Immunity, Vol. 12, 241–250, March, 2000, Copyright ©2000 by Cell Press

The Relationship of MHC-Peptide Binding and T Cell Activation Probed Using Chemically Defined MHC Class II Oligomers

Jennifer R. Cochran, Thomas O. Cameron, and Lawrence J. Stern*
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

A series of novel chemically defined soluble oligomers of the human MHC class II protein HLA-DR1 was constructed to probe the molecular requirements for initiation of T cell activation. MHC dimers, trimers, and tetramers stimulated T cells, as measured by upregulation of the activation markers CD69 and CD25, and by internalization of activated T cell receptor subunits. Monomeric MHC-peptide complexes engaged T cell receptors but did not induce activation. For a given amount of receptor engagement, the extent of activation was equivalent for each of the oligomers and correlated with the number of T cell receptor cross-links induced. These results suggest that formation or rearrangement of a T cell receptor dimer is necessary and sufficient for initiation of T cell signaling.

Introduction

T cells are activated upon encounter with another cell that carries on its surface appropriate complexes of major histocompatibility complex (MHC) protein and peptide antigen (Germain, 1994; Davis et al., 1998). The interaction involves specific recognition of the MHC-peptide complexes by clonotypic T cell receptor (TCR) subunits, as well as antigen-independent interactions between adhesion molecules and other cell surface receptors from the T cell and the antigen-presenting cell (Alberola-Ila et al., 1997; Germain and Stefanova, 1999). Downstream signaling pathways, including formation of large multicomponent activating clusters (Monks et al., 1998; Grakoui et al., 1999; Rudd, 1999), activation of cytoplasmic kinase cascades (Wange and Samelson, 1996; Cantrell, 1996; Qian and Weiss, 1997), and specific transcriptional activation leading to cytokine secretion and T cell proliferation (Ullman et al., 1990; Glimcher and Singh, 1999), have been characterized in detail, but the initial molecular events occurring at the juxtaposed membranes that lead to T cell activation are not well understood. Extracellular antibody-induced clustering of TCR complexes (Bekoff et al., 1986; Janeway, 1995) or of chimeric constructs carrying TCR cytoplasmic domains (Irving and Weiss, 1991; Letourneur and Klausner, 1991; Romeo et al., 1992) has been shown to induce T cell processes characteristic of interaction with an antigen-presenting cell. MHC-peptide complexes incorporated into planar membranes (Watts et al., 1984; Quill and Schwartz, 1987) or coated onto plastic surfaces (Wettstein et al., 1991; van Schooten et al., 1992; Kozono et al., 1994) also have been shown to activate T cell signal transduction pathways. These experiments have led to the hypothesis that clustering or aggregation of T cell receptor components is important for initiation of T cell activation, but the mechanism of clustering and the number and conformation of TCR components in the activating complex are not clear.

Several studies have attempted to determine the minimal requirements for T cell activation. In one approach, the effects of varying antigen density on T cell activation was investigated for MHC-peptide complexes incorporated into planar bilayers (Fox et al., 1987; Watts, 1988) and for soluble antigen arrays (Symer et al., 1992). These studies helped define a threshold antigen density required for activation but could not address local valency requirements at activated T cell receptors. More recently, soluble dimeric MHC-peptide complexes prepared by antibody-mediated oligomerization of soluble MHC molecules (Abastado et al., 1995) or by fusion of MHC extracellular domains with antibody Fc portions (Casares et al., 1999; Hamad et al., 1998) were shown to induce various T cell activation or differentiation processes, suggesting that dimers could be sufficient for activation. However, using a series of oligomeric MHC-peptide complexes produced by streptavidin-mediated cross-linking of biotinylated MHC proteins and measuring extracellular acidification and Ca^{2+} flux, Boniface et al. (1998) reported that trimers were the minimal activating species.

At odds with these results, some studies have suggested that under certain conditions monomeric MHC-peptide complexes can trigger T cell activation (Janeway, 1995). Sykulev et al. (1996) used antigen-presenting cells loaded with defined numbers of peptides to activate $CD8^+$ T cells and concluded that number densities as low as one peptide per cell were sufficient to activate a cytolytic T cell response. Delon et al. (1998) reported that monomeric soluble MHC-peptide complexes were able to induce Ca^{2+} flux in T cells that expressed CD8.

Recent characterization of two phenomena associated with MHC-TCR interaction further complicate our view of T cell activation processes. Reich et al. (1997) observed that at high concentrations, MHC-TCR complexes showed a tendency to aggregate exhibited by neither MHC nor TCR protein alone, suggesting that binding of monomeric MHC-peptide complexes could trigger oligomerization of TCR on the surface of T cells. Valitutti et al. (1995) observed that at low antigen densities, single MHC-peptide complexes serially engaged multiple TCR molecules, using downmodulation of activated receptors (Zanders et al., 1983) as a way to count the number of engaged TCR (Itoh et al., 1999). In principle such serial triggering could allow a single MHC molecule on an antigen-presenting cell to cluster TCR on the T cell membrane (Sykulev et al., 1996).

In this study, the requirements for T cell activation were investigated in a systematic manner, using a series of chemically defined, soluble oligomers of a human

*To whom correspondence should be addressed (e-mail: stern@mit.edu).

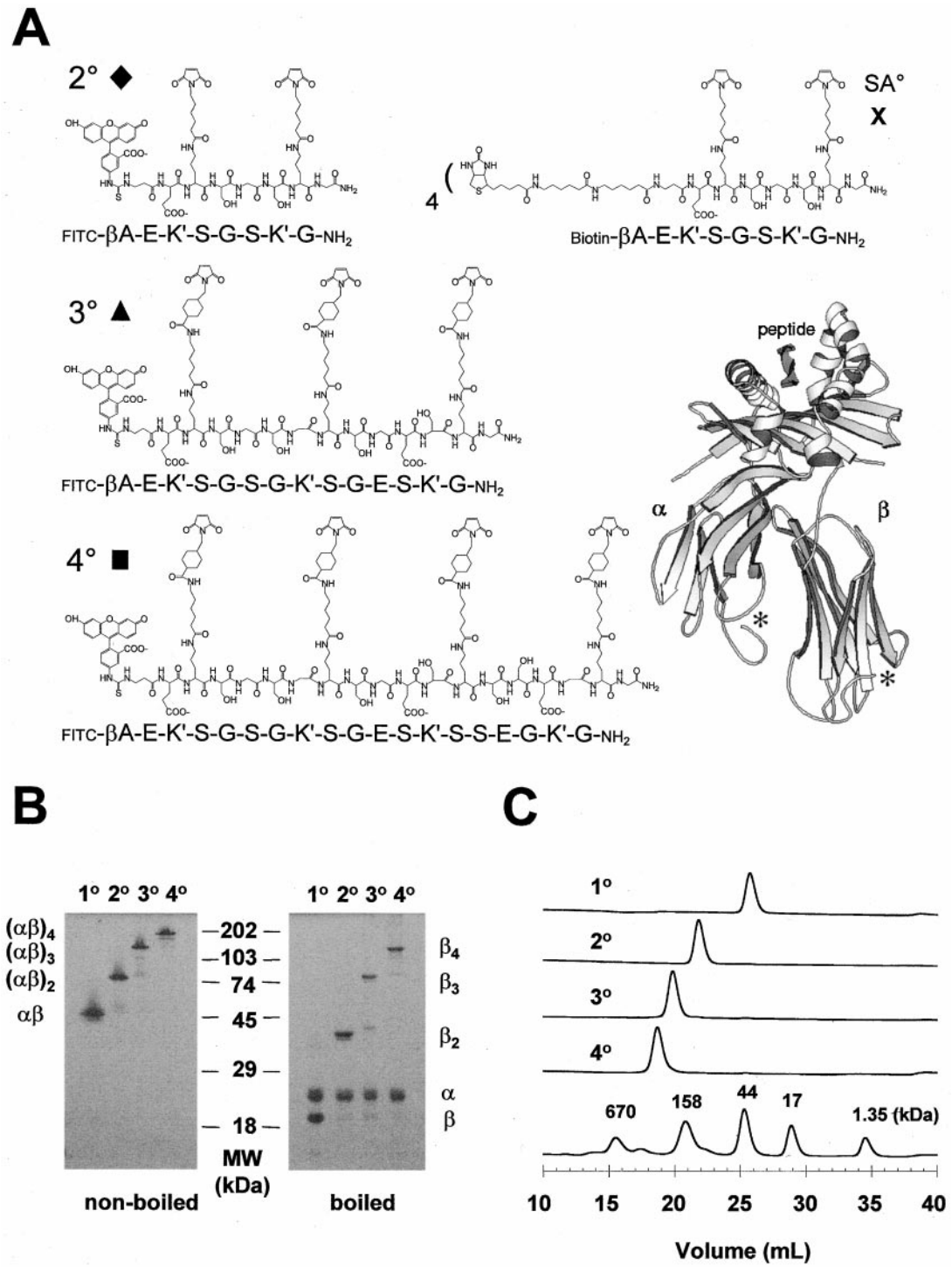

Figure 1. Design and Construction of Soluble MHC-Peptide Oligomers

(A) Chemical structures of cross-linking reagents used to make MHC-peptide oligomers. All cross-linkers carry maleimide groups for coupling to cysteine resides introduced into HLA-DR1. Left, fluorescein-labeled, peptide-based cross-linkers for production of MHC dimers (2°), trimers (3°), and tetramers (4°). Upper right, biotin-labeled dimeric cross-linker for production of higher order streptavidin-linked oligomers (SA°). Lower right, ribbon diagram of the HLA-DR1-peptide complex (Stern et al., 1994), showing the position of cysteine residues (asterisks) introduced at the end of either the α or β subunit connecting peptide region. The HLA-DR1 model is shown at approximately 80% of the scale of the chemical diagrams of the cross-linking reagents. The symbol used throughout the manuscript to refer to each complex is indicated.

(B) Oligomeric HLA-DR1-peptide complexes are stable to SDS-induced chain dissociation, indicating the presence of bound peptide (left panel), and contain the desired covalent bonds through the β chain as indicated by boiling in SDS (right panel).

(C) Gel filtration traces of purified oligomeric HLA-DR1-Ha peptide complexes exhibit the sizes expected for monomer (1°), dimer (2°), trimer (3°), and tetramer (4°). The profile for molecular weight standards is indicated below the HLA-DR1 traces.

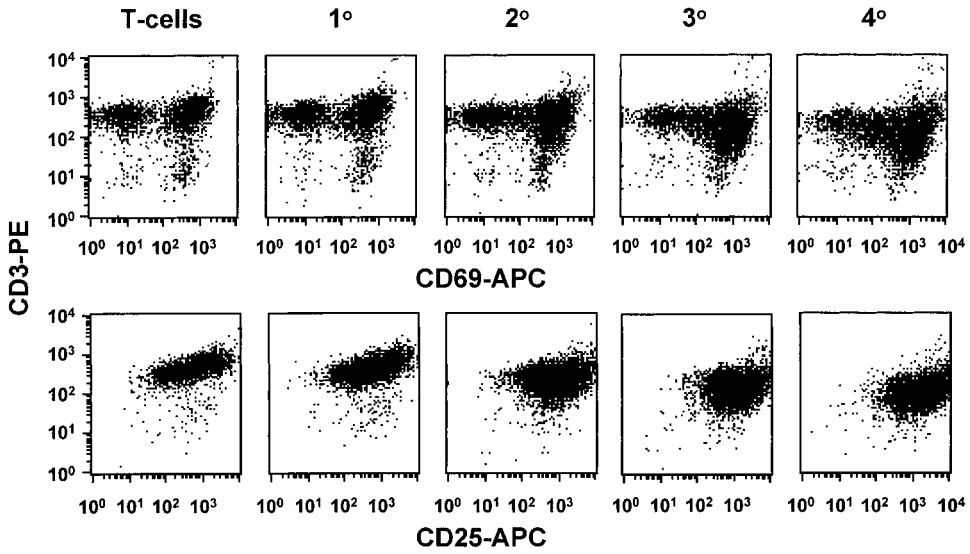

Figure 2. T Cell Activation by MHC Oligomers

Flow cytometry analysis of CD3 downregulation and CD69 upregulation at 12 hr (top panel) and of CD3 downregulation and CD25 upregulation at 27 hr (bottom panel) indicates that dimers (350 nM), trimers (460 nM), and tetramers (280 nM) of HLA-DR1-Ha induce activation of the HA1.7 T cell clone. Monomeric MHC-peptide complexes (1.4 μM) do not cause activation in any of the assays. Activation markers were detected using phycoerythrin-labeled CD3 (CD3-PE) and allophycocyanin-labeled CD69 or CD25 (CD69-APC, CD25-APC). The entire cell population was used to calculate the mean fluorescence values shown for these and other oligomer concentrations in Figure 3.

class II MHC protein. MHC binding was measured concurrently with several T cell activation processes, using fluorescent labels incorporated into oligomer cross-linking reagents. We find that a dimer of MHC-peptide complexes is necessary and sufficient for initiation of T cell activation. MHC-peptide monomers bind but do not activate T cells. Quantitative comparisons of binding and activation levels demonstrate that dimers, trimers, and tetramers activate identically per molecule of MHC bound. These results can be used to assess possible molecular models for initial T cell activation events and support a model in which formation or rearrangement of a dimer of TCR molecules in the membrane is the relevant molecular triggering event in T cell activation.

Results

Oligomer Design and Construction

To study the topological requirements for T cell activation a series of soluble oligomeric complexes of the human MHC class II protein, HLA-DR1 was constructed. For production of oligomers, we chemically synthesized peptide-based cross-linking reagents containing a fluorescent label (fluorescein) and two or more thiol-reactive maleimide groups linked to lysine residues spaced along a flexible backbone containing glycine, serine, and glutamic acid (Figure 1A). The nonrepeating backbone sequence was 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. HLA-DR1 was modified to introduce a cysteine residue at the C terminus of the extracellular domain of either the α or β subunit (Figure 1A). Cys-modified HLA-DR1 complexes were produced by in vitro folding of subunits expressed in *E. coli* (Frayser et al., 1999) in the presence of an antigenic peptide derived from influenza virus hemagglutinin (Ha) or a control endogenous peptide (A2). 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 β subunit (see Experimental Procedures). Purified cross-linked oligomers exhibited the expected β subunit covalent cross-links, as judged by SDS-PAGE (Figure 1B, right panel), and the sizes expected for soluble monomeric, dimeric, trimeric, and tetrameric MHC proteins with no tendency to aggregate, as judged by gel filtration chromatography (Figure 1C). MHC monomers and oligomers all retained the ability to tightly bind peptide antigens, as judged by resistance to SDS-induced chain dissociation at room temperature (Figure 1B, left panel).

T Cell Activation Induced by MHC-Peptide Oligomers

To evaluate the ability of soluble MHC class II oligomers to activate T cells, we used the well-characterized, Ha-peptide-specific human $CD4^+$ T cell clone HA1.7 (Lamb et al., 1982) and measured the ability of the MHC-peptide complexes to induce a variety of T cell activation processes, including upregulation of the early T cell activation marker CD69 (Testi et al., 1994), upregulation of the low-affinity IL-2 receptor CD25 (Waldmann, 1989), and downregulation of activated T cell receptor (TCR) CD3 components (Zanders et al., 1983; Valitutti et al., 1995). Dimeric, trimeric, and tetrameric complexes of HLA-DR1-Ha induced downregulation of CD3 and upregulation of both CD69 and CD25, as measured by flow cytometry (Figure 2). CD69 upregulation was measured at 12 hr, and CD25 upregulation at 27 hr, near the optimum levels as determined by a time course of the response

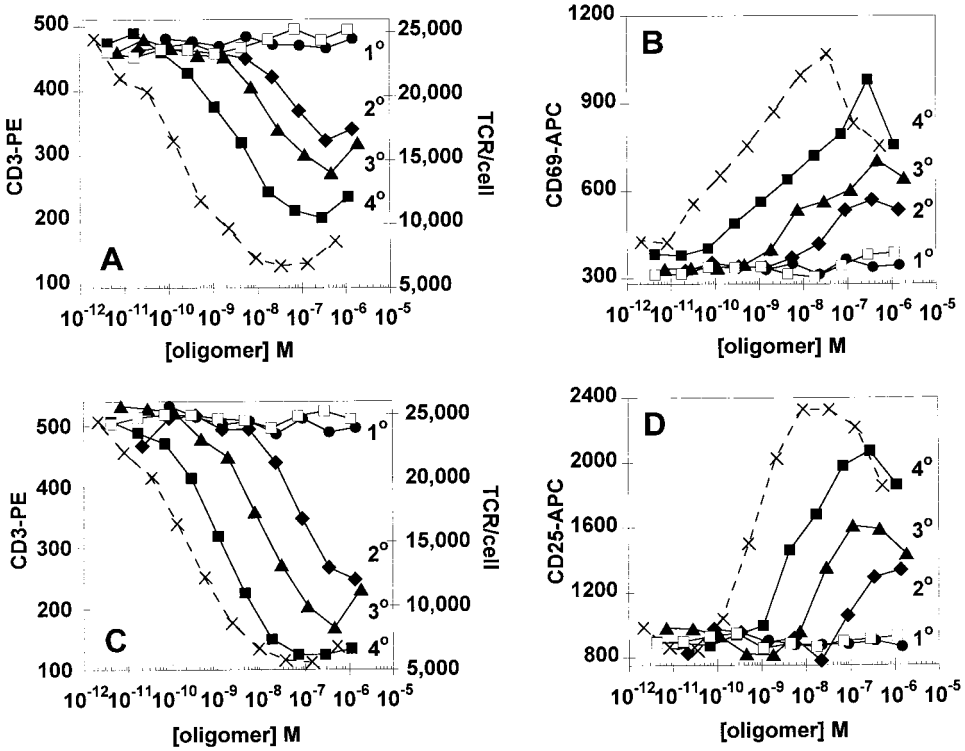

Figure 3. Concentration Dependence of T Cell Activation by Oligomeric MHC-Peptide Complexes

Dose response of CD3 downregulation (A) and CD69 upregulation (B) at 12 hr and of CD3 downregulation (C) and CD25 upregulation (D) at 27 hr by HLA-DR1-Ha monomers (closed circles), dimers (closed diamonds), trimers (closed triangles), and tetramers (closed squares), shown as mean fluorescence values and also as molecules per cell for CD3. The dose response of the higher order SA-linked oligomers (X) is indicated by a dotted line. The response is peptide-specific as tetramers carrying the endogenous peptide A2 (open squares) do not induce T cell activation. Monomeric HLA-DR1-Ha does not induce T cell activation even at high protein concentrations.

(J.R.C., unpublished data; Hara et al., 1986; Caruso et al., 1997). Significant CD3 downregulation was observed at these times as measured simultaneously by multicolor flow cytometry (Figure 2) and also as early as 5 hr (data not shown). Monomeric HLA-DR1-Ha complexes did not stimulate any of the activation responses in these assays. The monomer preparations were not damaged or otherwise intrinsically unable to activate T cells, as we observed strong T cell activation with HLA-DR1-Ha monomers aggregated by immobilization on plastic microtiter plates (data not shown), as previously observed in other systems (Wettstein et al., 1991; van Schooten et al., 1992; Kozono et al., 1994).

To determine the dose response of the activation, a wide range of oligomer concentrations was tested for each of the activation markers (Figure 3). In each assay, MHC-peptide tetramers (squares) activated more potently than trimers (triangles), and trimers more potently than dimers (diamonds). The oligomer concentration needed to induce a given level of response, as well as the maximum response observed, varied systematically with oligomer size. This pattern is consistent whether the oligomer concentration is considered in terms of the number of molecules of oligomers (Figure 3) or as the total number of MHC molecules (data not shown). The observed T cell responses to the oligomers were antigen specific, as tetramers complexed with the nonspecific endogenous peptide A2 showed no activation (open squares). Monomeric MHC-peptide complexes (circles) activated little or no CD3 downregulation, CD69 upregulation, or CD25 upregulation, even at concentrations up to 2 μM (100 μg/ml). T cell activation was not caused by the cross-linker itself, as demonstrated by the A2 peptide tetramer control. At higher concentrations, trace activation signals were observed for both specific (Ha peptide) and nonspecific (A2 peptide) MHC monomers (data not shown).

Oligomer Binding and Avidity

The more potent activation induced by the higher-order oligomers could be explained if they bound more efficiently to the T cell surface. Because avidity increases with increased valency of binding, at a given MHC concentration more MHC protein would be bound for a larger oligomer as compared to a smaller oligomer. Additionally, the inability of MHC monomers to induce activation could be the result of a very weak binding affinity, such that few monomers might be bound to the T cell surface even at the relatively high concentrations tested. To evaluate the relative ability of the various oligomers to bind to T cells, we measured oligomer binding concurrently with activation, using fluorescein incorporated into the cross-linking reagents. Oligomeric MHC-peptide complexes were observed to bind to HA1.7 T cells in a dose-dependent manner, with higher order oligomers exhibiting more binding at lower concentrations relative to smaller oligomers (Figure 4A). Fluorescein-labeled

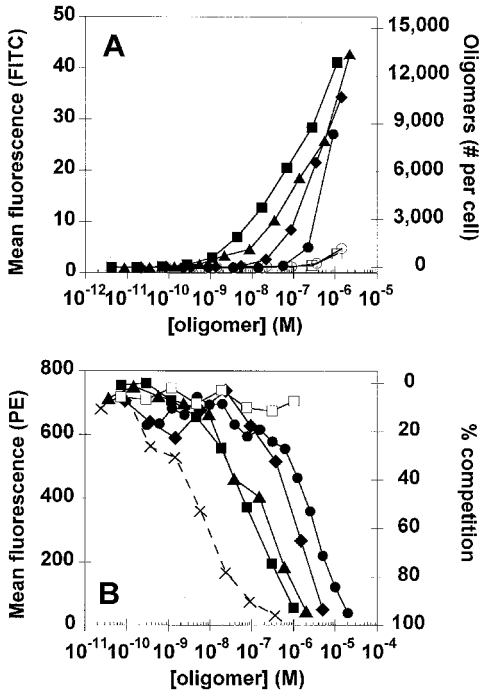

Figure 4. Oligomeric MHC-Peptide Complexes Bind to HA1.7 T Cells

(A) Direct binding of MHC-peptide oligomers, measured at 27 hr using fluorescein labels incorporated into cross-linking reagents. For this experiment, monomers were labeled using a thiol-specific fluorescein derivative.

(B) Competitive binding. Various concentrations of MHC-peptide oligomers or monomers were used to compete for binding in a 3 hr assay with R-phycoerythrin-labeled streptavidin (SA-PE) tetramers (35 nM) carrying HLA-DR1-Ha peptide complexes. In both (A) and (B), monomers (closed circles) were able to bind to HA1.7 T cells under conditions where no activation was observed. Tetramers (closed squares) bound better than trimers (closed triangles), and trimers bound better than dimers (closed diamonds). Higher order SA oligomer binding (X) is indicated by a dotted line. No binding was observed with tetramers or monomers carrying the nonspecific peptide A2 (open squares, open circles).

MHC monomers exhibited a clear antigen-specific binding signal. Tetramers and monomers carrying HLA-DR1 complexes of the nonspecific peptide A2 did not bind. Thus, MHC monomers and oligomers both bind efficiently to antigen-specific T cells under the conditions of the activation assays. However, quantitative interpretation of cross-linker fluorescence intensity in terms of MHC-TCR affinity was complicated by internalization of labeled MHC monomers and oligomers during the course of the assay (T. O. C., unpublished data). As a result of this internalization, the levels of cell-associated fluorescence did not saturate at high concentrations. Although we could not use the direct binding data to determine binding affinities, these data were very useful in correlating levels of T cell activation with numbers of MHC bound for the various oligomers (see below).

To measure the relative avidity of oligomer binding without complications arising from internalization, a competition assay was used (Figure 4B). R-phycoerythrin-labeled streptavidin-cross-linked (SA-PE) "tetramers" of biotinylated MHC-peptide complexes (Altman et al., 1996; Crawford et al., 1998) were used as a binding probe. These SA-PE tetramers exhibit strong, pH-independent fluorescence from the R-phycoerythrin conjugate and bind in an antigen-specific manner to HA1.7 T cells (T. O. C. unpublished data). We incubated T cells with a fixed concentration of SA-PE tetramers (35 nM) and varying concentrations of MHC-peptide oligomers and monomers in a 3 hr competitive binding assay (Figure 4B). Although the SA-PE tetramers may be internalized during even this brief incubation, the relative ability of soluble oligomers to compete for SA-PE tetramer binding sites will reflect their relative avidity. The chemically cross-linked tetramers competed most efficiently for binding (IC_{50} ~100 nM), followed by the trimers (~300 nM) and dimers (~1.8 μM). MHC tetramers carrying the nonspecific peptide A2 did not compete for binding. Monomeric HLA-DR1-Ha complexes exhibited clear competitive binding behavior, with an IC_{50} of ~3.4 μM. Thus, as observed in the direct binding assay, monomeric MHC molecules were able to occupy a substantial number of T cell receptors (>30% of the total TCR at 1 μM; Figures 4A and 4B) under conditions where essentially no T cell activation was observed (Figures 2 and 3).

Correlation of Activation Markers

To investigate the relative "quality" of the activation signal induced by oligomers of different sizes, we cross-correlated the levels of activation observed in the different activation assays. To extend the range of oligomer sizes investigated, we prepared higher order MHC oligomers using biotinylated MHC dimers (Figure 1A) clustered by streptavidin (SA). These SA oligomers were effective in inducing CD3 downregulation and CD69 and CD25 upregulation (Figure 3, dashed lines) and competed efficiently for T cell receptor binding with an IC_{50} of ~17 nM (Figure 4B, dashed line). Extrapolation of the log-linear relationship of IC_{50} with oligomer size observed for the chemically defined reagents suggests a nominal oligomer size of six for the SA oligomers (see Experimental Procedures). Figure 5A shows correlation of the levels of CD69 activation with TCR downregulation, and Figure 5B shows correlation of CD25 activation with TCR downregulation. In these plots we included data for streptavidin oligomers along with those for chemically defined dimers, trimers, and tetramers. The relationship between CD69 upregulation and TCR downregulation appears roughly linear (correlation coefficient 0.92), while the relationship between CD25 upregulation and TCR downregulation appears curved. Although there is substantial scatter in the plots, the points describing the activation induced by each of the different oligomers clearly follow the same relationship. This indicates that the relative activation of the various pathways is the same for each of the oligomers, with binding of a fewer number of larger oligomers indistinguishable from binding of a larger number of smaller oligomers. Thus, the quality of the activation signal induced by oligomer binding is analogous, regardless of the valency or chemical nature of the oligomer used to stimulate the T cell.

Correlation of Binding with Activation

Having measured binding data concurrently with T cell activation, we were able to differentiate avidity effects

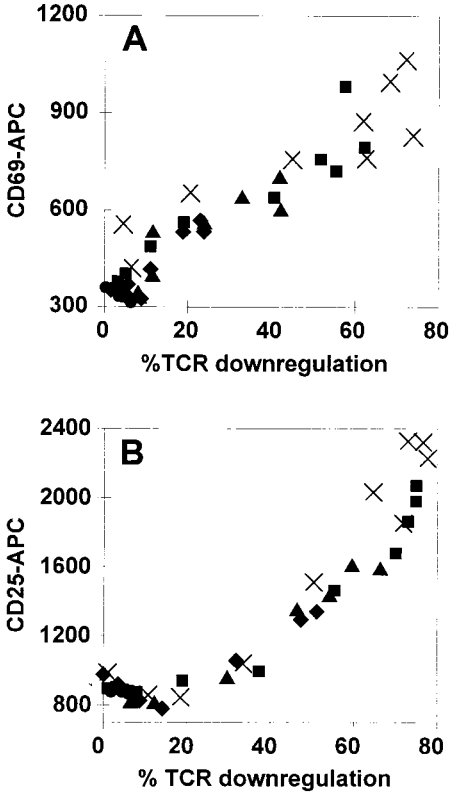

Figure 5. Correlation of T Cell Activation Markers

TCR downregulation plotted against the cell surface expression of CD69 (A) or CD25 (B) observed during the same experiment. Symbols for the various MHC oligomers are as used in the previous figures. Plots are representative of data from several experiments.

from any intrinsic differences in the ability of the bound oligomers to activate T cells. We observed a striking correlation between the level of activation and the number of MHC molecules engaged per cell, calculated from oligomer direct binding data and the number of MHC molecules per oligomer (Figure 6). For each of the T cell activation assays, the curves describing the binding activity relationships for MHC dimers, trimers, and tetramers nearly overlap. At high oligomer concentrations, the activation levels decreased with increasing oligomer concentration (Figure 3) as a result of a saturation effect that lowers the effective binding valency and avidity under conditions where a large fraction of the receptor are occupied (Lauffenburger and Linderman, 1993). The same phenomenon leads to monovalent antibody binding at high antibody/antigen ratios. These regimes are indicated with open symbols and dashed lines in Figure 6. For the monomers, the curves describing the binding activity relationships are essentially flat and clearly distinct from those of the other oligomers. The dramatically different dependence observed for the monomers confirms that monomers can bind to T cells without inducing activation. For the oligomers, the similarity of the curves for the various oligomers indicates that the T cell is responding to the number of MHC molecules engaged in oligomeric complexes, regardless of the size of the oligomer. These results can be used to evaluate possible molecular mechanisms for the T cell activation process, as discussed below.

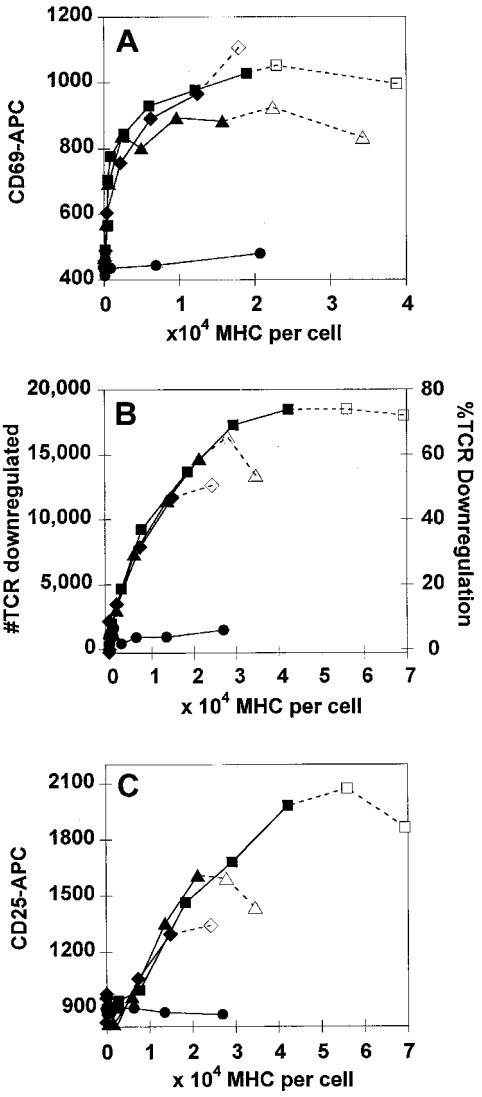

Figure 6. Correlation of MHC Binding and T Cell Activation

The number of MHC molecules bound (determined by direct binding measurement) was plotted against T cell activation for CD69 at 12 hr (A) and for CD3 (B) and CD25 (C) at 27 hr. Dotted lines and open symbols indicate regions (above 1 μM) of the activation plots (Figure 3) in which increasing oligomer concentration caused decreasing activation. At lower concentrations, tetramers (squares), trimers (triangles), and dimers (diamonds) appeared to follow the same dependence. Monomers (circles) bound but did not cause substantial activation. For oligomers, binding was measured concurrently with activation markers. For monomers, levels of binding for labeled preparations were compared with the activation level induced by unlabeled preparations at the same concentration. Data are representative of several experiments.

Discussion

Dimer Formation Is Necessary and Sufficient for Activation of HA1.7 CD4+ T Cells by MHC Oligomers

T cell activation in vivo involves many coupled cellular processes occurring over time scales from seconds to days (Ullman et al., 1990), and the nature of the activation response can vary depending on the history and developmental state of both the antigen-presenting cell and

the T cell (Constant and Bottomly, 1997; Dutton et al., 1998). In this work we have investigated the molecular requirements for the initial, membrane-proximal triggering event, using a well-characterized Th0 memory T cell clone specific for an influenza hemagglutinin peptide (Ha) bound to the human class II MHC molecule HLA-DR1 (Lamb et al., 1982). We find that soluble MHC oligomers are able to activate several conventional activation processes, including those that require membrane-to-nucleus signaling. Costimulation through other signaling pathways (Mueller et al., 1989) may be required in addition to the initial MHC-TCR triggering event for entry into the cell cycle and induction of the full range of effector responses characteristic of interaction with an antigen-presenting cell.

A dimer is the minimal oligomer size required for activation of HA1.7 T cells by soluble MHC oligomers, as judged by upregulation of the early activation marker CD69 and the low-affinity IL-2 receptor CD25, and internalization of activated TCR CD3 subunits. Monomers were inactive in all assays, although they were shown to bind to T cells in an antigen-specific manner and occupy a substantial fraction of T cell receptors. Although higher valency oligomers were more potent in activating T cells, this increased potency was due to their increased binding avidity. In a previous study (Boniface et al., 1998), the minimal T cell activity shown for streptavidin-linked MHC dimers as compared to trimers and tetramers may have been due to their much lower binding avidity. In our system, quantitative analysis demonstrated that per MHC molecule bound, the dimers were just as effective as the trimers or the tetramers in activating T cells. To the extent that induction of T cell activation by soluble MHC oligomers is representative of T cell activation in general, these results would suggest that bridging two T cell receptor molecules is a necessary and sufficient event for triggering T cell activation and that the bridged TCRs are equally efficient in stimulating T cell activation whether they are in a cluster of two, three, or four receptors.

The sets of dose-response curves for T cell activation by MHC oligomers (Figure 3) are similar to those predicted by a mathematical model of a system responsive to the number of receptor cross-links induced by multivalent ligands of varying sizes (Perelson and DeLisi, 1980; Lauffenburger and Linderman, 1993). In this model, a bound dimer contributes one cross-link, a bound trimer contributes two cross-links, and in general a bound N-mer contributes N-1 cross-links. In Figure 3, the distinct maximum observed in each curve, with decreasing activation observed at high concentration, the dependence of the half-maximal concentration on the oligomer size, and the dependence of the maximum level of activation on the oligomer size are all predicted by this model. What molecular event could correspond to the two "cross-links" induced by binding an MHC trimer? If engaged TCR molecules formed stable dimers, a bound MHC trimer could engage only one cross-linked TCR dimer. However, if TCR dimers were transient species, a bound MHC trimer could serially engage multiple TCR dimers. In this scenario, the T cell responds to the serial dimerization of its receptors on the bound MHC oligomer.

Implications for the Mechanism of T Cell Activation

The results presented in this manuscript can be used to evaluate possible molecular mechanisms for the initial triggering event in the activation of T cells by MHC-peptide complexes. T cell activation was not induced by MHC-peptide monomers, even under conditions where most of the TCR molecules were engaged by MHC-peptide complexes. Thus, processes activated by formation of the MHC-TCR complex per se appear to be ruled out or made less likely as key components of the triggering mechanism. These include conformational (Janeway, 1995), allosteric (Alam et al., 1999), or oligomeric (Reich et al., 1997) changes that may be induced in TCR molecules as a result of binding monomeric MHC-peptide complexes. Similarly, heterooligomerization processes, in which cytoplasmic signaling events are activated by simultaneous binding of a single MHC-peptide complex to a TCR molecule carrying signaling targets (such as CD3 $\gamma\delta\epsilon$ and ζ_2 ITAMs) and to another T cell protein carrying an enzymatic activity (such as CD4-lck) (Chan et al., 1994; Delon et al., 1998), appear to be ruled out as triggering mechanisms by the inability of bound MHC monomers to initiate activation. However, it is conceivable that monomeric binding on a cell surface might initiate partial signals not sufficient to initiate activation processes unless coupled with other stimuli. While CD8 might be participating in heterooligomerization of MHC-TCR in class I MHC-restricted T cell responses reported to be initiated by monomeric engagement (Sykulev et al., 1996; Delon et al., 1998), CD4 does not appear to have such a role in the $CD4^+$ HA1.7 T cells that we investigated here. In this system, our results demonstrate that monomeric engagement alone clearly is not sufficient to initiate T cell activation.

Large scale reorganizations of T cell membrane protein and lipid components have been shown to accompany T cell activation (Monks et al., 1998; Moran and Miceli, 1998; Xavier et al., 1998; Grakoui et al., 1999; Janes et al., 1999; Viola et al., 1999). In one particular model proposed for the coupling of such membrane reorganizations to molecular activation processes, close apposition of T cell and antigen-presenting cell membranes, bridged by the relatively small MHC and TCR molecules, acts to exclude large negative regulators such as the tyrosine phosphatase CD45, resulting in increased receptor phosphorylation (Shaw and Dustin, 1997). Our data would appear to rule out such molecular exclusions as the proximal triggering event, at least for the T cell activation processes investigated here, as it is not likely that the binding of soluble MHC dimers would exclude other proteins from the local region of membrane. If supramolecular receptor clustering and interaction with lipid microdomains do play a key role in the initial signaling processes that we examined, they must occur downstream of dimerization-initiated signaling events and by a mechanism available to soluble oligomers in the absence of costimulatory molecules.

Our results are consistent with a mechanism for an initial activation process in which multivalent engagement of MHC-peptide complexes causes dimerization of TCR molecules (Brown et al., 1993; Germain, 1993) or rearrangement of existing TCR dimers (Fernandez-Miguel et al., 1999). This could occur without significant

conformational change within an individual TCR molecule but rather by intermolecular reorientation, as suggested in a recent structural characterization of MHC-TCR complexes (Ding et al., 1999). Binding of MHC oligomers, or MHC molecules constrained to be in the same membrane as on the surface an antigen-presenting cell, could allow preexisting TCR dimers to reorganize into an active form by allosteric rearrangement of TCR subunits. For either preexisting TCR dimers or ligand-induced dimerization, the binding-dependent rearrangement could promote tyrosine phosphorylation of TCR subunits or transphosphorylation of associated kinases, as observed in other dimerization-responsive systems (Klemm et al., 1998).

At low oligomer concentrations, we observed downregulation of multiple TCR per MHC molecule bound (Figure 6B), in an apparent serial triggering phenomenon as first observed by Valitutti et al. (1995). Serial triggering provides greater sensitivity to the system at lower ligand levels (Valitutti and Lanzavecchia, 1997), which leads to a hyperbolic binding versus activation profile, as observed for CD3 downregulation (Figure 6B). This effect is particularly prominent in the CD69 upregulation profile (Figure 6A). The molecular mechanism for serial engagement by soluble MHC oligomers would appear to involve release and rebinding of TCR by MHC molecules that are constrained to the surface through interactions of other components in the same oligomer. We note that such a mechanism would allow "herding" of receptors into an activated cluster and might be similar to processes occurring at the juxtaposed membranes of an antigen-presenting cell and a T cell.

In conclusion, we have used a series of chemically defined oligomers of MHC-peptide complexes to probe the requirements for T cell activation. We find that monomers are unable to activate T cells even under conditions where they engage a substantial fraction of available T cell receptors, whereas MHC dimers, trimers, and tetramers all activate efficiently. The extent and nature of the activation signal induced by binding of these oligomers appears to be equivalent regardless of the oligomer size, suggesting that an initial step in the T cell activation mechanism is the formation or rearrangement of a dimer of T cell receptor molecules, and that a T cell responds to the number of TCR cross-links induced at the surface.

Experimental Procedures

MHC-Peptide Complexes

Soluble MHC-peptide complexes were produced by expression of the extracellular portions of *HLA-DRA1**0101 (α1–182) and *HLA-DRB1**0101 (β1–190) (SwissProt: P01903, P13758) in *E. coli* followed by folding in vitro in the presence of peptide (Frayser et al., 1999). Antigenic peptide Ha[306–318] (PKYVKQNTLKLAT), derived from influenza virus hemagglutinin, and control endogenous peptide HLA-A2[103–117] (VGSDWRFLRGYHQYA), were synthesized using FMOC chemistry, purified by reverse phase HPLC, and verified by mass spectrometry (MALDI-TOF). For cross-linking, Ala–Cys sequences were added individually after the "connecting peptide" regions α183–192 or β191–198. Cysteine modified complexes were purified in the presence of 5 mM dithiothreitol, which was removed immediately prior to cross-linking. The introduced cysteine residues are uniquely reactive, as the other seven HLA-DR1 cysteines are involved in disulfide bonds (α108–164, β16–79, β118–174) or sequestered from solvent (β30) by the bound peptide and are unreactive with thiol-specific maleimide or pyridyl disulfide probes (J. R. C., unpublished data). The introduced cysteines undergo facile reaction with thiol-specific reagents, allowing specific cross-linking at the α or β subunit termini.

Peptide-Based Cross-Linking Reagents

Peptides were synthesized by FMOC chemistry using an Advanced ChemTech 357 FBS combinatorial peptide synthesizer. After synthesis, the N terminus was capped by reaction with fluorescein isothiocyanate (FITC) or a long-chain succinimide ester of biotin (NHS-LC-LC-biotin, Pierce). Peptides were purified by reverse phase HPLC using a C4 or C18 column (Vydac) and verified using MALDI-TOF. Purified peptides were reacted through the ϵ-amino group of lysine residues with either *N*-[ϵ-Maleimidocaproyloxy]succinimide ester (EMCS, Pierce) or succinimidyl-4-[*N*-maleimidomethyl]-cyclohexane-1-carboxy-[6- amidocaproate] (LC-SMCC, Pierce) by dissolving excess reagent in DMF and adding it to peptide in 10 mM Na-phosphate buffer (pH 7) and 150 mM NaCl. After 1.5 hr at room temperature, the modified peptide was purified by reverse-phase HPLC. The presence of the appropriate number of maleimide functional groups to make dimers, trimers, and tetramers was confirmed by MALDI-TOF. Estimated maleimide-to-maleimide distances for the cross-linkers in a fully extended conformation would be approximately 20 Å for the dimeric cross-linker and 28 Å for the trimeric and tetrameric cross-linkers.

Cross-Linking of Soluble MHC-Peptide Complexes

Chemical cross-links were made through the introduced sulfhydryl group on the β chain of the MHC-peptide complex and the thiol-specific maleimide group on the peptide-based cross-linkers. Cross-linker was titrated step-wise into purified cysteine-modified HLA-DR1 in 10 mM Na-phosphate buffer (pH 7), 150 mM NaCl, and 5 mM EDTA. Extent of cross-linking, measured by SDS-PAGE, varied from approximately 60% for the dimers to 25% for the tetramers. Cross-linked MHC-peptide complexes were isolated by gel filtration using two Superdex 200 FPLC columns (Pharmacia) in series. Purity was determined by 10% SDS-PAGE.

Streptavidin-Based Oligomers

For R-phycoerythrin-labeled streptavidin-linked (SA-PE) tetramers, the free sulfhydryl on the α chain of the DR1 peptide complex was reacted with biotin PEO-maleimide (Pierce) and the excess biotin removed by gel filtration. The biotinylated MHC-peptide complex was then reacted in a slight molar excess with R-PE-labeled streptavidin (Sigma) to give a mixture of mostly tetramer and trimer. For higher order streptavidin-linked oligomers, a biotinylated dimer of MHC-peptide complexes was made using the peptide-based cross-linker shown in Figure 1B and the free sulfhydryl on the α chain. Biotinylated dimer was separated from free monomer and cross-linker using Superdex 200 FPLC and was reacted with streptavidin as above. The effective oligomer size of the streptavidin-linked oligomer of MHC dimers was approximately six, as estimated by extrapolation of the binding IC_{50} versus oligomer size dependence (see below). Estimated maleimide-to-biotin distances are approximately 35 Å for the biotinylated dimer.

MHC-Peptide Monomers

In activation and competitive binding experiments, we used soluble HLA-DR1 that did not carry an introduced cysteine residue. In preparations of monomers carrying the introduced cysteine, we could not avoid adventitious dimerization of a small fraction of molecules ($<$1%) due to air oxidation and disulfide bond formation, which complicated activation experiments at high monomer concentrations. For the direct binding experiments, FITC-labeled monomer was prepared by reaction of HLA-DR1 carrying an α chain free cysteine with fluorescein-maleimide (Pierce) followed by purification with gel filtration chromatography. Any possible dimerized fraction in this preparation would not be labeled.

T Cell Clones

The human $CD4^+$ T cell clone HA1.7 (Lamb et al., 1982) was cultured in RPMI, 5% human serum, 5% fetal bovine serum, 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. T cells were rested a minimum of 12 days before use in activation assays.

T Cell Activation Assays

MHC-peptide oligomers were added to 7.5×10^4 HA1.7 T cells in round-bottom 96-well plates and incubated at 37°C, 5% CO_2. After the indicated times, cells were placed in ice and stained concurrently with R-phycoerythrin-labeled CD3 (UCHT-1, Sigma) and either allophycocyanin-labeled CD69 or CD25 (FN50 or M-A251, PharMingen) for 1 hr 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 FACScalibur flow cytometer and analyzed using Cell Quest software. Number of CD3 molecules per cell was converted from mean fluorescence PE using SPHERO Rainbow Calibration particles (Spherotech) containing known amounts of PE equivalents.

Oligomer Binding Assay

For the direct binding assay, levels of cell-associated fluorescence were measured simultaneously with the activation markers by multicolor flow cytometry using the fluorescein labels incorporated into the synthetic cross-linkers or monomeric DR1. Mean fluorescence intensity was converted to numbers of fluorescein molecules per cell using SPHERO Rainbow Calibration particles (Spherotech). For comparison with activation levels, concentrations of bound oligomers were converted to concentration of bound MHC molecules using the known valency of the cross-linkers. Fluorescence levels in this assay reflect the number of molecules bound at the cell surface as well as those taken up into the cell (T. O. C., unpublished data). Fluorescence signals from molecules internalized to acidic compartments may have reduced intensity due to the intrinsic pH dependence of fluorescein fluorescence intensity. This effect was not considered in the calibration procedure but is presumed to be equivalent for all of the oligomers.

For the competition assay, various concentrations of MHC oligomers or unlabeled MHC monomer were added to 7.5×10^4 HA1.7 T cells containing a constant amount of phycoerythrin-labeled SA tetramer (SA-PE, 35 nM) in 96-well round-bottom plates and incubated for 3 hr at 37°C, 5% CO_2. Fluorescence arising from bound SA-PE was measured by flow cytometry. IC_{50} values were determined as the concentration of unlabeled oligomer required to reduce the level of SA-PE tetramer staining by one-half. For the chemically defined MHC oligomers and monomer, the IC_{50} values were logarithmically related to the oligomer size n as follows: $-\log IC_{50} = 0.54n + 4.84$ ($R = 0.986$). Upon extrapolation of this dependence, the measured IC_{50} for the SA-linked oligomer of MHC dimers yields an effective oligomer size of six (5.4 ± 0.8).

Acknowledgments

We thank Glen Paradis and the MIT Flow Cytometry Core Facility for expert assistance and advice in flow cytometry; Jonathan Lamb for the HA1.7 T cell clone; L. Wedderburn, L. Korb, and S. Sadegh-Nasseri for advice and materials used in growing T cell clones; J. Lubetsky for construction of HLA-DR1 carrying cysteine mutations; and J. Zarutskie for modeling the peptide-based cross-linkers. Supported by NSF-MCB-9506893 (L. J. S.), NIH-N01AI95361 (L. J. S.), and NIH-T32 GM08334 (J. R. C. and T. O. C.).

Received September 29, 1999; revised January 28, 2000.

References

Abastado, J.P., Lone, Y.C., Casrouge, A., Boulot, G., and Kourilsky, P. (1995). Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. J. Exp. Med. *182*, 439–447.

Alam, S.M., Davies, G.M., Lin, C.M., Zal, T., Nasholds, W., Jameson, S.C., Hogquist, K.A., Gascoigne, N.R., and Travers, P.J. (1999). Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. Immunity *10*, 227–237.

Alberola-Ila, J., Takaki, S., Kerner, J.D., and Perlmutter, R.M. (1997). Differential signaling by lymphocyte antigen receptors. Annu. Rev. Immunol. *15*, 125–54.

Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96.

Bekoff, M., Kubo, R., and Grey, H.M. (1986). Activation requirements for normal T cells: accessory cell-dependent and -independent stimulation by anti-receptor antibodies. J. Immunol. *137*, 1411–1419.

Boniface, J.J., Rabinowitz, J.D., Wulfing, C., Hampl, J., Reich, Z., Altman, J.D., Kantor, R.M., Beeson, C., McConnell, H.M., and Davis, M.M. (1998). Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. Immunity *9*, 459–466.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature *364*, 33–39.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annu. Rev. Immunol. *14*, 259–274.

Caruso, A., Licenziati, S., Corulli, M., Canaris, A.D., De Francesco, M.A., Fiorentini, S., Peroni, L., Fallacara, F., Dima, F., Balsari, A., and Turano, A. (1997). Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. Cytometry *27*, 71–76.

Casares, S., Zong, C.S., Radu, D.L., Miller, A., Bona, C.A., and Brumeanu, T.D. (1999). Antigen-specific signaling by a soluble, dimeric peptide/major histocompatibility complex class II/Fc chimera leading to T helper cell type 2 differentiation. J. Exp. Med. *190*, 543–554.

Chan, A.C., Desai, D.M., and Weiss, A. (1994). The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. Annu. Rev. Immunol. *12*, 555–592.

Constant, S.L., and Bottomly, K. (1997). Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu. Rev. Immunol. *15*, 297–322.

Crawford, F., Kozono, H., White, J., Marrack, P., and Kappler, J. (1998). Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. Immunity *8*, 675–682.

Davis, M.M., Boniface, J.J., Reich, Z., Lyons, D., Hampl, J., Arden, B., and Chien, Y. (1998). Ligand recognition by alpha beta T cell receptors. Annu. Rev. Immunol. *16*, 523–544.

Delon, J., Gregoire, C., Malissen, B., Darche, S., Lemaitre, F., Kourilsky, P., Abastado, J.P., and Trautmann, A. (1998). CD8 expression allows T cell signaling by monomeric peptide-MHC complexes. Immunity *9*, 467–473.

Ding, Y.H., Baker, B.M., Garboczi, D.N., Biddison, W.E., and Wiley, D.C. (1999). Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. Immunity *11*, 45–56.

Dutton, R.W., Bradley, L.M., and Swain, S.L. (1998). T cell memory. Annu. Rev. Immunol. *16*, 201–223.

Fernandez-Miguel, G., Alarcon, B., Iglesias, A., Bluethmann, H., Alvarez-Mon, M., Sanz, E., and de la Hera, A. (1999). Multivalent structure of an alpha-beta T cell receptor. Proc. Natl. Acad. Sci. USA *96*, 1547–1552.

Fox, B.S., Quill, H., Carlson, L., and Schwartz, R.H. (1987). Quantitative analysis of the T cell response to antigen and planar membranes containing purified Ia molecules. J. Immunol. *138*, 3367–3374.

Frayser, M., Sato, A.K., Xu, L., and Stern, L.J. (1999). Empty and peptide-loaded class II major histocompatibility complex proteins produced by expression in Escherichia coli and folding in vitro. Protein Expr. Purif. *15*, 105–114.

Germain, R.N. (1994). MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell *76*, 287–299.

Germain, R.N. (1993). Seeing double! Curr. Biol. *3*, 586–589.

Germain, R.N., and Stefanova, I. (1999). The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu. Rev. Immunol. *17*, 467–522.

Glimcher, L.H., and Singh, H. (1999). Transcription factors in lymphocyte development—T and B cells get together. Cell *96*, 13–23.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., and Dustin, M.L. (1999). The immunological synapse: a molecular machine controlling T cell activation. Science *285*, 221–227.

Hamad, A.R., O'Herrin, S.M., Lebowitz, M.S., Srikrishnan, A., Bieler, J., Schneck, J., and Pardoll, D. (1998). Potent T cell activation with dimeric peptide-major histocompatibility complex class II ligand: the role of CD4 coreceptor. J. Exp. Med. *188*, 1633–1640.

Hara, T., Jung, L.K., Bjorndahl, J.M., and Fu, S.M. (1986). Human T cell activation. III. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-o- tetradecanoyl phorbol-13-acetate, mitogens, and antigens. J. Exp. Med. *164*, 1988–2005.

Irving, B.A., and Weiss, A. (1991). The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. Cell *64*, 891–901.

Itoh, Y., Hemmer, B., Martin, R., and Germain, R.N. (1999). Serial TCR engagement and down-modulation by peptide:MHC molecule ligands: relationship to the quality of individual TCR signaling events. J. Immunol. *162*, 2073–2080.

Janes, P.W., Ley, S.C., and Magee, A.I. (1999). Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J. Cell Biol. *147*, 447–461.

Janeway, C.A., Jr. (1995). Ligands for the T-cell receptor: hard times for avidity models. Immunol. Today *16*, 223–225.

Klemm, J.D., Schreiber, S.L., and Crabtree, G.R. (1998). Dimerization as a regulatory mechanism in signal transduction. Annu. Rev. Immunol. *16*, 569–592.

Kozono, H., White, J., Clements, J., Marrack, P., and Kappler, J. (1994). Production of soluble MHC class II proteins with covalently bound single peptides. Nature *369*, 151–154.

Lamb, J.R., Eckels, D.D., Lake, P., Woody, J.N., and Green, N. (1982). Human T-cell clones recognize chemically synthesized peptides of influenza haemagglutinin. Nature *300*, 66–69.

Lauffenburger, D.A., and Linderman, J.J. (1993). Physical aspects of receptor/ligand binding and trafficking processes. In Receptors: Models for Binding, Trafficking, and Signaling (New York: Oxford University Press), pp. 133–180.

Letourneur, F., and Klausner, R.D. (1991). T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor zeta family proteins. Proc. Natl. Acad. Sci. USA *88*, 8905–8909.

Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. Nature *395*, 82–86.

Moran, M., and Miceli, M.C. (1998). Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. Immunity *9*, 787–796.

Mueller, D.L., Jenkins, M.K., and Schwartz, R.H. (1989). Clonal expansion versus functional clonal inactivation: a costimulatory signaling pathway determines the outcome of T cell antigen receptor occupancy. Annu. Rev. Immunol. *7*, 445–480.

Perelson, A.S., and DeLisi, C. (1980). Receptor clustering on a cell surface. I. Theory of receptor crosslinking by ligands bearing two chemically identical functional groups. Math. Biosci. *48*, 71–110.

Qian, D., and Weiss, A. (1997). T cell antigen receptor signal transduction. Curr. Opin. Cell Biol. *9*, 205–212.

Quill, H., and Schwartz, R.H. (1987). Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J. Immunol. *138*, 3704–3712.

Reich, Z., Boniface, J.J., Lyons, D.S., Borochov, N., Wachtel, E.J., and Davis, M.M. (1997). Ligand-specific oligomerization of T-cell receptor molecules. Nature *387*, 617–620.

Romeo, C., Amiot, M., and Seed, B. (1992). Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor zeta chain. Cell *68*, 889–897.

Rudd, C.E. (1999). Adaptors and molecular scaffolds in immune cell signaling. Cell *96*, 5–8.

Shaw, A.S., and Dustin, M.L. (1997). Making the T cell receptor go the distance: a topological view of T cell activation. Immunity *6*, 361–369.

Stern, L.J., Brown, J.H., Jardetzky, T.S., Gorga, J.C., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1994). Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature *368*, 215–221.

Sykulev, Y., Joo, M., Vturina, I., Tsomides, T.J., and Eisen, H.N. (1996). Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity *4*, 565–571.

Symer, D.E., Dintzis, R.Z., Diamond, D.J., and Dintzis, H.M. (1992). Inhibition or activation of human T cell receptor transfectants is controlled by defined, soluble antigen arrays. J. Exp. Med. *176*, 1421–1430.

Testi, R., D'Ambrosio, D., De Maria, R., and Santoni, A. (1994). The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol. Today *15*, 479–483.

Ullman, K.S., Northrop, J.P., Verweij, C.L., and Crabtree, G.R. (1990). Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. Annu. Rev. Immunol. *8*, 421–452.

Valitutti, S., and Lanzavecchia, A. (1997). Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. Immunol. Today *18*, 299–304.

Valitutti, S., Muller, S., Cella, M., Padovan, E., and Lanzavecchia, A. (1995). Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature *375*, 148–151.

van Schooten, W.C., Maiore, T., Jones, K.R., and Paborsky, L. (1992). Purified HLA class II peptide complexes can induce adherence and activation of peptide-specific human T cell clones. J. Immunol. *148*, 1043–1048.

Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science *283*, 680–682.

Waldmann, T.A. (1989). The multi-subunit interleukin-2 receptor. Annu. Rev. Biochem. *58*, 875–911.

Wange, R.L., and Samelson, L.E. (1996). Complex complexes: signaling at the TCR. Immunity *5*, 197–205.

Watts, T.H. (1988). T cell activation by preformed, long-lived Ia-peptide complexes. Quantitative aspects. J. Immunol. *141*, 3708–3714.

Watts, T.H., Brian, A.A., Kappler, J.W., Marrack, P., and McConnell, H.M. (1984). Antigen presentation by supported planar membranes containing affinity-purified I-Ad. Proc. Natl. Acad. Sci. USA *81*, 7564–7568.

Wettstein, D.A., Boniface, J.J., Reay, P.A., Schild, H., and Davis, M.M. (1991). Expression of a class II major histocompatibility complex (MHC) heterodimer in a lipid-linked form with enhanced peptide/soluble MHC complex formation at low pH. J. Exp. Med. *174*, 219–228.

Xavier, R., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998). Membrane compartmentation is required for efficient T cell activation. Immunity *8*, 723–732.

Zanders, E.D., Lamb, J.R., Feldmann, M., Green, N., and Beverley, P.C. (1983). Tolerance of T-cell clones is associated with membrane antigen changes. Nature *303*, 625–627.