A diverse set of oligomeric class II MHC-peptide complexes for probing T-cell receptor interactions

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Background: T-cells are activated by engagement of their clonotypic cell surface receptors with peptide complexes of major histocompatibility complex (MHC) proteins, in a poorly understood process that involves receptor clustering on the membrane surface. Few tools are available to study the molecular mechanisms responsible for initiation of activation processes in T-cells.

Results: A topologically diverse set of oligomers of the human MHC protein HLA-DR1, varying in size from dimers to tetramers, was produced by varying the location of an introduced cysteine residue and the number and spacing of sulfhydryl-reactive groups carried on novel and commercially available cross-linking reagents. Fluorescent probes incorporated into the cross-linking reagents facilitated measurement of oligomer binding to the T-cell surface. Oligomeric MHC-peptide complexes, including a variety of MHC dimers, trimers and tetramers, bound to T-cells and initiated T-cell activation processes in an antigen-specific manner.

Conclusion: T-cell receptor dimerization on the cell surface is sufficient to initiate intracellular signaling processes, as a variety of MHC-peptide dimers differing in intramolecular spacing and orientation were each able to trigger early T-cell activation events. The relative binding affinities within a homologous series of MHC-peptide oligomers suggest that T-cell receptors may rearrange in the plane of the membrane concurrent with oligomer binding.

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Introduction

Major histocompatibility complex (MHC) proteins are cell surface αβ heterodimeric glycoproteins (molecular weight (MW) ~60 kDa) that bind peptides within the cell and present them at the cell surface for recognition by antigen-specific T-cells [1,2]. MHC-peptide complexes on the surface of antigen-presenting cells are bound by receptors on the surface of T-cells, in a process that leads to activation of T-cell effector functions and initiation of immune responses [3]. T-cell receptors (TCRs) are multi-subunit membrane glycoproteins that include clonally expressed antigen binding αβ subunits (MW ~60 kDa) [4]. Three-dimensional structures have been determined by X-ray crystallography for the extracellular domains of many class I and class II MHC proteins in complex with defined peptides [5], and for the antigen binding domains of several TCRs and TCR/MHC co-complexes [6].

The molecular events occurring at the interface between a T-cell and an antigen-presenting cell that lead to MHC-stimulated T-cell activation are unknown and of considerable interest. Oligomerization of TCR molecules at the T-cell surface is believed to be the proximal stimulus for triggering T-cell cytoplasmic signaling cascades [7], but molecular characteristics of the putative TCR activating oligomer are not known. Alternative mechanisms involving MHC-induced TCR conformational or allosteric changes also have been proposed [8–11]. There are many factors complicating investigation of MHC/TCR interactions. The association of MHC-peptide complexes with TCR is a weak interaction, with affinities (K_d) for soluble extracellular domains typically ranging from 10^{-6} to 10^{-4} M [12,13]. MHC and TCR proteins are anchored in different cell membranes that must be juxtaposed during activation, in a process that can include other components from both cells [14]. MHC proteins on the surface of antigen-presenting cells typically will be occupied with a large number of different peptides, although recombinant sources of soluble MHC complexes with defined peptides are available [15–17]. Finally, the antigen binding TCR αβ subunits contain very short cytoplasmic domains not believed to participate in T-cell signaling, and activation signals are transmitted into the T-cell through association with TCR CD3γδε and ζ_2 subunits, which possess multiple signaling motifs [18].

Soluble MHC-peptide complexes have been used to investigate the molecular requirements for activation of T-cells. In this approach, arrays of purified MHC-peptide complexes are used to mimic the physiological effect of an antigen-presenting cell (Figure 1). Experiments with dimeric chimeras of MHC-peptide complexes fused to

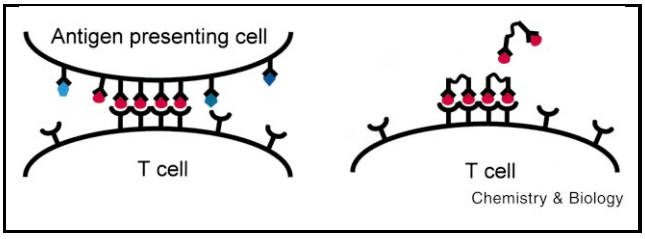

Figure 1. Arrays of MHC-peptide complexes can act as mimics of an antigen-presenting cell. T-cells are activated by multivalent engagement of their receptors [7].

antibody Fc portions [19–21], antibody-linked MHC dimers [22], and a series of streptavidin-based oligomers of biotinylated MHC-peptide complexes [23], have yielded conflicting measures of the minimum MHC oligomer size required for activation of T-cells. One constraint of these approaches is that the intramolecular valency, orientation and spacing are controlled by the antibody or streptavidin moieties used to form the oligomers. Difficulties in preparing homogenous oligomer species further limit the applications of these reagents in probing MHC/TCR interactions.

We have developed a series of novel, chemically defined, oligomeric complexes of the human MHC class II protein HLA-DR1 for investigation of the molecular mechanism of T-cell activation. MHC-peptide complexes were modified to carry an introduced cysteine residue at the C-terminus of the α subunit or the β subunit of the MHC molecule and were oligomerized with cross-linking reagents carrying two, three or four thiol-specific reactive sites with varying inter-site spacing and flexibility. Fluorescent labels incorporated into the cross-linking reagents allowed detection of MHC-peptide oligomer binding to T-cells. In contrast to previous approaches, our strategy permits construction of a range of novel MHC-peptide oligomers with varying valency, orientation and topological constraints. These oligomers offer a unique set of topologies for the presentation of peptide to T-cells and for investigation of the effect of MHC geometry on T-cell signaling.

Results

Cysteine modifications of HLA-DR1

Our oligomerization strategy was based on introduction of specific cross-links through reactive sulfhydryl groups introduced separately at the C-terminus of the α or β subunit of HLA-DR1. To generate the shorter Cys-modified subunits α_{cys} and β_{cys}, Cys residues were introduced after residues α182 or β190, at the end of the lower immunoglobulin domains that support the peptide binding site and immediately after the last well-ordered residues visible in the crystal structures [24,25] (Figure 2A,B). To generate the longer Cys-modified subunits α_{Lcys} and β_{Lcys}, Ala-Cys sequences were introduced individually after the 'connecting peptide' regions α183–192 or β191–198, just before the predicted start of the transmembrane portions (Figure 2A,B). HLA-DR1 has seven other cysteine residues, forming three disulfide bonds and one free cysteine. The HLA-DR1 intramolecular disulfide bonds (α108–164, β16–79, β118–174) are buried within the folded protein and are stable to at least 100 mM dithiothreitol [16]. Control experiments established that the free cysteine (β30) is buried underneath the bound peptide and unreactive to soluble thiol-specific maleimide or pyridyl disulfide probes in the folded MHC-peptide complex (JRC, not shown). The introduced sulfhydryl residues undergo facile reaction with thiol-specific reagents, allowing specific cross-linking at the α or β subunit termini.

Folded HLA-DR1 αβ complexes carrying the introduced cysteine residues were produced by dilution of urea-solubilized α and β subunits into denaturant-free buffer in the presence of antigenic viral peptide (Ha) or control endogenous peptide (A2), using a previously described protocol [16]. Both peptides bind tightly to HLA-DR1 with $K_d \sim 10$–50 nM [26] and dissociation half-life ~100–300 h [27]. Introduction of the cysteine residues did not affect protein folding, as assayed by reactivity with antibodies specific for the folded DR1 αβ heterodimer (not shown), or with the ability of the folded complexes to bind peptide, as determined by resistance to sodium dodecyl sulfate (SDS)-induced subunit dissociation (Figure 2C). Cysteine-modified MHC-peptide complexes were purified in the presence of 5 mM dithiothreitol, which was removed immediately prior to cross-linking reactions. Using this procedure, greater than 90% of the purified folded peptide complexes carried an unmodified, reactive thiol, as determined using a fluorescent sulfhydryl-reactive probe (see Materials and methods).

MHC oligomers prepared using commercially available cross-linking reagents

Homobifunctional cross-linking reagents are readily available carrying a variety of sulfhydryl-reactive groups such as pyridyl disulfides, maleimides and vinyl sulfones. A sampling of commercially available homobifunctional thiol-specific cross-linking reagents was chosen to test the cross-linking efficiencies of the cysteine-modified MHC-peptide complexes (Figure 3A). Pyridyl disulfides are very reactive towards thiols, and unlike the other sulfhydryl-reactive species the S–S bond that is formed can be reversibly cleaved with a reducing agent for ease in characterization or recovery of cross-linked molecules [28]. Maleimides also are highly reactive to thiols, but hydrolysis of the cross-linking reagent competes with thiol reaction [29], and the reaction can be (slowly) reversible. Vinyl sulfones suffer much less from competing hydrolysis and form stable link-

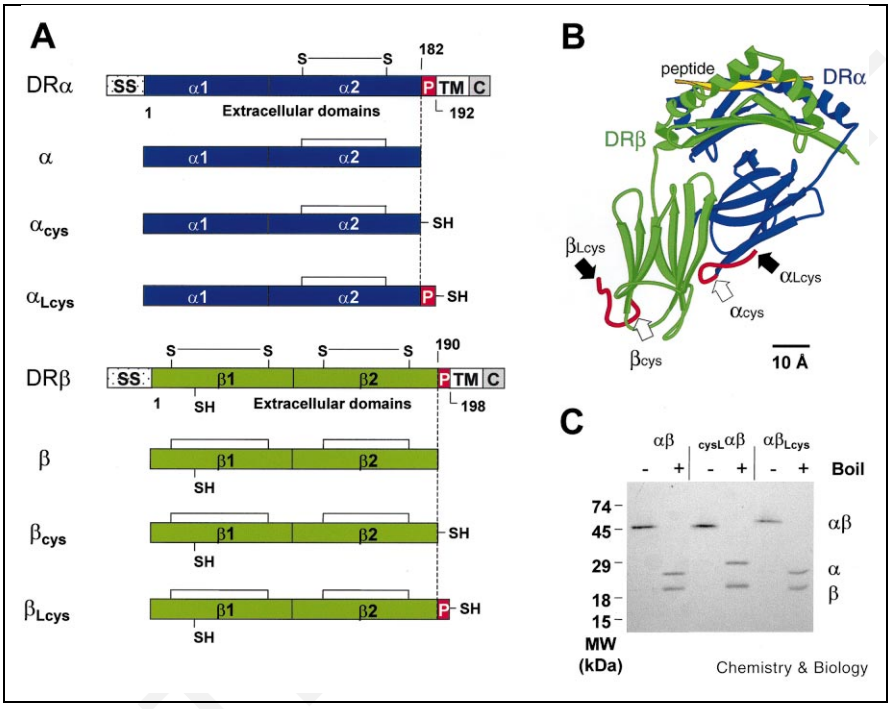

Figure 2. Design and construction of cysteine-modified class II MHC proteins. (**A**) Diagram of wild type and cysteine-modified α and β polypeptide chains of HLA-DR1. The positions of the signal sequences (SS), flexible 'connecting peptide' regions (P), transmembrane domains (TM) and cytoplasmic domains (C) are indicated on the full length DRα and DRβ genes. Cysteine residues were introduced individually at the C-terminus of the extracellular portions of the polypeptide chains after α182 (α_{cys}) or β190 (β_{cys}), or Ala-Cys sequences were added individually after the 'connecting peptide' regions at α192 (α_{Lcys}) or β198 (β_{Lcys}). Native disulfide bonds produced upon folding are indicated by S–S. DRβ carries a free cysteine residue (β30) that is sequestered in the peptide binding site in a folded MHC-peptide complex. (**B**) Ribbon diagram of HLA-DR1 with α subunit (DRα, blue), β subunit (DRβ, green) and bound peptide (yellow) from the crystal structure [25]. Open arrows indicate the termini of the short constructions α_{cys} and β_{cys}. Filled arrows indicate the termini of the long constructions α_{Lcys} and β_{Lcys}. The connecting peptide regions (red) are poorly defined by the crystal structure and can adopt different conformations [25]. (**C**) Non-reducing SDS–PAGE (12.5%) of purified MHC-peptide complexes of wild type DR1 (αβ), and DR1 with the introduced cysteine residue on the C-terminus of the connecting peptide region of the α chain ($_{cysL}\alpha\beta$) or of the β chain ($\alpha\beta_{Lcys}$). The additional cysteine residues do not affect protein folding or the ability of the MHC to bind the Ha peptide antigen, as indicated by the presence of SDS-resistant αβ heterodimer bands (−boil), that dissociate into α and β chains upon

ages [30], but do not appear to be as reactive towards thiols as maleimides or pyridyl disulfides (JRC, not shown).

Dimeric MHC-peptide complexes were prepared with the free thiol on the long version of the α subunit of HLA-DR1-Ha ($_{cysL}\alpha\beta$) using 1,4-di-[3′-(2′-pyridyldithio)-propionamido]butane (DPDPB) or 1,6-hexane-bis-vinyl sulfone (HBVS) (Figure 3A,B). Cross-linker and protein concentrations in the reaction mixture were varied to obtain optimum cross-linking efficiency. DPDPB cross-linking could be used to produce dimers with an efficiency of approximately 60%, while HBVS was less effective, producing dimers with ~15% efficiency, as estimated by SDS–PAGE and gel filtration chromatography (Figure 3B,C). Much higher efficiencies of cross-linking (~90%) were obtained through formation of direct disulfide bonds between the introduced thiols, using copper 1,10-phenanthroline to catalyze the oxidation (Figure 3B,C) [31]. Trimeric MHC-peptide complexes were formed with tris-2[-maleimidoethyl]amine (TMEA), a commercially available sulfhydryl-reactive trimeric cross-linking reagent (Figure 2A,B). However, the cross-linking yield with this reagent was extremely low (<10%), perhaps due to steric hindrance between the three cross-linked MHC molecules (Figure 3B,C).

Oligomerized $_{cysL}\alpha\beta$ MHC-peptide complexes were purified by gel filtration chromatography (Figure 3C). SDS–PAGE analysis showed that the purified cross-linked MHC-peptide complexes had formed the correct α subunit ($_{Lcys}\alpha$-$_{Lcys}\alpha$) covalent cross-links and retained their bound peptide (Figure 3B). The purified complexes exhibited the sizes expected for soluble dimeric and trimeric MHC proteins with no tendency to aggregate, as judged by gel filtration chromatography (Figure 3D).

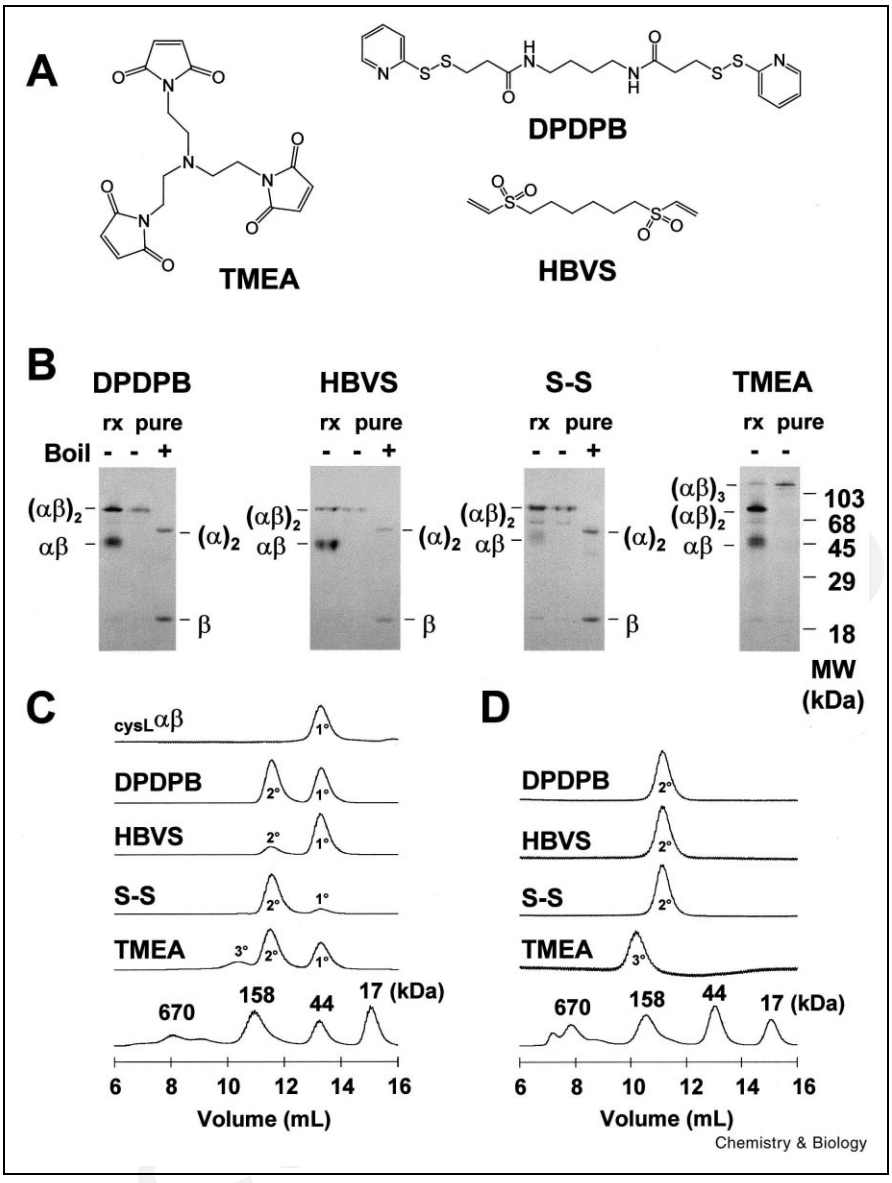

Figure 3. Oligomerization of MHC-peptide complexes with commercially available cross-linking reagents. (**A**) Chemical structures of the dimeric cross-linking reagents DPDPB and HBVS, and the trimeric cross-linker TMEA, carrying a variety of sulfhydryl-reactive functional groups. (**B**) Non-reducing 12.5% SDS–PAGE of cross-linking reaction mixtures (rx) and purified oligomers (pure) of Cys-modified HLA-DR1 ($_{cysL}\alpha\beta$) coupled with DPDPB, HBVS, S–S and TMEA. The position of unreacted monomer ($\alpha\beta$), and cross-linked dimers ($(\alpha\beta)_2$) and trimer ($(\alpha\beta)_3$) are indicated. cross-linked MHC-peptide dimers exhibit the correct covalent bond through the α subunits ($(\alpha)_2$), as shown by stability to boiling in SDS (+boil). (**C**) Gel filtration of reaction mixtures of MHC-peptide oligomers coupled through the α subunit ($_{cysL}\alpha\beta$) demonstrates the relative coupling efficiencies of the various cross-linking reagents. Data are representative of several experiments. (**D**) Gel filtration traces of purified MHC-peptide complexes exhibit the sizes expected for monomer (1°), dimers (2°) and TMEA trimer (3°). The profile for MW standards is indicated below the oligomer traces in **C** and **D**.

Commercially available cross-linking reagents can be used to prepare MHC dimers and trimers, but they have several limitations. These reagents are not fluorescent or otherwise labeled, and therefore cannot be used in direct binding or T-cell detection assays. Furthermore, the thiol-reactive homotrimeric cross-linking reagent TMEA exhibits a low cross-linking efficiency, and a homotetrameric reagent is not available. Cross-linking reagents that incorporate detection labels, and are available in a wide range of oligomeric sizes, would be preferred for high-affinity T-cell binding and to probe the effects of oligomer size on MHC/TCR interactions. Thus we developed an alternate cross-linking strategy, based on polypeptides carrying pendant thiol-reactive groups.

MHC oligomers prepared using novel peptide-based synthetic cross-linking reagents

For production of a set of fluorescently labeled oligomers with variable valency, we designed and chemically synthesized peptide-based cross-linking reagents containing a fluorescent label (fluorescein) and two or more thiol-reactive maleimide groups introduced at lysine residues spaced along a flexible backbone composed of glycine, serine and glutamic acid (Figure 4A). The non-repeating backbone amino acid sequences were designed to be water-soluble with little propensity to form an ordered structure, and to provide sufficient length and flexibility to allow pendant MHC molecules to bind simultaneously to a cell surface. Maleimide to maleimide distances for the cross-linkers, in extended conformations that would allow pendant MHC

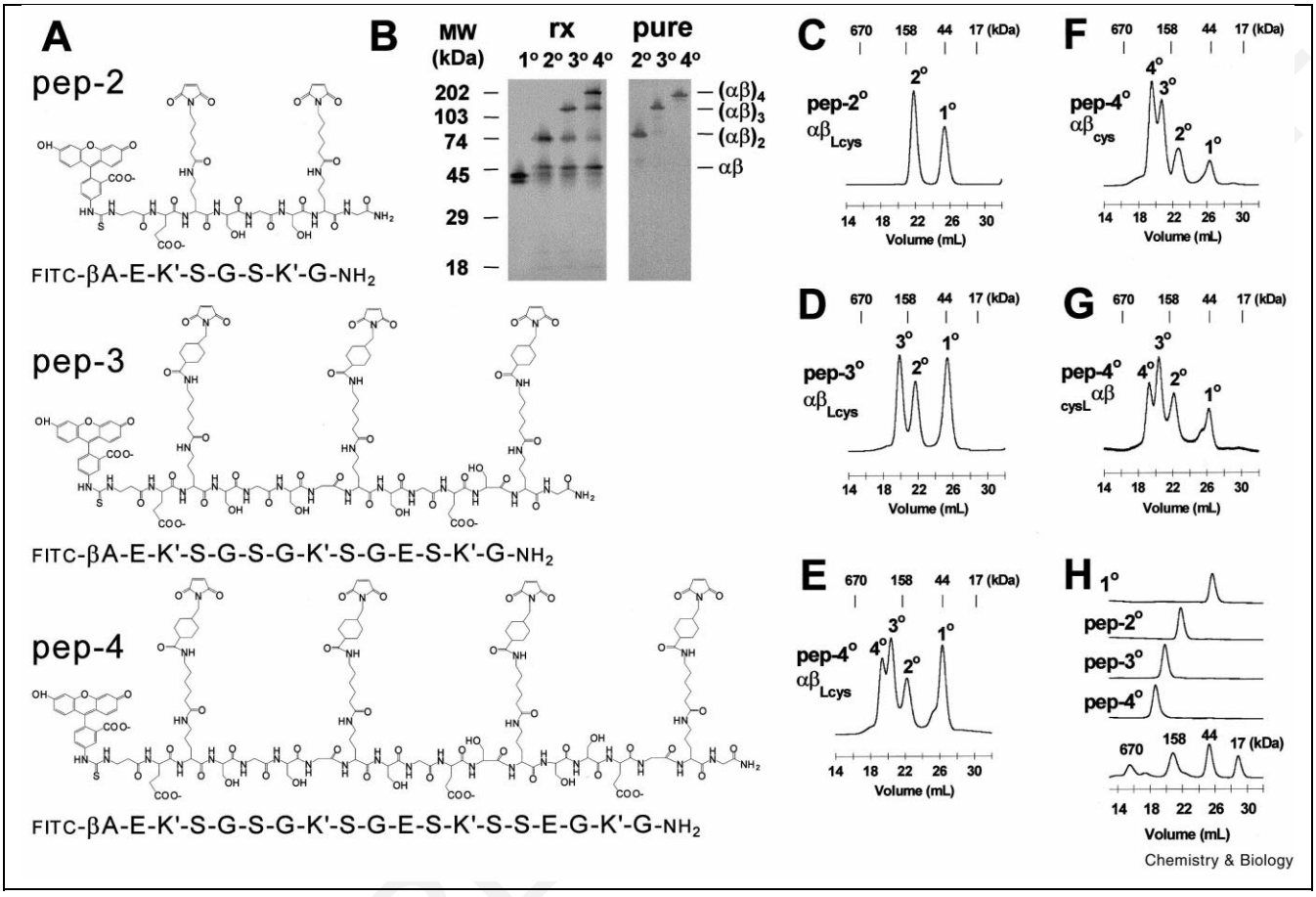

Figure 4. Oligomerization of MHC-peptide complexes using synthetic fluorescent cross-linking reagents. (**A**) Chemical structures of fluorescein-labeled, peptide-based cross-linkers for production of MHC dimers (pep-2), trimers (pep-3) and tetramers (pep-4). All cross-linkers carry maleimide groups for coupling to cysteine residues introduced into HLA-DR1. (**B**) 10% SDS–PAGE of cross-linking reactions (rx) and purified oligomeric complexes (pure) of Cys-modified HLA-DR1-Ha coupled through the β subunit ($\alpha\beta_{Lcys}$). (**C–G**) Gel filtration traces of reaction mixtures of (**C**) $\alpha\beta_{Lcys}$ pep-2°, (**D**) $\alpha\beta_{Lcys}$ pep-3°, (**E**) $\alpha\beta_{Lcys}$ pep-4°, (**F**) $\alpha\beta_{cys}$ pep-4° and (**G**) $_{cysL}\alpha\beta$ pep-4°. The position of MW standards is indicated by lines above the traces. (**H**) Gel filtration traces of purified MHC oligomers exhibit the sizes expected for monomer (1°), dimer (pep-2°), trimer (pep-3°) and tetramer (pep-4°). The profile of MW standards is indicated below the oligomer traces.

molecules to present peptides in the same plane, are approximately 45 Å for the dimeric cross-linkers (pep-2), and 50 Å for the trimeric (pep-3) and tetrameric (pep-4) cross-linkers, as estimated from molecular models.

Dimeric, trimeric and tetrameric complexes of HLA-DR1 were formed through reaction of the synthetic cross-linkers with MHC proteins carrying the introduced cysteine residue on the long version of the β subunit ($\alpha\beta_{Lcys}$) (Figure 4B). The cross-linking efficiency for the dimeric cross-linker pep-2 was comparable to that observed for DPDPB (~60%) (Figure 4B,C), while the cross-linking efficiency for the trimeric pep-3 was approximately 35% (Figure 4B,D), significantly greater than that observed for TMEA. The tetrameric cross-linker pep-4 was able to couple $\alpha\beta_{Lcys}$ with an efficiency of approximately 25% (Figure 4B,E). The cross-linked MHC-peptide complexes were purified by gel filtration chromatography (Figure 4C–G). Purified oligomeric MHC-peptide complexes exhibited the expected β subunit covalent cross-links as analyzed by SDS–PAGE (not shown) and had no tendency to aggregate as judged by gel filtration chromatography (Figure 4H). Tetrameric oligomers were also prepared with the MHC-peptide complexes carrying cysteine residues on the shorter version of the β subunit ($\alpha\beta_{cys}$) (Figure 4F), and with MHC-peptide complexes carrying an unmodified β subunit and the cysteine introduced on the longer α subunit ($_{cysL}\alpha\beta$) (Figure 4G). Tetramer cross-linking efficiencies for these constructions were approximately 40% and 45%, respectively, somewhat higher than obtained with $\alpha\beta_{Lcys}$.

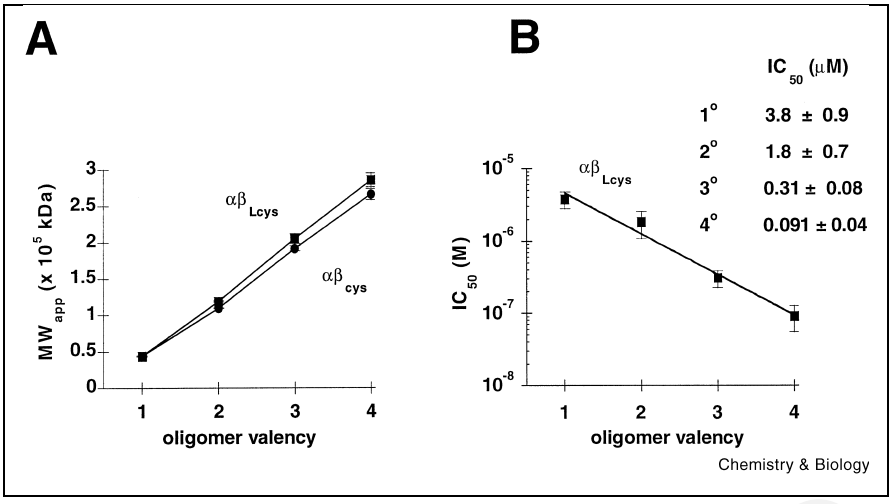

Figure 5. Characterization of MHC-peptide oligomers. (**A**) Oligomer valency versus apparent MW (MW_{app}) determined from data in Figure 4. (**B**) Oligomer valency versus relative T-cell binding affinity, determined by a competitive binding assay. IC_{50} values for the competition binding are plotted against the oligomer valency, revealing the approximate relationship log $IC_{50} = -5.6n - 4.8$, $R = 0.97$. Inset, IC_{50} values and standard deviations. Error bars represent the standard deviation of IC_{50} values and MW_{app} calculated from three separate experiments.

Purified oligomeric MHC-peptide complexes exhibited increasing apparent MW with increasing oligomer size, in a roughly linear relationship, as expected for a series of concatenated particles (Figure 5A). Oligomers prepared from MHC-peptide complexes containing the connecting peptide region ($\alpha\beta_{Lcys}$) exhibited apparent MW larger than the corresponding oligomers lacking the connecting peptide ($\alpha\beta_{cys}$) (Table 1). The difference was greater than that predicted from the actual MW increase of the connecting peptide region (Table 1), suggesting that the connecting peptide regions of the oligomer are in an extended rather than compact conformation (the apparent MW is calculated using a compact, spherical particle model [26]). Hydrodynamic Stokes radii (R_s), calculated from the apparent MW (Table 1), indicate that inclusion of the β subunit connecting peptide region causes a small increase in the particle radius (0.9–1.2 Å), consistent with the addition of eight amino acid residues.

Relative binding affinities were determined using a competitive binding assay, in which purified oligomeric MHC-peptide complexes were used to compete for binding to receptors on the surface of T-cells with tetramers formed by oligomerization of biotinylated MHC monomers with phycoerythrin-labeled streptavidin [32]. Half-maximal inhibitory concentrations (IC_{50}) varied systematically with

Table 1
Hydrodynamic behavior of MHC-peptide oligomers.

MHC-peptide	Cross-link	MW[a]	MW^b_{app} (kDa) gel filtration	MW_{app}/MW	R^c_s (Å)
αβ	monomer	44 820	41 ± 0.7	0.91	27.2 ± 0.15
αβ	crystal (calc)[d]				27.1
$_{cys}$αβ	S–S	89 843	94 ± 1	1.05	34.3 ± 0.14
$_{cys}$αβ	pep-2°	91 380	99 ± 1	1.08	34.9 ± 0.07
αβ$_{cys}$	S–S	89 843	105 ± 1	1.17	35.6 ± 0.12
αβ$_{cys}$	pep-2°	91 380	110 ± 0.1	1.20	36.1 ± 0.03
$_{cysL}$αβ	S–S	92 118	112 ± 0.9	1.22	36.4 ± 0.14
$_{cysL}$αβ	pep-2°	93 654	119 ± 0.5	1.27	37.1 ± 0.07
αβ$_{Lcys}$	S–S	91 733	116 ± 1	1.26	36.8 ± 0.06
αβ$_{Lcys}$	pep-2°	93 270	119 ± 0.8	1.28	37.1 ± 0.07
$(\alpha\beta)_2$	crystal (calc)[d]				34.2
αβ$_{cys}$	pep-3°	137 460	192 ± 4	1.40	43.5 ± 0.23
αβ$_{cys}$	pep-4°	183 204	266 ± 8	1.45	48.5 ± 0.49
αβ$_{Lcys}$	pep-3°	140 295	206 ± 9	1.47	44.5 ± 0.46
αβ$_{Lcys}$	pep-4°	186 984	286 ± 6	1.53	49.7 ± 0.55
$_{cysL}$αβ	HBVS	92 350	117 ± 2	1.27	36.9 ± 0.15
$_{cysL}$αβ	DPDPB	92 380	118 ± 2	1.28	37.0 ± 0.15

[a]MW calculated from the amino acid sequence of the protein.
[b]Apparent MW obtained from gel filtration chromatography.
[c]Stokes radius extracted from gel filtration data as described [26].
[d]Calculated values from the DR1-Ha crystallographic structure [24].

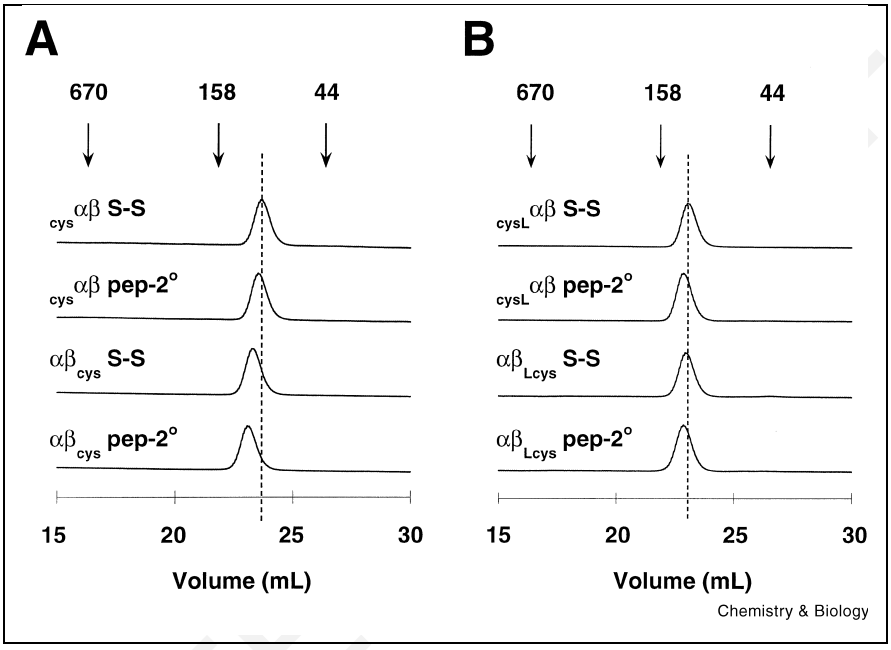

Figure 6. MHC-peptide oligomers in various orientations and topological constraints. Gel filtration chromatography of purified MHC-peptide dimers: (**A**) $_{cys}$αβ and αβ$_{cys}$, and (**B**) $_{cysL}$αβ and αβ$_{Lcys}$. The vertical reference line indicates the position of the midpoint of the dimer peak of $_{cys}$αβ S–S or $_{cysL}$αβ S–S. The α-coupled dimers of the short form $_{cys}$αβ S–S and $_{cys}$αβ pep-2° appear to be significantly more compact than the corresponding dimers coupled through the β subunit, αβ$_{cys}$ S–S and αβ$_{cys}$ pep-2°. For dimers of the long form $_{cysL}$αβ or αβ$_{Lcys}$, any differences in compactness are much less apparent. The elution positions of MW standards used to estimate apparent MW are indicated by arrows.

oligomer size, with larger oligomers binding more tightly. A plot of IC_{50} versus oligomer size (Figure 5B) revealed a log-linear dependence that does not cross the origin (log $IC_{50} = -5.6n - 4.8$, $R = 0.97$). This indicates that each additional concatenated MHC-peptide unit contributes a similar binding energy to the overall oligomer interaction, with the increment for each monomer significantly less than that contributed by binding a single MHC-peptide complex (see Discussion).

MHC-peptide dimers with varying intramolecular orientation and topological constraints

The availability of MHC proteins with cysteine residues at variable positions, along with cross-linkers of variable length and chemistry, provides a flexible system in which oligomers of varying orientation and topological constraints can be created. We exploited these characteristics to produce a series of dimeric MHC-peptide complexes that are connected through the α subunit or β subunit, by short or long cross-links. Eight topologically distinct MHC-peptide dimers were constructed, using direct disulfide bonds (S–S) or the peptide-based cross-linker (pep-2) to link MHC-peptide complexes carrying cysteine residues introduced before ($_{cys}$αβ, αβ$_{cys}$) or after ($_{cysL}$αβ, αβ$_{Lcys}$) the connecting peptide region, with cross-links between the α ($_{cys}$αβ, $_{cysL}$αβ) or β (αβ$_{Lcys}$, αβ$_{Lcys}$) subunits. The purified MHC-peptide dimers exhibited the correct covalent cross-links and the presence of tightly bound peptide antigen, as analyzed by SDS–PAGE (not shown), and gel filtration elution profiles consistent with those expected for MHC-peptide dimers (Figure 6).

Within this set of MHC-peptide dimers, apparent MW generally were larger for oligomers carrying the connecting peptide regions as compared to those without, and larger for oligomers linked by a peptide-based cross-linker as compared to those linked by a disulfide bond (Figure 6 and Table 1). Apparent MW for the dimeric complexes were approximately 20% greater than the actual MW (Table 1), consistent with an overall ellipsoidal rather than spherical shape as expected for concatenated particles. MHC-peptide monomers exhibited apparent MW similar to their actual MW, consistent with their roughly spherical shape, with calculated hydrodynamic radius (R_s) similar that observed in the crystals structure [26]. An exception to this general trend is the behavior exhibited by the MHC-peptide dimers linked through the shorter versions of the α subunit, $_{cys}$αβ S–S and $_{cys}$αβ pep-2°. These constructions are more compact than the corresponding oligomers linked through the β subunit (Figure 6A), with apparent MW similar to the actual MW, and hydrodynamic radii (R_s) similar to that of a $(\alpha\beta)_2$ dimer observed in several HLA-DR crystal structures (Table 1) (see Discussion).

MHC-peptide oligomers bind to and trigger activation in antigen-specific T-cells

We evaluated the utility of the MHC oligomers as immunological reagents by measuring their ability to induce antigen-specific upregulation of the low-affinity interleukin-2 (IL-2) receptor CD25, a well-characterized T-cell surface activation marker [33], using the HLA-DR1-restricted, Ha peptide-specific human $CD4^+$ T-cell clone HA1.7 [34] (Figure 7). MHC oligomers constructed using commercially available cross-linking reagents to link cysteine resi-

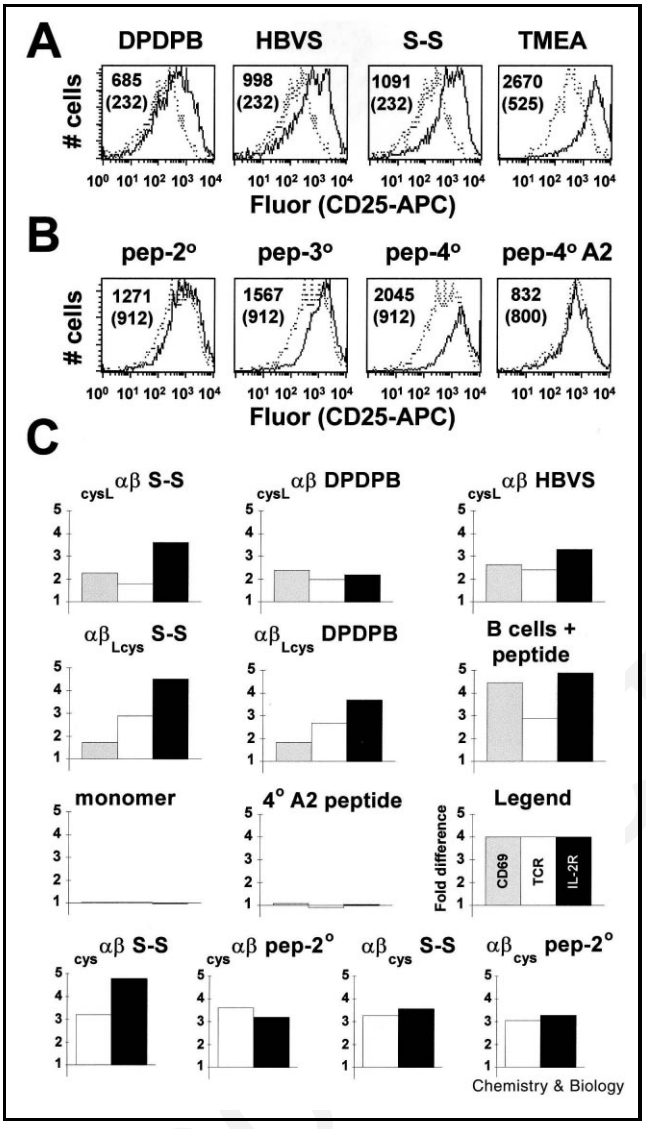

Figure 7. MHC-peptide oligomers induce activation of the antigen-specific T-cell clone HA1.7. (**A,B**) Upregulation of the IL-2 receptor (CD25) by (**A**) $_{cysL}\alpha\beta$ oligomers coupled through commercially available cross-linking reagents and (**B**) $\alpha\beta_{Lcys}$ oligomers linked with peptide-based cross-linkers. Dotted profiles show basal surface expression and solid profiles activated levels of CD25 detected by flow cytometry after 27 h. The activation is antigen-specific, as tetramers complexed with the endogenous peptide (pep-4° A2) do not induce activation. The mean fluorescence intensity for stimulated cells is shown in each panel, with the non-stimulated control value in parentheses. Oligomer concentrations: $_{cysL}\alpha\beta$ DPDPB (1.1 μM); $_{cysL}\alpha\beta$ HBVS (1.1 μM); $_{cysL}\alpha\beta$ S–S (1.1 μM); $_{cysL}\alpha\beta$ TMEA (185 nM); $\alpha\beta_{Lcys}$ pep-2° (350 nM); $\alpha\beta_{Lcys}$ pep-3° (460 nM); $\alpha\beta_{Lcys}$ pep-4° (280 nM) and $_{cysL}\alpha\beta$ pep-4° A2 (280 nM). (**C**) MHC-peptide dimers (1.1 μM), linked with a variety of cross-linking reagents through the α subunit or β subunit, are able to trigger T-cell activation in the HA1.7 T-cell clone as measured by upregulation of CD69 (shaded bars, 12 h), upregulation of CD25 (IL-2R) (solid bars, 27 h) or downregulation of activated CD3 (TCR) (open bars, 27 h). Activation is reported as the fold difference in levels of cell surface expression, calculated as the ratio of mean fluorescence intensity for stimulated versus non-stimulated cells. Antigen-presenting cells pulsed with 1 μM Ha peptide (B-cells+peptide) induce comparable levels of expression of cell surface markers. Monomeric MHC-peptide complexes (1.4 μM) did not stimulate activation in any of the assays tested.
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dues on the DRα subunit ($_{cysL}\alpha\beta$ DPDPB, $_{cysL}\alpha\beta$ HBVS, $_{cysL}\alpha\beta$ S–S and $_{cysL}\alpha\beta$ TMEA) all induced upregulation of CD25 in the HA1.7 T-cells, as detected by flow cytometry using an allophycocyanin (APC)-labeled monoclonal antibody against CD25 (Figure 7A). Relative cell surface expression levels of CD25 increased by 3–5-fold over the resting level. Dimers, trimers and tetramers constructed using the synthetic fluorescent peptide-based cross-linkers to link cysteine residues on the DRβ subunit ($\alpha\beta_{Lcys}$ pep-2°, $\alpha\beta_{Lcys}$ pep-3° and $\alpha\beta_{Lcys}$ pep-4°) also induced T-cell activation (Figure 7B). We tested MHC dimers linked in various ways for their ability to activate different T-cell responses. MHC-peptide dimers, linked through either subunit with a variety of cross-linking reagents, each induced upregulation of CD25, upregulation of the early activation marker CD69 [35], and downregulation of activated TCR (CD3) subunits [36] (Figure 7C). In many cases, the responses were comparable to those induced by peptide-pulsed antigen-presenting B-cells (B-cells+peptide). Monomeric MHC-peptide complexes did not stimulate the HA1.7 T-cells, even at concentrations up to 1.4 μM (Figure 7C). The observed T-cell response to the oligomers was antigen-specific, as tetramers complexed with the non-specific endogenous peptide A2 showed no activation (Figure 7B,C, pep-4° A2).

For the oligomers prepared with the fluorescent peptide-based cross-linkers ($\alpha\beta_{Lcys}$ pep-2°, $\alpha\beta_{Lcys}$ pep-3°, $\alpha\beta_{Lcys}$ pep-4°), binding to T-cells could be measured directly by flow cytometry. Fluorescent chemically cross-linked oligomers could be detected by flow cytometry as bound to the $CD4^+$ T-cell clone HA1.7 (Figure 8A), while tetramers carrying the non-specific peptide A2 (pep-4° A2) did not exhibit significant binding observable above the background fluorescence (Figure 8A). The ability of the chemically defined MHC-peptide oligomers to bind to T-cells was confirmed also in a short-term polyclonal T-cell line raised from the peripheral blood of a $HLA\text{-}DR1^+$ individual. Fluorescent chemically cross-linked oligomers prepared with $\alpha\beta_{cys}$ MHC-peptide complexes ($\alpha\beta_{cys}$ pep-2°, $\alpha\beta_{cys}$ pep-3°, $\alpha\beta_{cys}$ pep-4°) bound in an antigen-specific manner to the polyclonal T-cell line (Figure 8B), as observed with the T-cell clone HA1.7. These results demonstrate that the oligomeric MHC-peptide reagents could be useful tools for investigation of ongoing immune responses in primary T-cells as well as established clones.

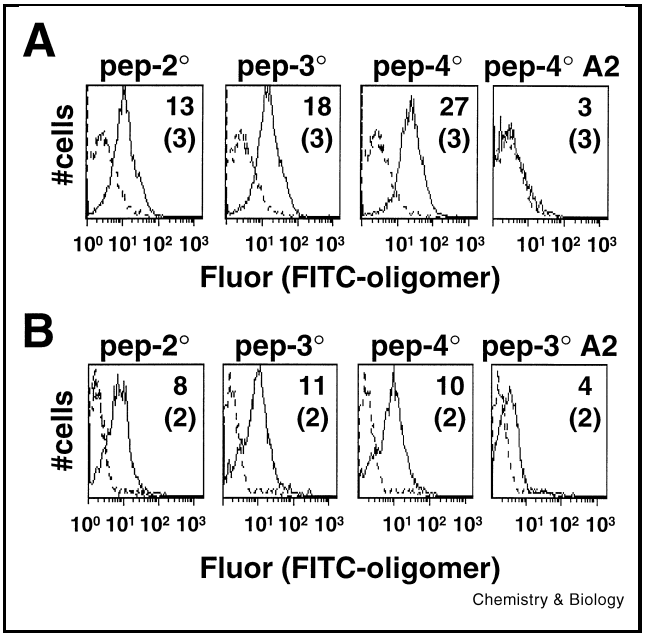

Figure 8. MHC-peptide oligomers exhibit antigen-specific binding to T-cells. Direct binding of (**A**) $\alpha\beta_{Lcys}$ oligomers to HA1.7 (5 h incubation), and (**B**) $\alpha\beta_{cys}$ oligomers to a short-term polyclonal T-cell line (12 h incubation) detected with fluorescein labels incorporated into the cross-linking reagents. Fluorescence of bound fluorescein oligomer is expressed in each panel (solid lines), with the background fluorescence of untreated T-cells indicated in parentheses (dotted lines). MHC oligomers carrying the Ha peptide bound specifically to the T-cells, whereas tetramers or trimers carrying the non-specific peptide (pep-4° A2; pep-3° A2) exhibited little or no binding. Concentrations for the $\alpha\beta_{Lcys}$ oligomers are as per Figure 7 or: $\alpha\beta_{cys}$ pep-2° (3.3 μM); $\alpha\beta_{cys}$ pep-3° (1.8 μM); $\alpha\beta_{cys}$ pep-4° (1.1 μM); and $\alpha\beta_{cys}$ pep-3° A2 (1.5 μM).

Discussion

Oligomeric MHC-peptide complexes as mechanistic probes and detection reagents

We report a strategy for production of a series of oligomeric MHC-peptide complexes with varying valency and topological constraints, by thiol-specific coupling through an introduced cysteine residue at the C-terminus of the α subunit or β subunit of the human class II protein HLA-DR1. We coupled these cysteine-modified complexes using direct disulfide bonds, commercially available cross-linking reagents, or a novel series of fluorescently labeled, peptide-based cross-linkers. Previous approaches to the production of MHC-peptide oligomers include antibody-mediated oligomerization of MHC-peptide complexes [22], formation of chimeric immunoglobulins as fusions of Fc domains with MHC α and β subunits [20,21], and incorporation of a target sequence for enzymatic biotinylation with subsequent oligomerization by streptavidin [23,37–39]. In all of these cases, the relative flexibility and orientation of monomer units within the oligomer, as well as the overall oligomer valency, clearly are constrained by the antibody or streptavidin component of the monomer. Both antibody molecules [40] and streptavidin [41] can have undesired binding to specific receptors on the surface of mammalian cells. Additionally, most of these reagents cannot be prepared in homogenous forms free from contamination with larger and smaller oligomers. Fluorescent labeling of these compounds can further alter their binding properties or oligomeric state. In contrast, our chemical oligomerization approach allows specific cross-linking anywhere in the protein at the introduced cysteine residue, the cross-linkers can be made in a variety of well-characterized lengths and topological constraints without the need for a macromolecular linker protein, direct incorporation of a fluorescent probe into the cross-linker facilitates measurement of oligomer binding to cell surfaces, and oligomers of various sizes and topological constraints all can be prepared in essentially homogenous form.

MHC-peptide dimers trigger T-cell activation

T-cells are activated in vivo by encounter with an antigen-presenting cell carrying appropriate complexes of surface MHC protein and peptide antigen. It has been widely recognized that artificial clustering of TCR components can also induce T-cell activation processes [7,42–44]. The valency of TCRs in the activating cluster has been the focus of much current investigation into the basic molecular mechanisms that underlie immune functions. MHC-peptide complexes incorporated into planar bilayers [43] or soluble antigen arrays [44] have been used to investigate the effects of varying antigen density on T-cell activation. These studies defined a threshold antigen density required for activation, but could not provide definitive insight into the molecular mechanism or nature of the activating species. Extracellular antibody-induced cross-linking of TCR complexes [45], or of chimeras carrying TCR cytoplasmic domains [46–48] has been shown to mimic T-cell activation processes induced by antigen-presenting cells, suggesting that receptor dimerization or aggregation might be necessary for T-cell activation. In several studies, soluble MHC-peptide multimers have been used to probe the molecular requirements for T-cell activation. Dimeric MHC-peptide complexes produced through antibody-mediated oligomerization [22], or fusion of MHC extracellular domains with antibody Fc portions [19–21], have been shown to induce a variety of T-cell activation or differentiation processes, providing evidence that MHC dimers could be sufficient for activation. In contrast, a series of streptavidin-based MHC oligomers constructed using biotinylated MHC monomers argued that trimeric complexes were required for efficient T-cell activation [23].

In our system, every MHC-peptide oligomer tested was able to induce T-cell activation processes. Dimers linked through the α or β subunits, with or without the flexible 'connecting peptide' regions, and linked through a variety

of coupling chemistries and linker lengths, each was able to activate T-cells in an antigen-specific manner as judged by upregulation of cell surface activation markers and downregulation of activated TCR receptor subunits. In all, 12 different MHC-peptide dimers (including those prepared by commercially available and novel peptide-based cross-linkers) were shown to induce a variety of T-cell activation markers, demonstrating that dimers are sufficient to stimulate activation processes in T-cells. In the physiological context, larger-order clustering of TCR and involvement of other cell surface proteins may contribute to the overall cellular activation program. The differential effects of the topologically distinct dimers on T-cell activation remain to be investigated in detail. In another study using these oligomeric MHC-peptide complexes, the dose-dependence of activation by monomers, dimers, trimers and tetramers was investigated [32]. Tetramers bound better than trimers, and trimers better than dimers, but all oligomers activated equivalently on the basis of MHC molecules bound.

X-ray crystallographic analyses of several class II MHC-peptide complexes have revealed a conserved non-crystallographic dimer of αβ heterodimers [49]. Since this discovery, there has been much speculation about the existence and functional role of these 'superdimers' or 'dimer of dimers' in mimicking an activating oligomeric species that forms transiently at the interface between antigen-presenting cells and T-cells [49–52]. Direct confirmation or contradiction of this hypothesis has proven to be difficult, but some evidence has been presented in support of the existence of these MHC-peptide 'superdimers' on the surface of living cells [53–55]. The crystallographic MHC-peptide dimer has a compact, spherical shape, with frictional coefficient = 1.0 and calculated hydrodynamic radius (R_s) = 34.2 Å (Table 1). In this dimer, the termini of the α subunits are substantially closer than those of the β subunit termini (13 versus 39 Å). In our series of topologically varied dimeric MHC-peptide complexes, dimers linked through shorter α subunits ($_{cys}$αβ S–S and $_{cys}$αβ pep-2°) exhibited substantially more compact conformations than the other dimers, with hydrodynamic radius similar to that calculated for the crystallographic dimer (Table 1). We hypothesize that these dimers may preferentially assemble into the close-packed MHC arrangement observed in the crystal structures. The MHC-peptide complexes and cross-linking strategy described herein will be useful in evaluating the physiological relevance of the crystallographic dimer, and in determining the effect of MHC orientation on T-cell activation.

Binding affinity and receptor rearrangement

Theoretically, the free energy of binding for an oligomer should equal the sum of (1) a favorable free energy reflecting the binding of each monomer unit, (2) a favorable entropic factor resulting from fewer rotational and translation degrees of freedom lost for binding an additional monomer in an already bound oligomer, and (3) an unfavorable term reflecting strain or distortion in the oligomer or its receptor required to achieve the bound state [56]. For example, for a flexible divalent ligand binding to a multivalent cell surface receptor, the free energy of binding should be greater than the sum of contributions of individual monomeric components, because once the oligomer has attached itself to a cell by one monomer, it will undergo a smaller entropy loss by binding through the second monomer [57]. These effects were demonstrated in the vancomycin system where a rigid trimeric ligand was designed to bind without distortion to a receptor pre-existing as a trimer [58]. Here, the binding affinity of the trimeric ligand ($K_d \sim 10^{-6}$ M) was approximately the cube of that of a monomeric ligand ($K_d \sim 10^{-17}$ M). The trimer free energy of binding (~ -22 kcal/mol) was then approximately the sum of the three monomer contributions (~ -8 kcal/mol), indicating that the 'strain or distortion' term was small, consistent with the pre-arranged ligand structure. In contrast, in the characterization of the binding of divalent antibody or monovalent Fab′ fragments to non-pre-arranged MHC class I molecules on a cell surface [59], the affinity of the dimer ($K_d \sim 10^{-7}$ M) was found to be tighter than that of the monomer ($K_d \sim 10^{-9}$ M) only by a factor of 50, corresponding to a dimer free energy of binding substantially lower than the sum of monomer contributions. For a case of streptavidin-coupled class II MHC oligomers [23], the affinity of a tetramer binding to immobilized monomeric TCR was estimated to be only ~170 times greater than that of a MHC monomer. Again, the relatively small affinity increase for the MHC monomer relative to the multivalent MHC tetramer is consistent with the lack of pre-arrangement in the dextran-immobilized TCR used in the plasmon resonance experiment.

In the case of our MHC-peptide oligomer series binding to T-cell surfaces, the relative binding affinity varied monotonically with oligomer size in a log-linear dependence (Figure 5B). The apparent free energy increment gained by adding a second, third or fourth component into the oligomer was approximately 0.8 kcal/mol, substantially less than the apparent binding free energy for the monomer, estimated to have a lower limit of approximately −7.7 kcal/mol (see Materials and methods). This indicates that MHC oligomer binding to T-cells induces strain or distortion in either the oligomers or T-cell membrane. As the MHC components in our oligomers would be expected to be fairly flexible, the strain could be due to rearrangements in the T-cell membrane necessary to cluster receptors in an arrangement suitable for oligomeric engagement. In support of this idea, binding of MHC tetramers is not observed at 4°C, a temperature at which membrane rearrangements are blocked, although this observation may reflect a block in TCR internalization at low temperature (T. Cameron and L. Stern, unpublished results). TCRs as

well as other components involved in interactions with an antigen-presenting cell are known to relocalize specifically to the membrane interface during the overall cell–cell interaction process, but the stoichiometry of these colocalized clusters is not known [60,61]. Whether TCRs exist as pre-formed dimers in the cell membrane or dimerize upon engagement with MHC is an outstanding question in T-cell biology [10,11,32,62]. The relatively small free energy increment observed with our oligomers and the similarity of monomer–dimer, dimer–trimer and trimer–tetramer free energy increments might argue against pre-existing TCR dimers in the membrane, unless binding of MHC oligomers to a pre-existing TCR dimer causes a gross receptor rearrangement that results in substantial energy loss. An oligomer series with linkers of varying flexibility should be examined to address this issue in a definitive manner.

Significance

Molecular details are not known for the activation of T-cells by MHC-peptide complexes binding to surface TCRs, and few tools are available to study these processes. We prepared chemically defined dimers, trimers and tetramers of class II MHC-peptide complexes with varying intermolecular spacing and orientation, through cysteine residues incorporated into the MHC protein and thiol-specific cross-linking reagents. Dimerization of TCR components was sufficient to trigger T-cell activation, as demonstrated by induction of T-cell processes with a diverse variety of cross-linked MHC-peptide dimers. Relative binding affinities within a homologous series of MHC monomer, dimer, trimer and tetramer complexes suggested that TCRs may need to rearrange in the membrane plane concurrent with oligomeric binding of MHC-peptide complexes. The capability to systematically vary oligomer size and intermolecular orientation, and to measure oligomer binding concurrent with activation events by flow cytometry, are useful tools for probing the molecular mechanism of T-cell activation. This type of approach could have widespread applications in other areas of protein biochemistry and signal transduction, as well as in developing general mechanistic probes of receptor–ligand interactions.

Materials and methods

Cysteine residues incorporated in HLA-DR1

Genes encoding the extracellular regions of the α (*HLA-DRA1*0101*, SwissProt: P01903) or β (*HLA-DRB1*0101*, P13758) subunits of HLA-DR1 were modified by polymerase chain reaction to include Ala-Cys (GCATGC) sequences immediately before a stop codon and *Hin*dIII site, and were transferred to *Escherichia coli* expression plasmids for T7-mediated expression as described [16]. For the shorter constructions $_{cys}\alpha$ and β_{cys}, the cysteine was introduced immediately after the last residue of the lower immunoglobulin domain Alaα182 or Alaβ190. For the longer constructions $_{cysL}\alpha$ and β_{Lcys}, Ala-Cys sequences were introduced after the 'connecting peptide' regions α183–192 (PSPLPETTEN) or β191–198 (RSESAQSK), immediately before the predicted transmembrane domains.

Preparation of MHC-peptide complexes

HLA-DRα and β subunits were expressed in *E. coli* BL21(DE3)pLysS cells as insoluble inclusion bodies, solubilized in 8 M urea, and purified as previously described [16]. Soluble MHC-peptide complexes were produced by in vitro folding of denatured α and β subunits of HLA-DR1 in the presence of peptide, by dilution of the denaturant under redox-controlled conditions [16]. Antigenic peptide Ha[306–318] (PKYVKQNTLKLAT), derived from influenza virus hemagglutinin [63], and control endogenous peptide A2[103–117] (VGSDWRFLRGYHQYA), derived from the class I MHC HLA-A2 [64], were synthesized using FMOC chemistry, purified by reverse phase high performance liquid chromatography (HPLC), and verified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF). MHC-peptide complexes carrying C-terminal cysteine residues could not be cross-linked efficiently after isolation using the standard refolding/purification protocol [16], apparently due to reversible modification of the introduced thiol, therefore were purified in the presence of 5 mM dithiothreitol which was removed immediately prior to cross-linking.

Thiol assays

The availability of unmodified, reactive sulfhydryl residues was determined by treatment of purified cysteine-modified MHC proteins with diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (Molecular Probes), a sensitive, thiol-specific, fluorescent probe. The amount of free thiol was quantitated by comparison of fluorescence maxima at 469 nm with a standard curve using β-mercaptoethanol. The presence of reactive sulfhydryl residues was also monitored by dimerization of the cysteine-modified MHC-peptide complexes through oxidation mediated by copper phenanthroline (see below). The amount of reactive thiol was quantitated by comparison of the resultant MHC-peptide dimer and monomer peaks on a Superdex 200 gel filtration column (Pharmacia).

Oligomerization using commercially available cross-linking reagents

Chemical cross-links were made between the long version of the α subunit of the cysteine-modified HLA-DR1 peptide complexes ($_{cysL}\alpha\beta$) and thiol-reactive groups on commercially available cross-linking reagents. To prepare dimers of DPDPB (Pierce) or HBVS (Pierce), and trimers of TMEA (Pierce), a 500 μM stock solution of cross-linker was prepared in dimethyl sulfoxide and added in small batches over 4 h to MHC-peptide (10–30 μM) in phosphate-buffered saline (PBS; 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl (pH 7.4)), with 1 mM EDTA in a final molar ratio of 1:2 cross-linker:MHC for dimers and 1:3 cross-linker:MHC for trimers. Cross-links were made through direct disulfide bonds between the introduced thiols by addition of 0.25 mM $CuSO_4$ and 1.3 mM 1,10-phenanthroline (Sigma-Aldrich) to MHC-peptide complexes in 50 mM HEPES (pH 8.0) [31]. The extent of cross-linking in reaction mixtures was determined by SDS–PAGE (12.5%) and quantitated with gel filtration chromatography (Superdex 200, Pharmacia) by integrating the peak areas. Cross-linked MHC-peptide complexes were purified by gel filtration on Superdex 200 (Pharmacia), and integrity of disulfide bonds and bound peptide was measured by SDS–PAGE (12.5%, non-reduced vs. reduced and non-boiled vs. boiled samples, respectively).

Synthesis of novel peptide-based cross-linking reagents

Peptides were synthesized by FMOC chemistry on a solid phase peptide synthesizer, and capped at their N-termini by reaction with fluorescein isothiocyanate. TentaGel S RAM FMOC resin (Advanced ChemTech) was used to give a C-terminal amide upon cleavage. β-Alanine was used as the N-terminal amino acid to prevent thiazolidone formation and release of fluorescein during peptide deprotection. Peptides were cleaved from the resin and side-chains were deprotected with 8% trifluoroacetic acid, 2% triisopropylsilane, 5% dithiothreitol, and the final product was recovered by ether precipitation. Peptides were purified by reverse phase HPLC using an acetonitrile gradient in 0.1% trifluoro-

acetic acid and a C4 or C18 column (Vydac) and verified using MALDI-TOF. To introduce the thiol-specific maleimide group, purified peptides (2–5 mg) were reacted through the lysine ε-amino group with either *N*-[ε-maleimidocaproyloxy] succinimide ester (Pierce) or succinimidyl-4-[*N*-maleimidomethyl]-cyclohexane-1-carboxy-[6-amidocaproate] (Pierce), by dissolving 5-fold molar excess reagent in *N,N*-dimethylformamide and adding it to peptide in 10 mM Na-phosphate buffer (pH 7), 150 mM NaCl [65]. After 1.5 h at room temperature, the modified peptide was purified by reverse phase HPLC on a C18 column (Vydac). Approximate yields of fully modified peptide were 60% for the dimeric cross-linker and 40% for the trimeric and tetrameric cross-linker. The presence of the appropriate number of maleimide functional groups was confirmed by MALDI-TOF.

Oligomerization using synthetic cross-linking reagents

Chemical cross-links were made through the introduced sulfhydryl on cysteine-modified HLA-DR1 and thiol-reactive maleimide groups on the synthetic peptide-based cross-linking reagents. Cross-linker was added in small batches to purified MHC-peptide complex in 10 mM Na-phosphate (pH 7), 150 mM NaCl, 5 mM EDTA over approximately 5 h at room temperature, to a final MHC:cross-linker ratio of 2:1 for dimers, 3:1 for trimers and 4:1 for tetramers. Extent of cross-linking was measured by SDS–PAGE (10%) and gel filtration chromatography as discussed above. Cross-linked MHC-peptide complexes were isolated by gel filtration using two Superdex 200 HR 10/30 FPLC columns (Pharmacia) in series. Purity was determined by SDS–PAGE (10%).

Hydrodynamic studies of MHC-peptide dimers

MW of MHC-peptide complexes were calculated using the amino acid sequences of protein, peptide and cross-linkers. Apparent MW (MW_{app}) of the MHC-peptide dimers were determined from elution volumes obtained by gel filtration chromatography on two Superdex 200 HR 10/30 columns (Pharmacia). Values were calculated by linear regression using a calibration curve obtained from the elution volumes of known MW standards: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa) (Bio-Rad). Confidence intervals ($\pm\sigma$) reported in Table 1 reflect the standard deviation from the mean elution volume of replicate samples from separate experiments. The hydrated volumes (not shown) and the Stokes radii (R_s) of the HLA-DR1-Ha dimers, trimers and tetramers were derived from the MW_{app} values as previously described [26], using a frictional coefficient of 1.0 as calculated for the structural dimensions of the HLA-DR1-Ha crystallographic dimer [24]. The HLA-DR1-Ha monomer has a frictional coefficient of 1.044 [26].

T-cell clones

The human $CD4^+$ T-cell clone HA1.7 [34] was cultured in RPMI (Gibco) containing 5% human serum (Sigma) and 5% fetal bovine serum (Sigma), and was stimulated every 2 weeks with 120 IU/ml IL-2 (Intergen) and an irradiated mixture of peripheral blood lymphocytes and EBV1.24, a $DR1^+$ B-cell line, pulsed with 1 μM Ha peptide. The polyclonal T-cell line was raised from the peripheral blood of a HLA-$DR1^+$ individual by in vitro stimulation with antigenic peptide and cultured as above for HA1.7. T-cells were rested a minimum of 7 days after stimulation before use in activation assays and binding experiments.

T-cell activation assays

For activation assays, MHC-peptide oligomers were added to 7.5×10^4 T-cells in complete medium in round bottom 96-well plates. In some assays, T-cells were added to 5×10^4 B-cells (EBV1.24) that had been pre-pulsed with 1 μM Ha peptide for 2 h at 37°C. After the indicated time at 37°C and 5% CO_2, cells were placed on ice and incubated with fluorescent monoclonal antibodies: APC-labeled anti-CD25 or anti-CD69 (M-A251 or FN50, Pharmingen), or phycoerythrin-labeled anti-CD3 (UCHT-1, Sigma). After 1 h at 4°C, cells were washed with PBS containing 1% fetal bovine serum and 0.1% sodium azide and analyzed by flow cytometry. Fluorescence data were obtained with a Becton-Dickinson FACS Calibur flow cytometer and analyzed using Cell Quest software. Some experiment to experiment variation in maximal activation levels was observed, therefore quantitative comparisons were made only for samples analyzed in the same experiment.

Oligomer binding assays

For the direct binding assay, levels of cell-associated fluorescence after oligomer incubation and wash were measured by flow cytometry using the fluorescein labels incorporated into the synthetic cross-linkers. MHC-peptide oligomers were added to T-cells as above and incubated for the indicated time at 37°C, 5% CO_2. Cells were placed on ice and washed with PBS containing 1% fetal bovine serum and 0.1% sodium azide and analyzed by flow cytometry. For the competitive binding assay, phycoerythrin-labeled, streptavidin-coupled MHC tetramer (SA-PE) [32] was used as a probe, with competition by unlabeled MHC-peptide oligomers. These SA-PE tetramers exhibit a strong fluorescence from the R-phycoerythrin conjugate, and bind to T-cells in an antigen-specific manner (T. Cameron and L. Stern, unpublished data). Various concentrations of MHC oligomers or monomers with a constant amount of SA-PE tetramer (35 nm) were added to 7.5×10^4 HA1.7 T-cells in 96-well round bottom plates and incubated for 3 h at 37°C, 5% CO_2. Cells were washed as described above and fluorescence arising from the bound SA-PE tetramer was measured by flow cytometry. IC_{50} values were determined as the concentration of oligomer or monomer required to reduce the level of SA-PE tetramer fluorescence to one-half its maximum value [32]. IC_{50} values are reported as oligomer concentrations; for total MHC concentration, the IC_{50} should be multiplied by the oligomer valency. Error bars represent replicate measurements from separate experiments. Binding free energies were estimated using the relationship $\Delta G = 2.3RT(\log K_d)$, with $\Delta G = \Delta G_{mon} + n\Delta G_{oligo}$, where n = oligomer size, as shown by the linear plot of IC_{50} versus n. In this analysis, we assume $K_d = IC_{50}$. This assumption will be true if the concentration of phycoerythrin-labeled streptavidin-cross-linked MHC, [SA-PE], is substantially less than its binding affinity $K_{d,SA-PE}$. If [SA-PE] is substantial, the experimental IC_{50} will overestimate the K_d by the factor $\alpha = 1/(1+[\text{SA-PE}]/K_{d,SA-PE})$, and the calculated ΔG_{mon} will underestimate the true ΔG_{mon} by the factor $\ln \alpha$.

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