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Soluble, High-Affinity Dimers of T-Cell Receptors and Class II Major Histocompatibility Complexes: Biochemical Probes for Analysis and Modulation of Immune Responses

Michael S. Lebowitz,*[,1] Sean M. O'Herrin,*[,1,2] Abdel-Rahim A. Hamad,† Tarek Fahmy,‡ Didier Marguet,‡ Nicholas C. Barnes,* Drew Pardoll,†[,‡] Joan G. Bieler,* and Jonathan P. Schneck*

**Department of Pathology and †Department of Oncology, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, Maryland; and ‡Department of Biology, Johns Hopkins University, Baltimore, Maryland 21205*

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T cell receptors (TCR) and major histocompatibility complex (MHC) molecules are integral membrane proteins that have central roles in cell-mediated immune recognition. Therefore, soluble analogs of these molecules would be useful for analyzing and possibly modulating antigen-specific immune responses. However, due to the intrinsic low-affinity and inherent solubility problems, it has been difficult to produce soluble high-affinity analogs of TCR and class II MHC molecules. This report describes a general approach which solves this intrinsic low-affinity by constructing soluble divalent analogs using IgG as a molecular scaffold. The divalent nature of the complexes increases the avidity of the chimeric molecules for cognate ligands. The generality of this approach was studied by making soluble divalent analogs of two different classes of proteins, a TCR (2C TCR_2Ig) and a class II MHC ($^{MCC}I\text{-}E^k{}_2Ig$) molecule. Direct flow cytometry assays demonstrate that the divalent 2C TCR_2Ig chimera retained the specificity of the native 2C TCR, while displaying increased avidity for cognate peptide/MHC ligands, resulting in a high-affinity probe capable of detecting interactions that heretofore have only been detected using surface plasmon resonance. TCR_2IgG was also used in immunofluorescence studies to show ER localization of intracellular peptide–MHC complexes after peptide feeding. $^{MCC}I\text{-}E^k{}_2Ig$ chimeras were able to both stain and activate an MCC-specific T cell hybridoma. Construction and expression of these two diverse heterodimers demonstrate the generality of this approach. Furthermore, the increased avidity of these soluble divalent proteins makes these chimeric molecules potentially useful in clinical settings for probing and modulating *in vivo* cellular responses. © 1999 Academic Press

INTRODUCTION

Specificity of recognition is a cardinal feature of the immune system. This specificity is dictated, at the molecular level, by the interactions between T cell receptors (TCR) on T cells and peptide/major histocompatibility complexes (pepMHC) on antigen-presenting cells. A variety of assays including both site-directed mutagenesis and crystal structure analyses indicate that specific TCR recognition of pepMHC is based on direct contacts with both the MHC and the antigenic peptide it binds (1–3). This recognition is the first step in the initiation of the T cell arm of the immune response. Therefore, TCR/pepMHC interactions are crucial in influencing effector T cell responses.

Reagents able to detect and/or regulate cells bearing specific TCR or peptide/MHC complexes would be of great utility given the centrality of TCR–pepMHC interactions in antigen-specific cell-mediated immunity. Inherent specificity and the potential ability to engage cognate ligands in a physiologically relevant manner suggest that soluble analogs of TCR and pepMHC complexes could be ideal probes to study immune responses. However, there are two problems with the generation and use of soluble analogs of TCR and class II MHC molecules. First, soluble analogs of TCR and class II MHC are difficult to produce since these protein complexes consist of two polypeptides each of which have transmembrane domains. These transmembrane domains are important in facilitating pairing and hence proper folding of the α and β polypeptides. The second problem with the use of soluble analogs of TCR and class II MHC molecules is that the ability to use these probes is limited by their intrinsic low affinity.

Despite the technical difficulties involved in generating such complexes, in several instances soluble complexes have been produced (4–12). However, the utility of these probes is limited by their intrinsic low

[1] These authors both contributed equally to the work.
[2] Current address: University of Chicago, AMB S-315, MC 1089, 5841 S. Maryland Ave., Chicago, IL 60637.

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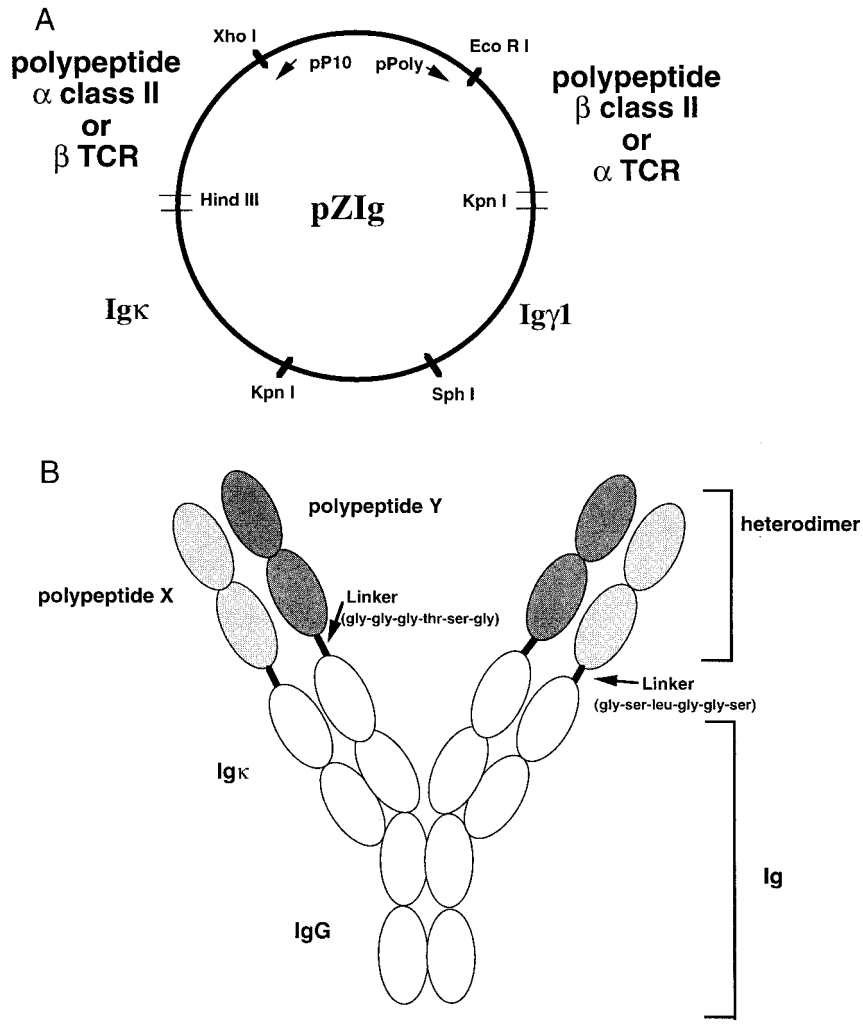

FIG. 1. Expression vector and theoretical structure of X/Ig chimeric polypeptide chains. (A) In the generic cloning vector, DNA-encoding α and β chains of extracellular domains of either class II or TCR proteins were ligated to DNA-encoding Ig heavy and light chains using the restriction endonuclease sites shown. Subsequently, these chimeras were ligated sequentially to a modified pAcUW51 vector (18). (B) Theoretical structure of a soluble, divalent Ig chimeric complex. (C) Computer-generated model of the peptide backbone of 2C TCR_2Ig. The model is based on the crystal structures of an intact IgG1 (42) and the 2C TCR (3). The six amino acid linkers have been modeled into the structure. The IgG1 heavy chains are shown in green; the light chains, in yellow; the 2C TCR α chains, in blue; the 2C TCR β chains, in red; and the linker peptides, in white. The arrows point to (from top to bottom) the pepMHC-binding site on the TCR, the linker region, and the IgG hinge region.

affinity for cognate ligands. In contrast, due to their increased avidity, multivalent constructs of TCR or peptide/MHC are valuable probes for detection of their ligands (13–16).

In this report we describe the construction and activity of soluble, divalent TCR and MHC class II analogs. Biochemical and biological analysis of these soluble, divalent TCR (TCR_2Ig) and MHC class II (class II_2Ig) analogs suggests that they may be of great utility as specific, high-affinity probes of the immune system. Furthermore, the potentially broad applicability of this expression system for making divalent, soluble analogs of heterodimeric integral membrane proteins with increased avidity for cognate ligand suggest its use in a range of biological systems.

MATERIAL AND METHODS

Construction of the Soluble Divalent Molecules

The genes encoding the chimeric 2C TCR_2Ig molecule were constructed by inserting cDNA encoding the extracellular domains of the TCR α and β chains upstream of cDNA encoding the murine IgG1 heavy, 93G7, and light chain, 91A3, respectively (Fig. 1A) (17). A *Hin*dIII restriction enzyme site and linker were inserted immediately 5′ of the codon for Asp at the start of the mature κ protein in clone 91A3. A *Kpn*I restriction enzyme site was introduced 3′ of the stop codon in the κ polypeptide. A *Kpn*I restriction site and linker were inserted immediately 5′ of the codon for Glu lo-

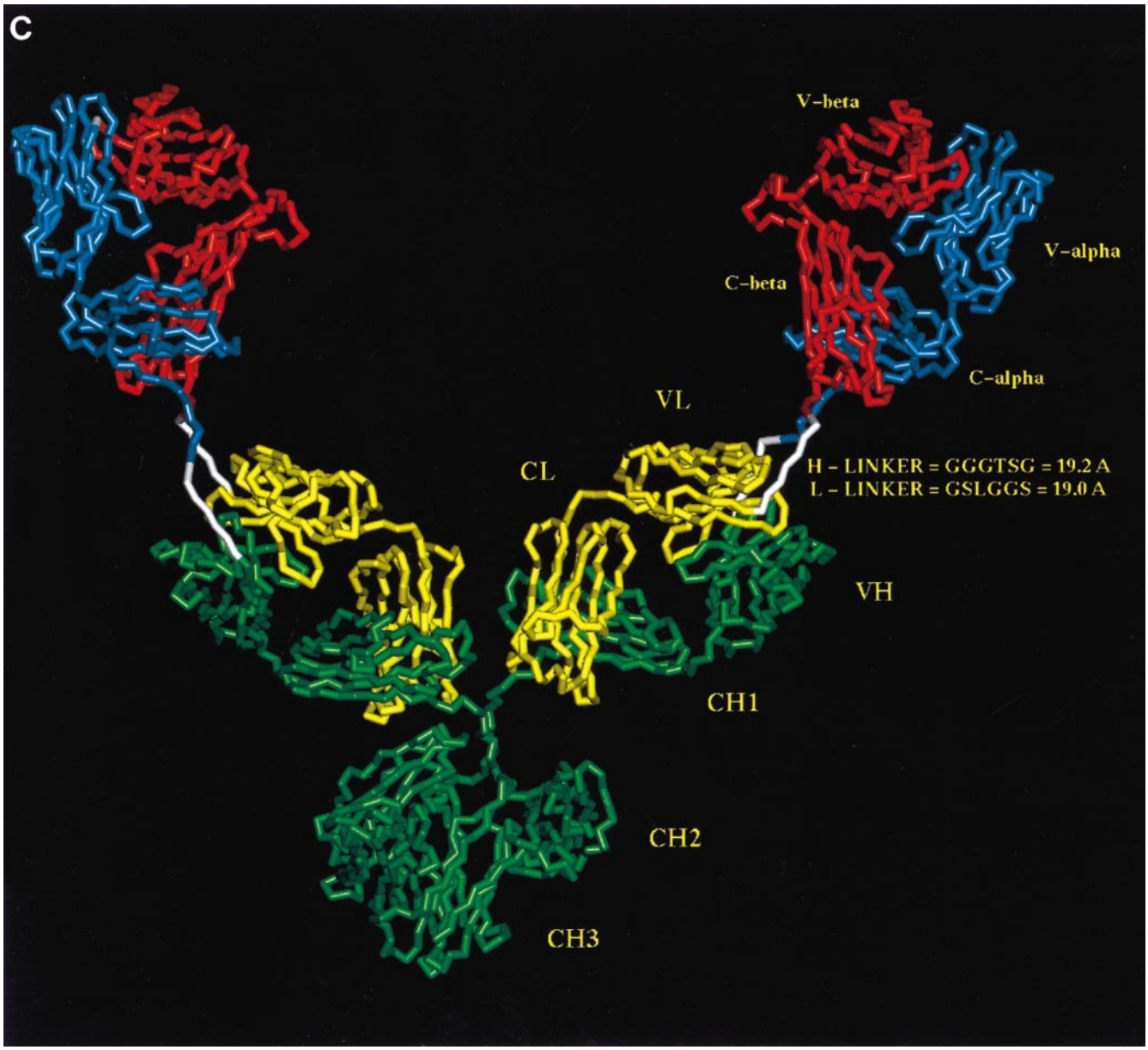

FIG. 1—*Continued*

cated at the start of the mature Igγ_1 polypeptide in clone 93G7. An *Sph*I restriction site was introduced 3′ to the stop codon in the γ_1 polypeptide.

The genes encoding the 2C TCR α and β chains have previously been described (4). A *Kpn*I restriction site and linker was inserted immediately 3′ to the codon for the Gln residue at the interface between the extracellular and transmembrane domains of the 2C TCR α polypeptide. The 5′ regions of the gene already expressed an appropriate restriction enzyme endonuclease site, *Eco*RI. A *Xho*1 site was introduced 5′ to the start of the signal sequence in the 2C TCR β chain and a *Hin*dIII restriction enzyme endonuclease site immediately 3′ to the codon for the Ile residue at the interface between the extracellular and transmembrane domains of the β polypeptide. In the construction of the chimeric proteins, linkers of six amino acid residues were introduced at the junctions between the end of the TCR α and β and the mature γ and κ polypeptides, respectively (Fig. 1B).

A similar approach was used to modify the genes encoding the I-E α and β chains. A *Kpn*I restriction site and linker was inserted immediately 3′ to the interface between the extracellular and transmembrane domains of the I-E β polypeptide. The 5′ regions of the gene had already been modified to encode the MCC peptide (18) and also already expressed an *Eco*RI site. The I-Eα chain was modified by introduction of a *Hin*dIII

restriction enzyme endonuclease site immediately 3′ to the codon at the interface between the extracellular and transmembrane domains.

The baculovirus expression vector used was previously described (18). This vector has two separate viral promoters, polyhedron and P10, allowing simultaneous expression of both chimeric polypeptide chains in the same cell (Fig. 1A). The expression vector was digested with *Xho*I and *Kpn*I and 2C TCR_β/Ig_κ or I-E_α/Igκ was inserted downstream of the P10 promoter (Fig. 1A). Subsequently, the 2C TCR_α/Igγ1 or ^{MCC}I-E_β/Igγ1 was inserted into *Eco*RI/*Sph*I sites downstream of the polyhedron promoter. The signal sequence for 2C TCR_2IgG and ^{MCC}I-$E^k{}_2$IgG, ER localization came from the endogenous signal sequences associated with the native TCR and I-E proteins, respectively.

Mutagenesis

For mutagenesis cDNA encoding the individual polypeptides were subcloned into pSP72 and pSP73 vectors (Promega, Madison, WI). Oligonucleotide-directed mutagenesis was performed using the Chameleon kit (Stratagene, La Jolla, CA). All mutations were confirmed by sequencing.

Oligo nucleotides used to introduce the above mutations were 5′ IgG1 mutation, ctgtcagtaactgcaggtgtccactctggtaccagcggtgaggttcagcttcagcagtctggagc; 3′ IgG1 mutation, agcctctcccactctcctggtaaatgagcatgctctcagtgtccttggagccctctggtc; 5′ Igk mutation, ctgttgctctgttttcaaggtaccaggtgtggaagcttgggaggatctgatatccagatgacgcaaatccatcc; 3′ Igk mutation, gtcaagagcttcaacaggaatgagtgttagggtaccagacaaaggtcctgagacgccaccaccagc; 3′ 2C TCR α mutation, cagatatgaacctaaactttcaaggaggaggtacctgtcagttatgggactccgaatc; 5′ 2C TCR β mutation, ccaaagagaccagtatcctgactcgaggaagcatgtctaacactgccttc; 3′ 2C TCRβ mutation, ctgcaaccatcctctatgagatcggaagcttaggatctggtacctactggggaaggccaccctatatgc; 3′ I-$E^d\alpha$ mutation, ggtagcgaccggcgctcagctggaattcaagcttccattctctttagtttctgggaggaggt; and 3′ I-$E^k\beta$ mutation, gcacagtccacatctgcacagaacaagggaggaggtaccggggatccggttattagtacatttattaag.

Molecular Modeling

The 2C TCR_2IgG structure was modeled and linkers regions regularized using Quanta 4.1/CHARMm (Molecular Simulations Inc.). Coordinates for the IgG1 were obtained from the PDB (1IgGY) and for the 2C TCR (1TCR), respectively.

Biochemical Analysis of Chimeras

The conformational integrity of the chimeric molecules was assayed by ELISA using antibodies specific for each moiety of the protein. The primary antibody used was specific for murine IgG1 Fc. The secondary antibody used was either a biotinylated H57, a hamster mAb specific for a conformational epitope expressed on the β chain of murine TCR (19), or 1B2 (20, 21), a murine mAb specific for a clonotypic epitope expressed on 2C TCR. I-$E^k{}_2$Ig was assayed using biotinylated 14.4.4, an anti-I-E_α chain-specific mAb, as the secondary antibody. SDS–PAGE analysis of the chimeric protein was preformed as described (22). Samples were electrophoresed through a 10% SDS–polyacrylamide gel.

Peptide Loading of Cells, Flow Microfluorometry, and Immunohistochemistry

RMA-S L^d cells, a cell line that is defective in antigen processing and expresses functionally "empty" class I MHC on the cell surface, were loaded with peptides as described (22). For analysis of 2C TCR_2IgG, RMA-S L^d cells were loaded with high-affinity cognate peptides, QL9 (QLSPFPFDL); intermediate-affinity cognate peptides, p2Ca (LSPFPFDL); and p2Ca-substituted peptides that have previously been characterized with varying affinities for 2C TCR (23). The p2Ca substituted peptides include: A1 (ASPFPFDL), A2 (LAPFPFDL), A3 (LSAFPFDL), A4 (LSPAPFDL), A5 (LSPFAFDL), A7 (LSPFPFAL), D1 (DSPFPFDL), L4 (LSPLPFDL), and Y4 (LSPYPFDL), peptides. Approximately 1×10^6 cells, either peptide-loaded or not loaded, were incubated for 60 min at 4°C with 30 μl of the anti-L^d-specific mAb, 30.5.7 (10μg/ml) culture supernatants, or 2C TCR_2IgG culture supernatants, 10 μg/ml final concentration. Cells were washed twice, stained with fluorescent phycoerythrin-labeled $F(ab')_2$ goat anti-mouse IgG (Cappel Laboratories, Costa Mesa, CA), and analyzed by flow cytometry.

For staining of MCC-specific hybridoma cells, ^{MCC}I-$E^k{}_2$Ig (5 μg) was incubated with 1×10^6 cells for 1 h at 4°C and washed, followed by incubation with goat anti-mouse IgG_1 conjugated to RPE for an additional 1 h. Hybridoma cells were then washed 2× and analyzed by flow cytometry.

For immunohistochemistry experiments, L^d-expressing L cells were plated on coverslips overnight. Cells were incubated with peptides QL9 or MCMV (100 μM) for 1 h, washed with HBSS, and fixed in 4% PFA for 30 min. Slides were quenched in 0.25% ammonium chloride for 5 min, fixed in methanol/acetone, and blocked with 1% BSA overnight. For staining 2C TCR_2IgG was "airfuged," 80K for 1 h, and diluted to 10 μg/ml in 1% BSA. Coverslips were incubated with 2C TCR_2IgG for 90 min at 4°C, washed, and then incubated with Cy-3-conjugated donkey anti-mouse Ig.

T Cell Stimulation Assay

Various concentrations of soluble ^{MCC}I-$E^k{}_2$Ig or the murine anti CD3-mAb, 2C11, were immobilized on sterile Immulon 4 plates (Dynatech, Burlington, MA) overnight at 4°C. Following two washes, either the MCC-specific 5KC cells or the control ovalbumin-specific DO11.10 cells

(1×10^5/well) were added in 250 μl of culture medium and incubated for 20–24 h at 37°C. IL-2 was measured by ELISA (R&D, Minneapolis, MN).

RESULTS

Construction of a Vector for Expressing Soluble Divalent Analogs of Heterodimeric Transmembrane Proteins

Characteristics of a general system for expression of soluble divalent analogs of heterodimeric proteins include relative simplicity and broad applicability. To accomplish this, IgG was chosen as a molecular scaffold because it is divalent by nature and can be simply modified to serve as a molecular scaffold (13, 24–26). Of further advantage is the fact that the IgG scaffold should facilitate subunit pairing, folding, secretion, and stability of the covalently linked heterodimeric polypeptides. cDNA encoding the Ig heavy and light chains was modified immediately 5′ of the coding region for the mature variable domains on the heavy and light chain polypeptides, respectively (Fig. 1). These modifications allow for genetic linkage of the cDNA encoding the polypeptides of interest to the amino terminal-encoding regions of the mature immunoglobulin polypeptides. Polypeptide X is attached via a short six amino acid linker (GSLGGS) to the amino terminal end of the variable region of the Igκ chain, while polypeptide Y is attached via another six amino acid linker (GGGTSG) to the amino terminus of the variable region of the Igγ chain (see Fig. 1B for a schematic representation of the chimeric molecule.)

We analyzed the utility of the Ig scaffold for producing two classes of heterodimers, TCR and class II α,β heterodimers. The TCR heterodimer was derived from the well-characterized alloreactive, class I-specific 2C CTL clone (21, 27) which is specific for a naturally processed endogenous peptide, p2Ca, derived from α-ketoglutarate dehydrogenase bound by the murine class I molecule H-2 L^d (27). This system was of interest since both higher affinity, peptide QL9, and lower affinity, peptide variants of p2Ca have been characterized (see Materials and Methods) (23, 28, 29). The class II MHC heterodimer was derived from the murine class II molecule I-E^k that has been previously modified to also encode a nominal peptide antigen derived from moth cytochrome C (18, 30). Soluble divalent TCR (2C TCR_2Ig) was generated by linking cDNA encoding the extracellular domains of TCR α or β chains to cDNA encoding Igγ1 heavy and κ light chain polypeptides, respectively (Fig. 1A). For expression of soluble divalent class II MHC molecules (^{MCC}I-$E^k{}_2$IgG), cDNAs encoding the extracellular domains of ^{MCC}I-$E^k{}_\beta$ and I-Eα chains were genetically linked to cDNA encoding the Igγ_1 heavy and κ light chain polypeptides, respectively. The constructs were cloned into a dual promoter baculovirus expression vector (Fig. 1A) (18).

Computer modeling of the chimeric 2C TCR_2Ig protein indicates several interesting features (Fig. 1C). First, assuming an extended linker, there is no steric hindrance imposed by linking the TCR molecule to the Ig polypeptides. The approximate mean distance between the TCR and Ig polypeptides is 22 Å. There is also a fair amount of flexibility associated with the linker which is probably critical for its function. In addition the model highlights the importance of the Ig hinge region in conferring flexibility in binding of cognate cell surface ligands.

Biochemical Characterization of Divalent Chimeras

Expression and conformational integrity of the chimeric molecules was assayed by ELISA. Cells infected with baculovirus viral stocks containing the transfer vectors encoding the 2C TCR_2IgG and ^{MCC}I-$E^k{}_2$IgG constructs secrete 0.5–2 μg/ml of an Ig-like material 3–4 days postinfection. Conformational epitopes of 2C TCR_2IgG are intact as detected by a mAb H57, specific for a TCR Cβ epitope, as well as with an anticlonotypic mAb, 1B2, specific for the 2C TCR (Fig. 2A; top and middle panels). Soluble divalent class II molecules, ^{MCC}I-$E^k{}_2$IgG, are recognized by the conformationally dependent mAb, 14.4.4, specific for an α chain determinant only expressed on intact I-E molecules (Fig. 2A; bottom panel). The purified chimeric Ig proteins also have the expected molecular weights as analyzed by SDS–PAGE (Fig. 2B). Taken together, these data indicate that 2C TCR_2IgG and ^{MCC}I-$E^k{}_2$IgG are intact chimeric molecules that in solution possess conformational epitopes associated with the native transmembrane proteins.

Peptide Specificity of 2C TCR_2IgG

To define the specificity and sensitivity of 2C TCR_2IgG recognition for peptide/MHC complexes, we compared the staining efficacy of soluble divalent 2C TCR_2IgG to soluble monovalent 2C TCR in flow cytometry. Binding of 2C TCR_2IgG to a high-affinity cognate ligand, QL9-loaded L^d molecules, was very sensitive and could be detected even at the lowest concentration tested, 1 nM (Fig. 3A). In contrast greater than 100 nM soluble monovalent 2C TCR was required to detect binding to QL9-loaded L^d molecules. The difference in "relative affinity" had an even more dramatic impact on the ability to detect an intermediate-affinity cognate peptide MHC ligand, p2Ca-loaded L^d molecules. Approximately 3 nM 2C TCR_2IgG was required to detect p2Ca-loaded L^d molecules. Even at the highest concentrations tested, 3000 nM, soluble monovalent 2C TCR could not detect p2Ca-loaded L^d molecules. L^d molecules loaded with a control peptide, MCMV, were not recognized at any concentration by either soluble monovalent or divalent 2C TCR.

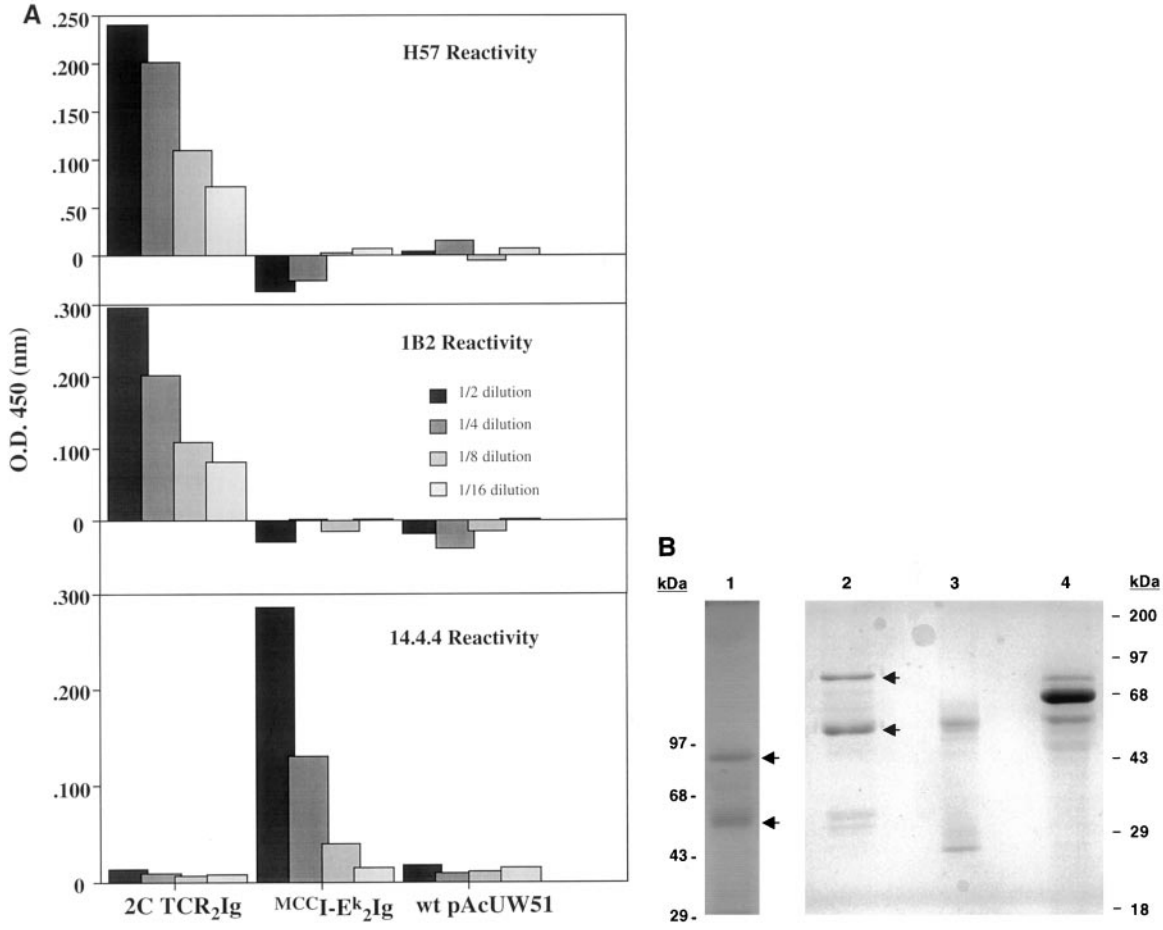

FIG. 2. Biochemical characterization of TCR_2IgG, MHC Class II_2Ig. (A) Detection of chimeras in baculovirus supernatants. Plates were coated with goat anti-mouse Fc. For detection of 2C TCR_2Ig, the secondary antibody was either biotinylated H57 (top) or the anti 2C mAb 1B2 (middle), followed by streptavidin–HRP. For detecting $^{MCC}I\text{-}E^k{}_2IgG$, the secondary antibody was biotinylated 14.4.4 (bottom). When using the biotinylated second antibody 1B2, wells were incubated with 10% mouse serum for an additional 1 h, after washing out sample, to reduce background reactivity. After a 1-h incubation with the biotinylated antibody, the plates were washed and incubated with HRP-conjugated strepavidin for 1 hour, washed, and developed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride substrate for 3–5 min. The reaction was stopped by the addition of 1 M H_2SO_4 and optical density was measured at 450 nm. The assay was linear over the range of 1–20 ng/ml of purified IgG. (B) SDS–PAGE analysis of affinity chimeric proteins. For protein production, *Trichoplusia ni* (Invitrogen, San Diego, CA) cells were infected with virus, m.o.i. 5–10, and supernatants were harvested after 72 h of infection. The chimeric protein was purified from 1 liter of culture supernatants passed over a 2.5-ml affinity column of protein G–Sepharose. Fractions were pooled, concentrated in an Amicon concentrator (50 kDa MW cutoff), and washed with PBS. Purified proteins were analyzed by 10% SDSPAGE. Purified 2C TCR_2Ig (lane 1); $^{MCC}I\text{-}E^k{}_2IgG$ (lane 2) control IgG_1 protein (lane 3) and unpurified supernatant from *T. ni* cells infected with wild-type baculovirus (lane 4) are shown for comparison.

To further demonstrate the efficacy of 2C TCR_2IgG in analyzing pepMHC complexes, an array of p2Ca peptide variants bound to L^d were tested in the direct flow cytometry assay (Figs. 3B–3E) (see Material and Methods for list of peptides). As expected QL9-loaded H-2L^d-expressing cells had the highest MCF while p2Ca elicited a signal approximately 10-fold lower (Figs. 3B and 3D). 2C TCR_2IgG demonstrated differential binding to L^d molecules loaded with p2Ca and its variants, A1-A5, A7, D1, L4, and Y4 peptides (Figs. 3D and 3E). Specifically, peptide variants A1-, A2-, D1-, L4-, and Y4- stabilized L^d molecule were all recognized to varying extents by 2C TCR_2IgG. Other peptide variants, A3-, A4-, A5-, and A7-stabilized L^d molecules, could not be detected by 2C TCR_2IgG even though these peptides all stabilized L^d as measured by 30.5.7 binding. These data are similar to the previously published data based on surface plasmon resonance (SPR) (23). Thus, there were no peptides detected by SPR that were not also recognized by 2C TCR_2IgG in the flow cytometry based assay. Together these data indicate that 2C TCR_2IgG is both a specific and sensitive probe for cognate ligands.

Intracellular Localization of Peptide MHC Complexes

In addition to using TCR_2IgG to stain cells by flow cytometry we also analyzed the ability to use TCR_2IgG

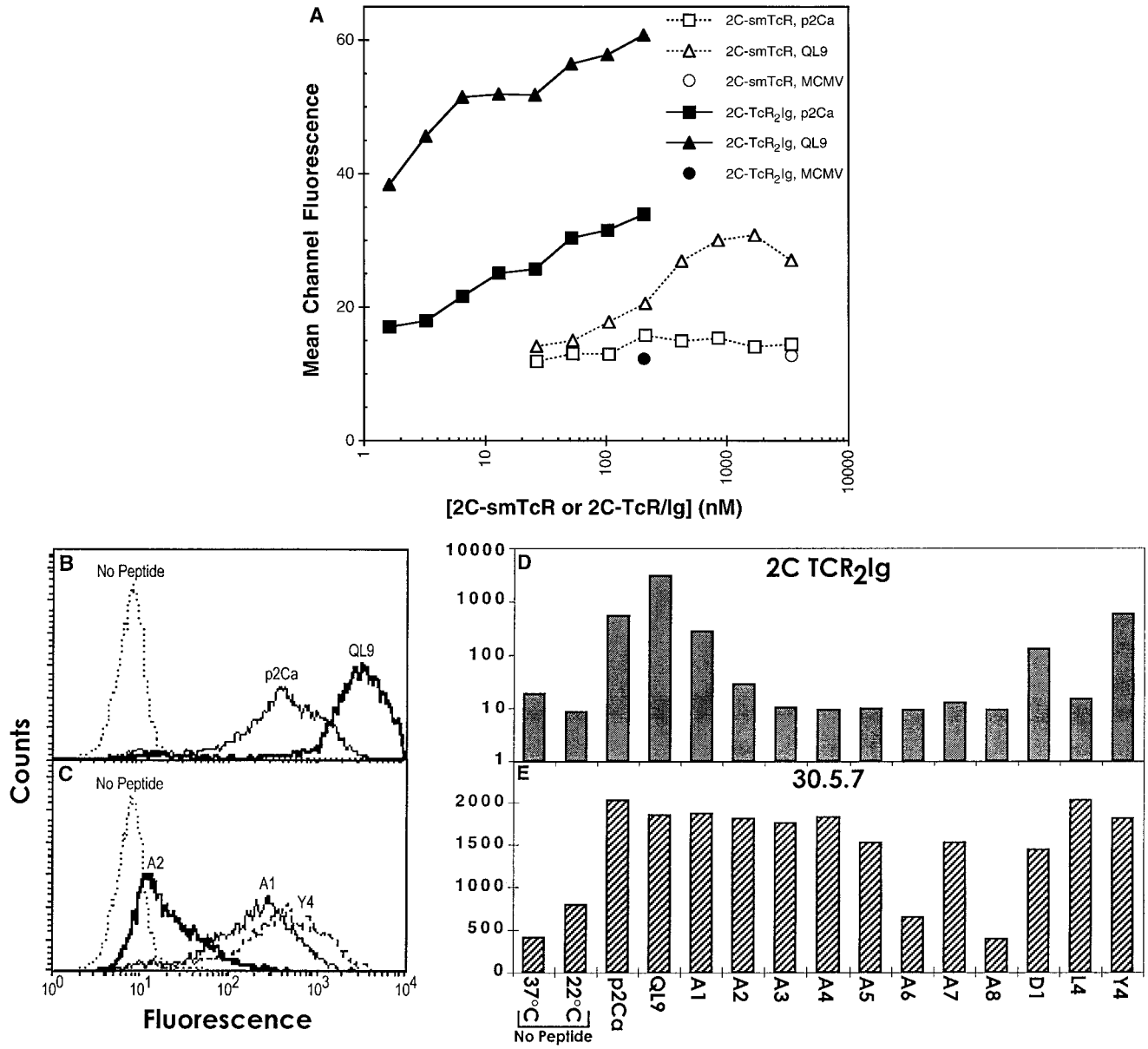

FIG. 3. Staining of peptide-loaded L^d molecules. (A) A comparison of the reactivity of soluble 2C TCR_2Ig to that of soluble monovalent 2C TCR with peptide-stabilized H-2 L^d molecules. RMA-S L^d cells were incubated at 27°C overnight as described. Subsequently, peptides QL9 (△), p2Ca (□), or MCMV (○) (100 μM) were added to cultures and cells were processed as previously described. Cells were stained with serial twofold dilution of either soluble divalent 2C TCR_2Ig (solid lines) or soluble monovalent 2C TCR (dashed lines),washed once in FACS wash buffer, and then stained with saturating amounts of H57–FITC. Cells were incubated for an additional 1 h and processed as described. To facilitate comparison of cells stained with either 2C TCR_2Ig or soluble monovalent 2C TCR, data are presented as mean channel fluorescence. Data shown are from a representative experiment that has been repeated at least three times. (B–E) 2C TCR_2IgG is able to detect p2Ca variant peptides on RMAS-L^d cells. RMAS-L^d cells were loaded with the indicated peptide (100 μM) as in A. Peptide-loaded cells were subsequently incubated with either 2C TCR_2IgG (15μg/ml) (B–D) or 30.5.7 (15 μg/ml) (E) and then subsequently with GAM/IgG-PE (25μg/ml) as described for A and analyzed by flow cytometry. (B,C) Raw FACS data are plotted for the indicated peptides. (D, E) Mean channel fluorescence of 2C TCR_2IgG (D) and 30.5.7 (E) stained cells is plotted in bar graph form for indicated peptides. (Note: Mean channel fluorescence is plotted on a log scale in (D) and a linear scale in (E).)

in immunofluorescence studies. Specifically, we analyzed immunohistochemical staining by 2C TCR_2IgG to address the question of where exogenously added peptides load onto MHC molecules. While peptide loading of class I MHC can be easily performed by coculturing exogenously added peptides with antigen-presenting cells, the site of loading, plasma membrane or ER, has not been clearly defined (31). To address this question peptides were added to L^d-expressing L cells and cells were analyzed by immunofluorescence with 2C

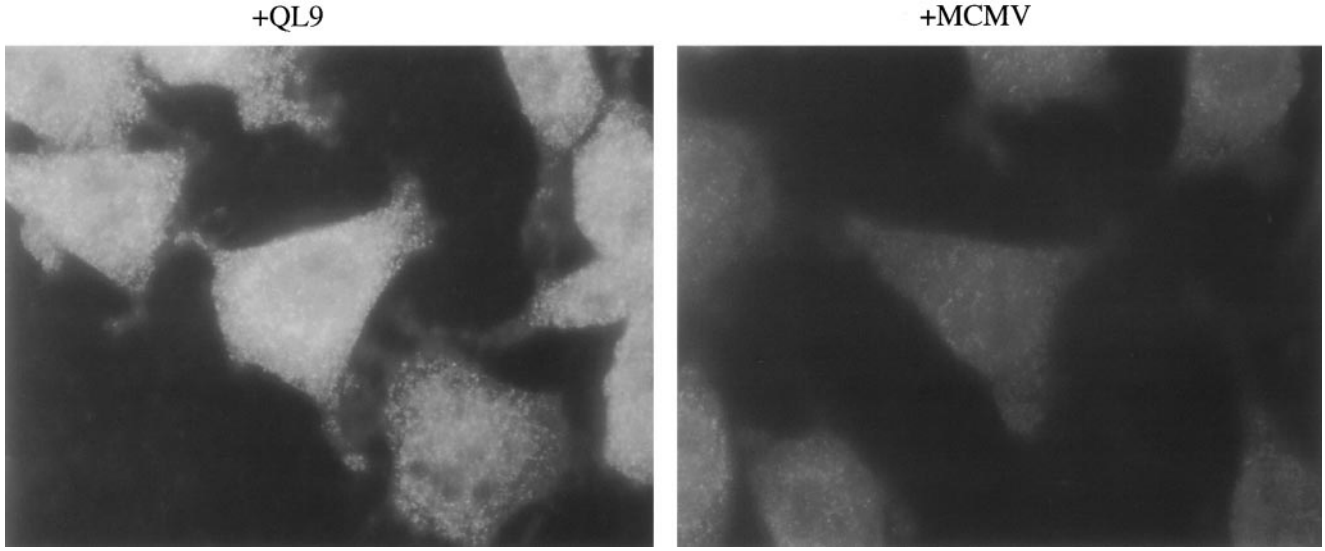

FIG. 4. Intracellular staining of peptide-loaded MHC molecules. For immunohistochemistry experiments, L^d-expressing L cells were plated on coverslips overnight. Cells were incubated either with peptides QL9 or MCMV (100 μM). Cells were incubated with 2C TCR_2IgG (10 μg/ml in 1% BSA) and stained with Cy-3-conjugated donkey anti-mouse Ig. The vesicular immunofluorescence pattern in cells treated with QL9 is characteristic of ER staining in these cells.

TCR_2IgG. Prominent staining activity was seen in the ER as evidenced by vesicular staining pattern, characteristic of ER localization (Fig. 4). Control peptide, MCMV, pulsed cells showed only background 2C TCR_2IgG staining. 2C TCR_2IgG staining was largely restricted to intracellular sites where MHC molecules was found (data not shown). These data suggest that peptide loading primarily occurs in the ER. This finding extends other experiments using peptide-specific MHC-dependent mAb that also indicate that exogenously added peptides load MHC molecules in the ER (31) and show the potential for using these reagents in immunofluorescence studies.

^{MCC}I-$E^k{}_2$IgG Binds and Activates Cognate T Cell Hybridoma

To assess the interaction of soluble divalent class II I-E analogs with antigen-specific T cells, we determined whether ^{MCC}I-$E^k{}_2$IgG could stain antigen-specific T cell hybridomas. ^{MCC}I-$E^k{}_2$IgG binds specifically to 5KC, an MCC-specific, I-E^k-restricted T cell hybridoma (Fig. 5). Mean channel florescence of 5KC cells stained with ^{MCC}I-$E^k{}_2$IgG increased approximately 15-fold. Specific staining of 5KC cells was seen with as little as 5 nM ^{MCC}I-$E^k{}_2$IgG complexes (data not shown). In contrast ^{MCC}I-$E^k{}_2$IgG complexes did not react with DO11.10, an irrelevant control T cell hybridoma specific for ovalbumin peptide in the context of I-A^d (Fig. 5).

The biological activity of ^{MCC}I-$E^k{}_2$IgG was further assessed by the ability of ^{MCC}I-$E^k{}_2$IgG to stimulate T cells. For these assays, proteins were immobilized on plastic and activation of 5KC or DO11.10 cells was assayed by lymphokine secretion. Immobilized ^{MCC}I-$E^k{}_2$IgG stimulated IL-2 production by 5KC but not DO11.10 (Table 1). ^{MCC}I-$E^k{}_2$IgG stimulated 5KC to produce IL-2 at a level comparable to or slightly better than did anti-CD3 mAb. At the lowest concentration tested, approximately 10-fold greater stimulation was achieved with ^{MCC}I-$E^k{}_2$IgG over anti-CD3 mAb (data not shown). These results demonstrate that even when

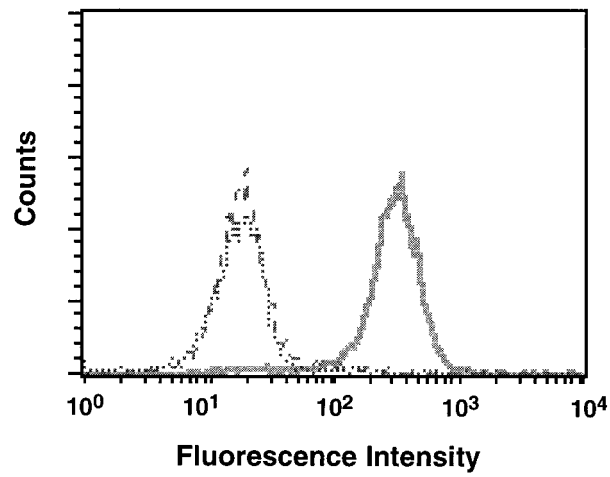

FIG. 5. ^{MCC}I-$E^k{}_2$IgG binds 5KC cells but not DO11.10 cells. T hybridoma cells, 5KC, and DO11.10 were stained with ^{MCC}I-$E^k{}_2$IgG (10 μg/sample) for 1 h at 4°C. Cells were washed and incubated with GAM/IgG_1–PE for an additional 1 h at 4°C. Cells were then washed again and analyzed by flow cytometry. The histograms of 5KC cells stained either with ^{MCC}I-$E^k{}_2$IgG (solid line) or without any primary reagent (dotted line) and of D011.10 cells stained with ^{MCC}I-$E^k{}_2$IgG (dashed line) are shown.

TABLE 1
Immobilized ^{MCC}I-E$^{k}_{2}$IgG Stimulates IL-2 Production by ^{MCC}I-E^{k}-Specific Hybridoma

Stimulating ligand	[Ligand] (pM)	Cell line	IL-2 production (pg/ml)
^{MCC}I-E$^{k}_{2}$IgG	10,000	5KC	49.5
	1,100		20.6
	10,000	DO11.10	<1
	1,100		<1
Anti-CD3	6,600	5KC	31
	750		13
	6,600	DO11.10	31.8
	750		31.8

Note. Soluble proteins, ^{MCC}I-E$^{k}_{2}$IgG or anti-CD3, were immobilized on Immunlon 4 plates at various concentrations, overnight at 4°C. Wells were washed thoroughly and 5KC or DO11.10 T hybridoma cells were (1×10^5) incubated overnight at 37°C. T cell activation was measured by IL-2 production.

immobilized on a plate soluble divalent ^{MCC}I-E$^{k}_{2}$/IgG retains its specificity for its cell surface cognate TCR and minimally activates antigen-specific T cells as efficiently as anti-CD3 mAb.

DISCUSSION

To enhance our ability to analyze and regulate antigen-specific immune responses we have designed a general system for expression of soluble divalent analogs of both MHC class II and TCR molecules, using Ig as a molecular scaffolding structure. One important role the IgG scaffold serves is appositioning the two heterodimers so that the complex can engage its cognate ligands. It does this with the intrinsic flexibility afforded both by the immunoglobulin hinge region and potentially by the linker which likely facilitate TCR or MHC moieties binding their cognate ligands on cell surface membranes.

Other approaches have been developed to generate probes for antigen-specific T cells. One approach has been to generate high-affinity, anticlonotypic mAb. Anticlonotypic mAb discriminate on the basis of specific TCR Vα and Vβ conformational determinants that are not directly related to antigenic specificity. Therefore, an anticlonotypic antibody will interact with only one of potentially many different clonotypic T cells specific for the antigen that develop during an immune response. In contrast multivalent peptide/class II MHC complexes, as reported here, exploit the natural antigenic specificity and will detect many different clonotypic T cells specific for a given antigen.

An alternative approach for expressing multivalent class I MHC molecules using strepavidin has been reported (15). A distinct advantage of the strepavidin system is that it permits coordination of theoretically up to four biotinylated peptide/MHC complexes. This approach has been developed for peptide/class I MHC complexes (15) and recently also reported for class II complexes (32, 33). In this system there appears to be a requirement for four peptide/MHC complexes although the definition of the minimal requirement for stable association has not yet been reported. Using Ig as a scaffolding structure allows expression of two peptide/MHC complexes which are sufficient for stable binding to MCC-specific T cells; a system where the TCR interaction with monovalent peptide/MHC complexes has been characterized as an intrinsically low-affinity one, 50 μM (34, 35). The intrinsic flexibility associated with the Ig hinge region, a region that has been adapted during evolution to maximize binding of each Ig arm to its cognate ligand, may explain why stable association of soluble divalent ^{pep}MHC$_2$Ig complexes occurs when using Ig as a scaffolding structure.

The development of reagents that differentiate between specific peptide/MHC complexes has also been an area of extensive research. Recently investigators have used soluble monovalent TCR to stain cells by crosslinking them with avidin after they have been bound to the cell (7). Another approach has been to generate mAb that differentiate between MHC molecules on the basis of peptides resident in the groove. While conventional approaches have produced only a few such antibodies with anti-peptide/MHC specificity (36–38), two new approaches have been developed to obtain peptide-specific, MHC-dependent mAb (39–41). Where reported, the affinity of these mAb for specific peptide/MHC complexes has been of intermediate strength (10–100 nM) (39, 40) comparable to those measured using TCR$_2$IgG (14).

A distinct advantage of soluble TCR$_2$IgG complexes over generation of mAb that differentiate between specific peptide/MHC complexes is that TCR$_2$IgG complexes may be useful in defining specific ligands recognized by T cells, as their production does not require *a priori* knowledge of their ligand. They could have potential uses in defining ligands of γ/δ TCR or of undefined tumor-specific T cells. Furthermore, since T cell activation requires crosslinking of multiple TCR, interaction of TCR$_2$IgG may mimic natural T cell activation. This could facilitate elucidation of biochemical interactions involved in TCR recognition of peptide/MHC complexes.

Soluble divalent class II MHC analog, ^{MCC}I-E$^{k}_{2}$IgG, was able to stimulate cognate T cells. ^{MCC}I-E$^{k}_{2}$IgG was at least as efficient, if not more efficient, than anti-CD3 mAbs in eliciting IL-2 production and had a much greater efficiency than immobilized monovalent ^{MCC}I-E^{k} (data not shown). However, unlike anti-CD3 mAbs the ^{MCC}I-E$^{k}_{2}$IgG was highly specific for its cognate T cell, suggesting that soluble divalent MHC analogs may be able to regulate antigen-specific T-cells and have a role in adoptive immunotherapy.

We have presented a general approach for producing soluble divalent versions of heterodimeric proteins, such as T cell receptors and class II MHC molecules. The experimental system described here outlines an approach using divalent high-affinity ligands to study cell–cell interactions, driven by multivalent ligand–receptor interactions. Our work suggests that divalent chimeric molecules are good candidates for soluble, high-avidity analogs of proteins that could be used to probe and selectively regulate cellular responses.

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