IA from stably transfected cell lines were between 10 and 50 µg per liter of supernatant, and purification was not feasible (L. Teyton, unpublished observation). The strategy of fusing peptide residues that correspond to the antigenic peptide sequence to the NH<sub>2</sub>-terminus of the β chain has been recently described (5); however, this approach did not increase levels of production. Promiscuous chain pairing by IA and HLA-DQ has been demonstrated by the existence of mixed isotype

<sup>1</sup>Abbreviations used in this paper: HA, hemagglutinin; NEPHGE, nonequilibrium pH gradient gel electrophoresis.



**Table 1.** Sequencing of Empty and Peptide-loaded IA<sup>d</sup> Molecules

IA <sup>d</sup> molecule	Chain or peptide	Cycle						
		2	3	4	5	6	7	8
Empty IA <sup>d</sup>	α	D(8)	D(12)	I(8)	/	A(8)	D(8)	/
	β	N(16)	/	E(12)	/	H(6)	F(8)	/
	p	/	/	/	/	/	/	/
IA <sup>d</sup> -OVA	α	D(22)	D(27)	I(7)	E(19)	A(19)	D(15)	H(12)
	β	N(25)	/	E(15)	/	H(16)	F(11)	V(21)
	p	/	Q(22)	A(13)	V(29)	H(16)	A(12)	A(18)
IA <sup>d</sup> -HA	α	D(23)	D(31)	I(9)	E(14)	A(15)	/	H(7)
	β	N(20)	/	E(21)	G(16)	H(9)	F(11)	V(16)
	p	N(21)	T(18)	N(16)	G(16)	V(14)	T(7)	/

For each sequence the amount recovered per cycle is indicated in parenthesis in picomoles. The slashes indicate residues that did not appear in the sequencing. α stands for the sequence of the α chain, β for the sequence of the β chain, and p for the sequence of the peptide. Amino acids are in single-letter code.

We noticed that the β chain thrombin site was consistently more accessible than its α chain counterpart and that it was readily removed by a 10-min protease digestion. On the other hand, the α chain site needed 30–45 min to be cleaved. Endoglycosidase-F (or PNGase; data not shown) was used to assess the state of glycosylation of the expressed IA molecules. Partial digest with endoglycosidase-F removed the 2 N-linked sugars of the α chain and the single site of the β chain (Fig. 1 B, panel 3).

A native, folded conformation of the purified IA molecules was then confirmed by antibody reactivity and functional studies. The following antibodies, as shown by ELISA (data not shown), all reacted with the recombinant molecules: MKD6 (16), M5-114 (17), 40-B (19), and K24-199 (18).

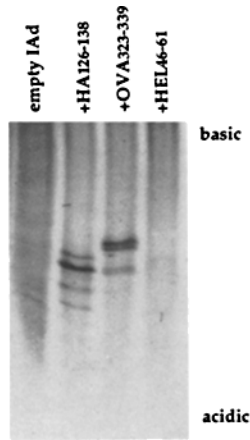
In addition, to confirm the correct folding of the IA dimers, we were able to demonstrate their ability to associate with the invariant chain (Ii) (Fig. 1 B, panels 4 and 5). A human soluble form of Iip31 (14), extended at its NH<sub>2</sub>-terminus by a stretch of six histidines, was cotransfected into SC2 cells together with IA α and β leucine zipper cDNAs, and the association of their products was tested by immunoprecipitating complexes through the Ii histidines by use of nickel-agarose beads. As seen in Fig. 1 B, panel 4, IA α, β, and Ii did associate together, showing that the conformation of the IA dimer was comparable to that of normal IA molecules. Furthermore, the leucine zipper could be removed from these complexes by thrombin digestion without cleaving Ii (Fig. 1 B, panel 5). The equimolar ratio

among α, β, and Ii chains retained in these complexes was confirmed by protein sequencing of size-purified material.

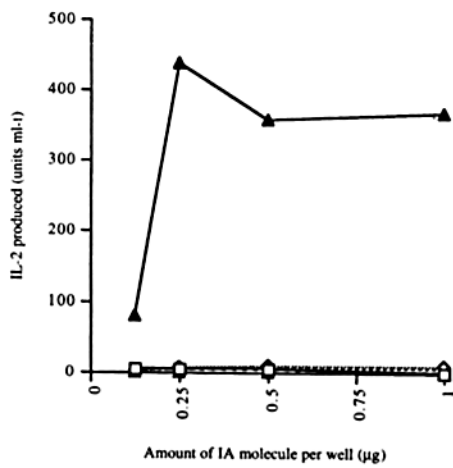
The next step was to show that the recombinant molecules were empty when secreted and were able to bind peptides. The absence of peptide in the purified material was confirmed by protein sequencing. The first 12 cycles could only detect the sequences of IA α and β chains without the presence of other sequences (Table 1). In addition, no SDS stable dimers (23–25) could be detected by SDS-PAGE of the purified material. However, we cannot exclude definitely that some low abundance, non-SDS-stabilizing peptides were present in these preparations of purified IA<sup>d</sup> molecules and fell below the level of detection of the techniques that we used to determine the emptiness. These molecules could be loaded efficiently with single peptides immediately after the affinity purification step to avoid the possibility of denaturation and aggregation (26). Since the two peptides we used, OVA<sub>323–339</sub> and HA<sub>126–138</sub>, do not confer SDS stability to the dimers (L. Teyton, unpublished observation), we examined peptide loading by native IEF. Fig. 2 A shows the shift in pI of the IA molecule induced by the binding of the two peptides (pIs of 5.2 and 5.4 for HA and OVA peptides, respectively). The appearance of a limited number of sharp bands in these samples corresponds to the peptide-loaded IA<sup>d</sup> molecules, whereas empty IA molecules and IA molecules plus a non-IA<sup>d</sup>-binding peptide (HEL<sub>46–61</sub> peptide) do not focus within a narrow range. The broad focusing range is explained by the partial denaturation of the empty dimers and/or by the loading of a

**Figure 2.** Peptide loading of empty IA<sup>d</sup> molecules and stimulation of T cell hybridomas by purified, immobilized IA-peptide complexes. (A) Empty IA<sup>d</sup> molecules were loaded with single peptides after elution from the affinity column and analyzed by native IEF electrophoresis (top). Sharp bands corresponding to peptide-loaded IA<sup>d</sup> molecules appeared only with HA<sub>126–138</sub> and OVA<sub>323–339</sub> peptides but not with non-IA<sup>d</sup>-binding peptides such as HEL<sub>46–61</sub>. These complexes were used to stimulate DO11.1.10 T cells and HA.126 T cells. Purified IA<sup>d</sup>-OVA complexes (solid squares) and purified IA<sup>d</sup>-HA complexes (solid triangles) were used in each experiment. Negative controls included empty IA<sup>d</sup> and empty IA<sup>d</sup> plus 10 μg of free peptide. They all gave background levels of stimulation, as the nonspecific IA<sup>d</sup>-peptide complex did. (B) Formation of functional IA<sup>d</sup>-peptide complexes by acidic treatment of IA<sup>d</sup>-

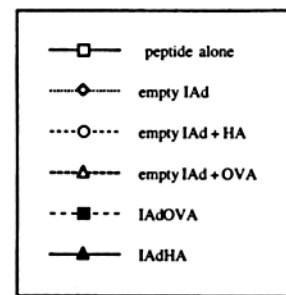
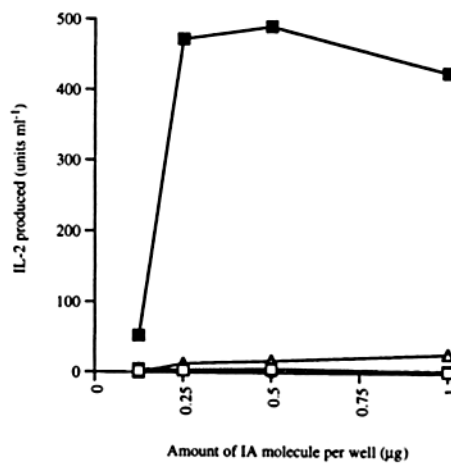
**A**



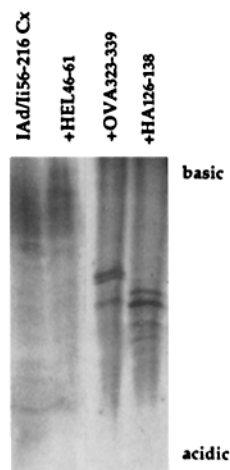
**HA.126 (IAd+HA)**



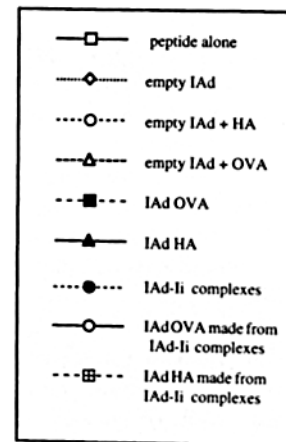
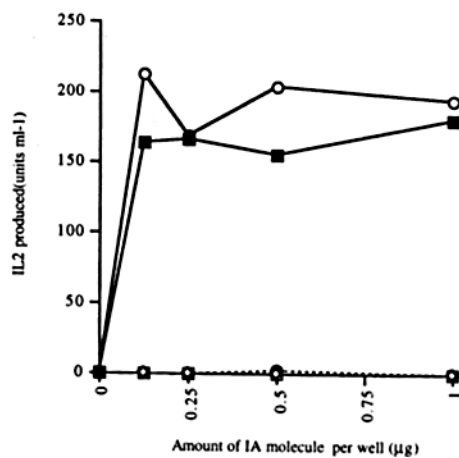
**DO11.1.10 (IAd+OVA)**



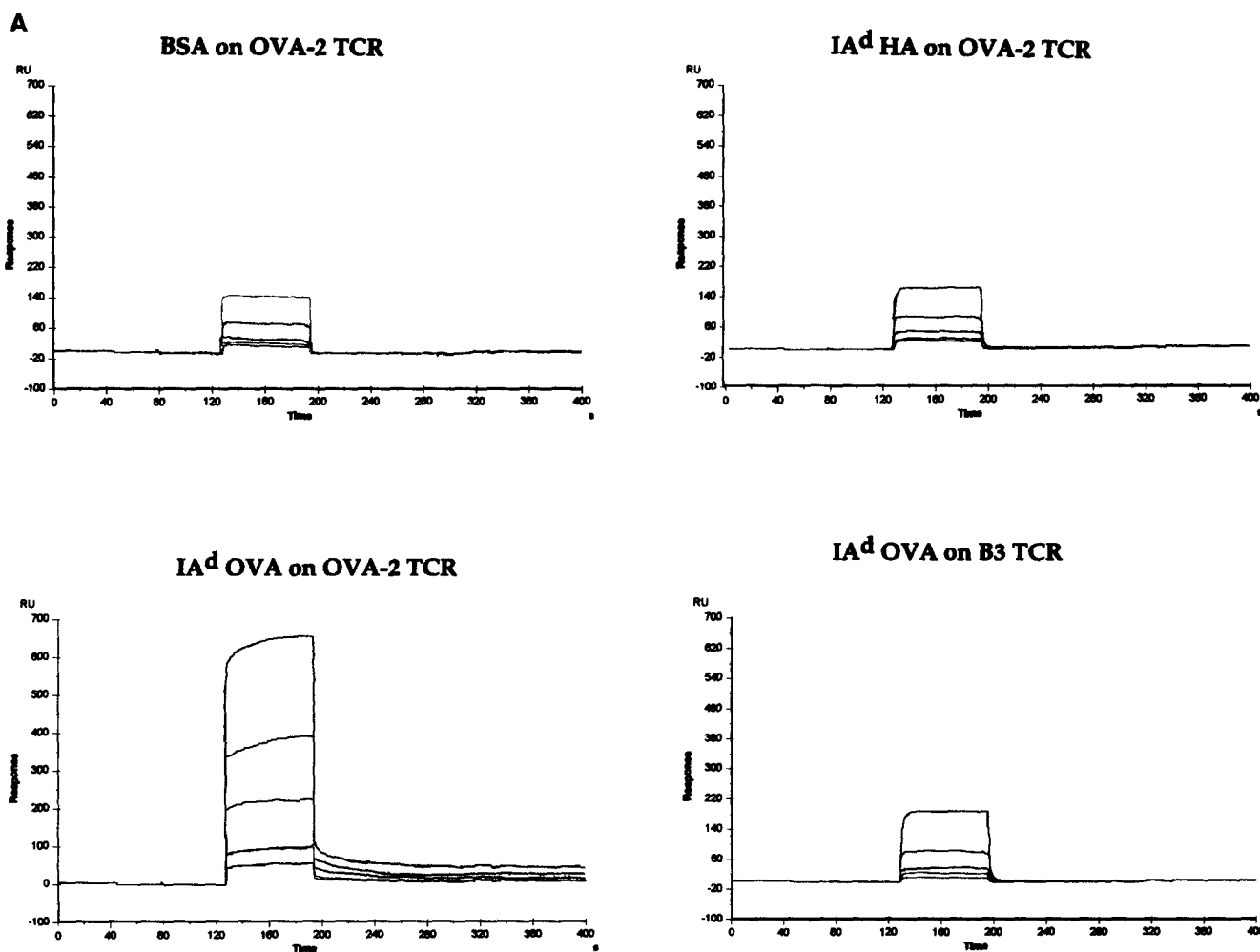
**B**



**DO11.1.10 (IAd+OVA)**



Ii complexes. IA<sup>d</sup>-Ii<sub>56-216</sub> complexes (Cx) were incubated with different peptides at pH 5.5 (see Materials and Methods for details) and analyzed by native IEF (left). These complexes were used to stimulate DO11.1.10 T cells (right). IA<sup>d</sup>-OVA complexes made from IA<sup>d</sup>-Ii complexes (open circles) and IA<sup>d</sup>-OVA complexes made from freshly eluted empty IA<sup>d</sup> molecules (solid squares) were equally efficient at stimulating DO11.1.10 T cells. Non-stimulatory ligands included empty IA<sup>d</sup> molecules, IA<sup>d</sup>-Ii complexes, IA<sup>d</sup>-HA complexes made from either empty IA<sup>d</sup> molecules or IA<sup>d</sup>-Ii complexes, and IA<sup>d</sup>-Ii complexes plus 10 µg of free peptide. They all gave background levels of stimulation.



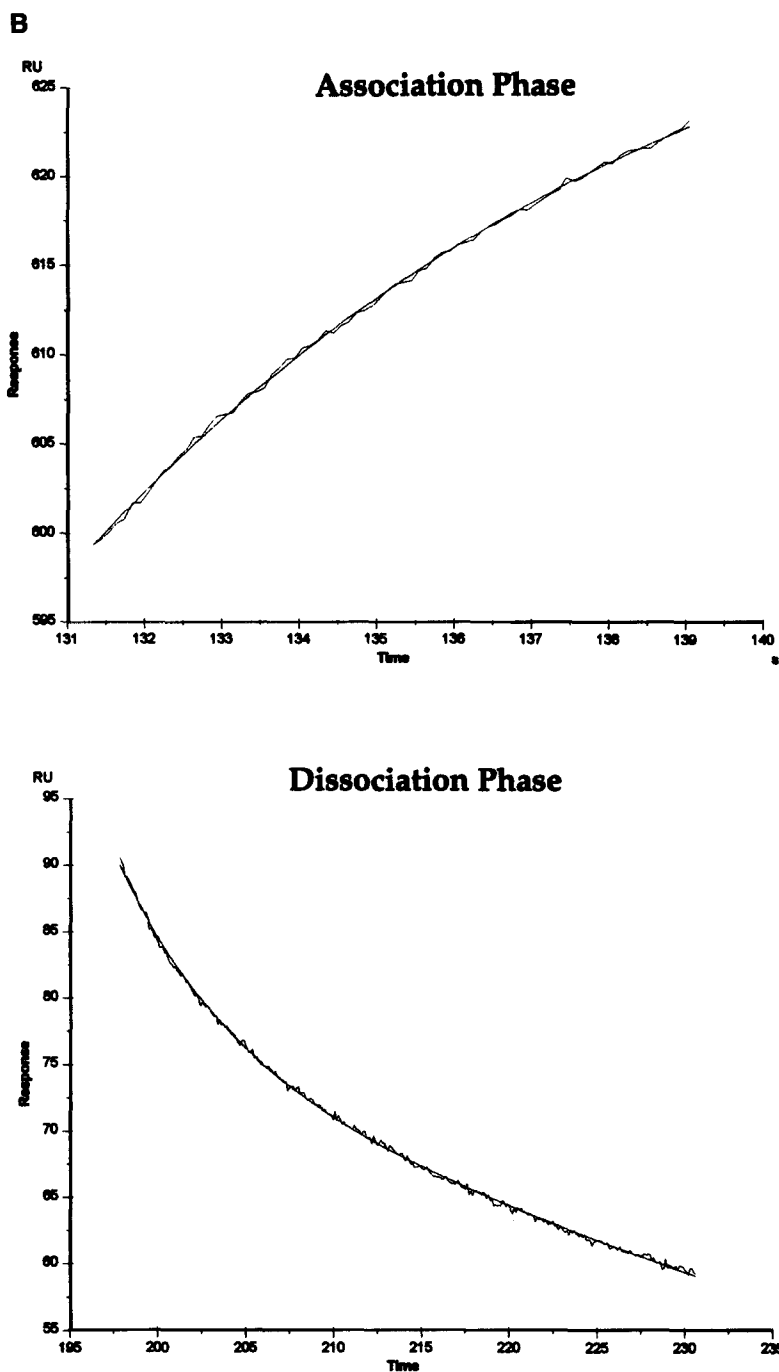
spectrum of low affinity peptides that are undetectable by protein sequencing, as mentioned above. The loading with single peptide was confirmed by  $\text{NH}_2$ -terminal protein sequencing and appeared to be complete and stoichiometric (equimolar ratios of  $\alpha$  and  $\beta$  chains and peptide; see Table 1). Peptide-loaded  $\text{IA}^d$  molecules were easily purified by ion exchange chromatography and eluted as a single peak or a close doublet of sharp peaks (data not shown).

The native structure and functionality of these  $\text{IA}$ -peptide complexes were confirmed by examining their interaction with the TCR. First, we examined this interaction *in vivo* by T cell assay; then, we looked at its binding characteristics *in vitro* by using surface plasmon resonance. The pre-formed  $\text{IA}^d$ -peptide complexes were adsorbed onto the plastic wells of 96-well microtiter plates at increasing concentrations ranging from 0.125  $\mu\text{g}$  to 1  $\mu\text{g}$  per well and used to stimulate T cell hybridomas. For both the  $\text{IA}^d$ -OVA-restricted hybridoma (DO.11.1.10 [22]) and the  $\text{IA}^d$ -HA-restricted hybridoma (HA-126 [21]), maximal stimulation was reached for doses of  $\sim 0.25$   $\mu\text{g}$  per well. This is considerably lower than the dose used for half-maximal stimulation for the published  $\text{IA}^d$   $\beta$  chain peptide hybrid molecules, which could not reach a plateau of stimula-

tion at 0.5  $\mu\text{g}$  per well (5). All of the following other combinations failed to induce T cell activation: empty  $\text{IA}^d$ , empty  $\text{IA}^d$  plus free peptide,  $\text{IA}^d$  plus non-specific peptide complex, and peptide alone. The removal of the leucine zipper had no effect on these T cell assays (data not shown). Empty  $\text{IA}^d$  molecules failed to bind free peptide when the binding was done more than 24 h after the purification of the MHC molecule (L. Teyton, manuscript in preparation).  $\text{IA}^d$  molecules complexed with  $\text{Ii}_{56-216}$  were loaded with single peptides by acidic treatment and used in an identical T cell assay. The stimulatory capacity of the  $\text{IA}^d$  molecules from either source was comparable (Fig. 3 B).

We directly measured the affinity of  $\text{IA}^d$ -OVA complexes for a soluble purified  $\text{IA}^d$ -OVA-restricted TCR (OVA-2,  $\text{V}\alpha 2$   $\text{V}\beta 5$ ; F. Carbone, C. Scott, and L. Teyton, unpublished sequences).

1,000 RUs of TCR were immobilized on the dextran layer of the CM5 chip, and concentrations of  $\text{IA}^d$  complexes ranging from 1.875 to 30  $\mu\text{M}$  were flowed over it. As seen in Fig. 3 A, only the  $\text{IA}^d$ -OVA complexes gave a measurable signal, whereas the  $\text{IA}^d$ -HA complexes gave background signal on this surface. On a control surface, coated with B3, an anti-Kb-OVA TCR (27), the  $\text{IA}^d$ -OVA com-



**Figure 3.** Binding of IA<sup>d</sup>-OVA complexes to immobilized OVA-2 soluble TCR analyzed by surface plasmon resonance. Purified soluble OVA-2 and B3 TCRs were coupled to a CM5 chip by amine chemistry. BSA, IA<sup>d</sup>-OVA complexes, and IA<sup>d</sup>-HA complexes, at successive twofold dilutions, ranging from 30 to 1.875 μM, were caused to flow through the flow cell at 20 μl/min. (A) The overlay of the different sensograms is shown for each analyte on the OVA-2 surface and for IA<sup>d</sup>-OVA complexes on the B3 surface. Only IA<sup>d</sup>-OVA complexes on the OVA-2 surface exhibited stigmates of binding. (B) Fit of the association and dissociation phases of IA<sup>d</sup>-OVA complexes (30 μM) binding to OVA-2 TCRs to theoretical models (*solid interpolated curve*). The association was fitted on a single association site model (A + B = AB), whereas the dissociation was fitted on a two parallel dissociations model (AiBj = Ai + Bj).

plexes exhibited no sign of binding. The association rate was derived by fitting the experimental curve on a single association site model (Fig. 3 B) and was relatively fast for a TCR-MHC interaction with a  $K_{on}$  of  $2.96 \times 10^4 \text{ M}^{-1}/\text{s}$  (28–30). The dissociation phase could be fitted satisfactorily on a single-site dissociation model, but the best fit was obtained on a model accounting for the parallel dissociation of two sites (Fig. 3 B) with a very slow  $K_d$  of  $6.19 \times 10^{-3} \text{ s}^{-1}$  and a very fast one at  $0.164 \text{ s}^{-1}$ . The slow component of this dissociation could be accounted for by some nonspe-

cific binding of the IA molecules to the dextran surface, whereas the fast one likely represents the dissociation of the specific TCR-MHC interaction.

In conclusion, we are able to produce empty and functional IA<sup>d</sup> molecules by improving the efficiency of correct pairing between the  $\alpha$  and  $\beta$  chains of the IA dimer, owing to the addition of a leucine zipper. The opportunity to load them efficiently with single peptides now allows us to study individual IA-peptide interactions as we have been able to do for MHC class I peptide complexes (31). In our experi-

ments, the addition of an antigenic peptide sequence to the NH<sub>2</sub>-terminus of the  $\beta$  chain did not increase the levels of production, suggesting that the presence of the peptide did not lead to an increase in correct folding and intracellular transport of IA<sup>d</sup> molecules. The limited efficiency of pair-

ing of IA chains appears to be the main limiting factor for their expression and transport. The availability of these larger quantities of purified MHC and TCR molecules should enable us to embark on X-ray crystallographic studies.

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