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

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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 has a transmembrane domain. 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 affinity.
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 (TCR2Ig) and MHC class II (class II2Ig) 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 TCR2Ig 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 and light chains using the restriction endonuclease sites shown. Subsequently, these chimeras were ligated sequentially to a modified pAcUW51 vector (18). A HindIII 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 KpnI restriction enzyme site and linker were inserted immediately 3' of the stop codon in the κ polypeptide. A KpnI restriction site and linker were inserted immediately 5' of the codon for Glu lo-
icated at the start of the mature Igγ1 polypeptide in clone 93G7. An SphI restriction site was introduced 3' to the stop codon in the γ1 polypeptide.

The genes encoding the 2C TCR α and β chains have previously been described (4). A KpnI 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, EcoRI. A XhoI site was introduced 5' to the start of the signal sequence in the 2C TCR β chain and a HindIII 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 KpnI 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 EcoRI site. The I-E α chain was modified by introduction of a HindIII
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 XhoI and KpnI and 2C TCR1IgG or 1-E8.IgG was inserted downstream of the P10 promoter (Fig. 1A). Subsequently, the 2C TCR1IgG or MCC1-I-E8.IgG1 was inserted into EcoRI/Sphi sites downstream of the polyhedron promoter. The signal sequence for 2C TCR1IgG and MCC1-I-E8.IgG1, 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.

Oligonucleotides used to introduce the above mutations were 5’ IgG1 mutation, ctgtagaatcagcgtctcacttctgttacagctcgtggagg; 3’ IgG1 mutation, aatcagcgtctcacttctgttacagctcgtggaggctg; 5’ IgG mutation, ctgtagaatcagcgtctcacttctgttacagctcgtggagg; 3’ IgG mutation, ctgtagaatcagcgtctcacttctgttacagctcgtggaggctg; 5’ I-E mutation, ccaaaga-agaattgcgtctcacttctgttacagctcgtggagg; 3’ I-E mutation, ccagaattgcgtctcacttctgttacagctcgtggaggctg; 5’ 2C TCR α mutation, cagatatgaaactcatcttacttacaagaggtagttcagct-ctgttacctgagctcgtggagg; 5’ 2C TCR β mutation, cccaaaga-agaattgcgtctcacttctgttacagctcgtggagg; 3’ 2C TCR β mutation, ccagaattgcgtctcacttctgttacagctcgtggagg; and 3’ I-E β mutation, gacacgtcactttacaagggcagcttgcgtcttacctgttacctgagctcgtggagg.

Molecular Modeling

The 2C TCR1IgG structure was modeled and linkers regions regularized using Quanta 4.1/CHARMM (Molecular Simulations Inc.). Coordinates for the IgG1 were obtained from the PDB (1IgG) 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 donotypic epitope expressed on 2C TCR. I-E8.Ig was assayed using biontinylated 14.4.4, an anti-I-E, chain-specific mAb, as the secondary antibody. SDS–PAGE analysis of the chimeric protein was performed as described (22). Samples were electrophoresed through a 10% SDS–polyacrylamide gel.

Peptide Loading of Cells, Flow Microfluorometry, and Immunohistochemistry

RMA-S Ld 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 TCR1IgG, RMA-S Ld 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 (NASDAQPFDL), A2 (LAPFPFDL), A3 (LSAFPFDL), A4 (LSPFPFDL), A7 (LSAFPDAL), D1 (DSPFPFDL), L4 (LSPLFPSL), and Y4 (LSPFPFDL), peptides. Approximately 1 x 106 cells, either peptide-loaded or not loaded, were incubated for 60 min at 4°C with 30 μl of the anti-Ld-specific mAb, 30.5.7 (10 μg/ml) culture supernatants, or 2C TCR-IgG 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, MCC1-I-E8.Ig (5 μg) was incubated with 1 x 106 cells for 1 h at 4°C and washed, followed by incubation with goat anti-mouse IgG1 conjugated to RPE for an additional 1 h. Hybridoma cells were then washed 2× and analyzed by flow cytometry.

For immunohistochemistry experiments, Ld-expressing L cells were plated on coverslips overnight. Cells were incubated with peptides QL9 or MCMV (100 μM) for 1 h, washed, fixed with 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 TCR1IgG was “airfuged,” 80K for 1 h, and diluted to 10 μg/ml BSA. Coverslips were incubated with 2C TCR1IgG 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 MCC1-I-E8.Ig or the murine anti CD3 mAb, 2Cl1, were immobilized on sterile Immulon 4 plates (Dynatech, Burlington, MA) overnight at 4°C. Following two washes, either the MCC-specific 5K cells or the control ovalbumin-specific DO11.10 cells
(1 × 10⁵/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 Analogos 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 IgG 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 done (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 Ld (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-Ek that has been previously modified to also encode a nominal peptide antigen derived from moth cytochrome C (18, 30). Soluble divalent TCR (2C TCR IgG) 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-Eκ IgG), cDNAs encoding the extracellular domains of MCC-I-Eκ and I-Eκ chains were genetically linked to cDNA encoding the Igγ1, 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 IgG 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 IgG and MCC-I-Eκ IgG constructs secrete 0.5–2 μg/ml of an Ig-like material 3–4 days postinfection. Conformational epitopes of 2C TCR IgG are intact as detected by a mAb H57, specific for a TCR Cβ epitope, as well as with an anticondonotypic mAb, 1B2, specific for the 2C TCR (Fig. 2A; top and middle panels). Soluble divalent class II molecules, MCC-I-Eκ 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 IgG proteins also have the expected molecular weights as analyzed by SDS–PAGE (Fig. 2B). Taken together, these data indicate that 2C TCR IgG and MCC-I-Eκ IgG are intact chimeric molecules that in solution possess conformational epitopes associated with the native transmembrane proteins.

Peptide Specificity of 2C TCR IgG

To define the specificity and sensitivity of 2C TCR IgG recognition for peptide/MHC complexes, we compared the staining efficacy of soluble divalent 2C TCR IgG to soluble monovalent 2C TCR in flow cytometry. Binding of 2C TCR IgG to a high-affinity cognate ligand, QL9-loaded Ld 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 Ld 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 Ld molecules. Approximately 3 nM 2C TCR IgG was required to detect p2Ca-loaded Ld molecules. Even at the highest concentrations tested, 3000 nM, soluble monovalent 2C TCR could not detect p2Ca-loaded Ld molecules. Ld molecules loaded with a control peptide, MCMV, were not recognized at any concentration by either soluble monovalent or divalent 2C TCR.
To further demonstrate the efficacy of 2C TCR2IgG in analyzing pepMHC complexes, an array of p2Ca peptide variants bound to Ld were tested in the direct flow cytometry assay (Figs. 3B–3E) (see Material and Methods for list of peptides). As expected, QL9-loaded H₂Ld-expressing cells had the highest MCF while p2Ca elicited a signal approximately 10-fold lower (Figs. 3B and 3D). 2C TCR2IgG demonstrated differential binding to Ld 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 Ld molecule were all recognized to varying extents by 2C TCR2IgG. Other peptide variants, A3-, A4-, A5-, and A7-stabilized Ld molecules, could not be detected by 2C TCR2IgG even though these peptides all stabilized Ld 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 TCR2IgG in the flow cytometry based assay. Together these data indicate that 2C TCR2IgG is both a specific and sensitive probe for cognate ligands.

Intracellular Localization of Peptide MHC Complexes

In addition to using TCR2IgG to stain cells by flow cytometry we also analyzed the ability to use TCR2IgG...
in immunofluorescence studies. Specifically, we analyzed immunohistochemical staining by 2C TCR1IgG 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...
TCR, IgG. 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, IgG staining. 2C TCR, IgG 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\textsuperscript{k} 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\textsuperscript{k} IgG could stain antigen-specific T cell hybridomas. MCC I-E\textsuperscript{k} IgG binds specifically to 5KC, an MCC-specific, I-E\textsuperscript{k}-restricted T cell hybridoma (Fig. 5). Mean channel fluorescence of 5KC cells stained with MCC I-E\textsuperscript{k} IgG increased approximately 15-fold. Specific staining of 5KC cells was seen with as little as 5 nM MCC I-E\textsuperscript{k} IgG complexes (data not shown). In contrast, MCC I-E\textsuperscript{k} IgG complexes did not react with DO11.10, an irrelevant control T cell hybridoma specific for ovalbumin peptide in the context of I-A\textsuperscript{k} (Fig. 5).

The biological activity of MCC I-E\textsuperscript{k} IgG was further assayed by the ability of MCC I-E\textsuperscript{k} 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\textsuperscript{k} IgG stimulated IL-2 production by 5KC but not DO11.10 (Table 1). MCC I-E\textsuperscript{k} 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\textsuperscript{k} IgG over anti-CD3 mAb (data not shown). These results demonstrate that even when
immobilized on a plate soluble divalent MCCI-Ek2IgG 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, antiidotypic mAb. Antidiotypic mAb discriminate on the basis of specific TCR Vα and Vβ conformational determinants that are not directly related to antigenic specificity. Therefore, an antidiotypic antibody will interact with only one of potentially many different donotypic 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 donotypic 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 mIgM/MHC 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 TCR1IgG (14).

A distinct advantage of soluble TCR1IgG complexes over generation of mAb that differentiate between specific peptide/MHC complexes is that TCR1IgG 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 γδ TCR or of undefined tumor-specific T cells. Furthermore, since T cell activation requires crosslinking of multiple TCR, interaction of TCR1IgG 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 MCCI-Ek2IgG, was able to stimulate cognate T cells. MCCI-Ek2IgG 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 MCCI-Ek (data not shown). However, unlike anti-CD3 mAbs the MCCI-Ek2IgG 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.

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<th>Stimulating ligand</th>
<th>[Ligand] (pM)</th>
<th>Cell line</th>
<th>IL-2 production (pg/ml)</th>
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<tr>
<td>MCCI-Ek2IgG</td>
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<td>5KC</td>
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Note: Soluble proteins, MCCI-Ek2IgG 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 × 10⁵) incubated overnight at 37°C. T cell activation was measured by IL-2 production.
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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