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MHC-Restricted, Glycopeptide-Specific T Cells Show Specificity for Both Carbohydrate and Peptide Residues[1]

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We examined the antigenic specificity of two T cell hybridomas elicited against the disaccharide galabiose attached to the fifth residue of the I-A^k binding peptide 52–61 of lysozyme. By making changes in the saccharide molecule and in the peptide, we conclude that the outer galactose residue of the galabiose moiety is directly recognized by the T cells together with the exposed side chains of the peptide. The overall spatial display of this galactose moiety on the 52–61 peptide is likewise important. ***The Journal of Immunology,*** **1999, 162: 4740–4744.**

The T cell Ag receptor recognizes MHC molecules and the peptides bound to them. The TCR establishes contact with amino acid residues located on the helices of the peptide-combining site of the MHC molecules, as well as with residues from the peptide that are solvent exposed (1–4). T cells also recognize small reactive chemicals that are attached to peptides bound to MHC molecules, or directly to the MHC molecules. In retrospect, the first appreciation of such T cells came from the reports of Leskowitz on contact sensitivity to azobenzene arsonate (5). Subsequently, detailed studies were conducted with several small chemicals, haptens, like fluorescein, and di- or trinitrochlorobenzene, all of which showed T cell reactivity to them (for example, Refs. 6–8). The importance of T cell recognition of haptens lies in the clinical reactions of contact sensitivity to small chemicals. Here, the interpretation is that the chemical binds to the MHC of skin APC to create a neo Ag recognized by T cells. These studies imply that the TCR can contact chemical moieties that may be linked to MHC bound structures. How the recognition of MHC, peptides, and hapten takes place is not entirely known.

Carbohydrates represent another set of molecules that can be recognized by T cells (Refs. 9–17; reviewed in Refs. 18 and 19). However, to elicit such responses, as with the haptens cited above, the carbohydrate needs to be linked to a peptide that binds to an MHC molecule. This is because many carbohydrates do not bind directly to MHC molecules (20), so the T cell epitope cannot be formed. The T cell anticarbohydrate response elicited by glycopeptides could be of importance in the response to viral or tumor Ags. Indeed, many eukaryotic and viral proteins carry covalently linked carbohydrates.

In two previous reports we analyzed the specificity of CD4 T cells directed to the disaccharide galabiose (Galα1–4Galβ) (Gal2)[4] bound to the peptide 52–61 of hen egg-white lysozyme (HEL) (10, 11) (Fig. 1). The 52–61 peptide (DYGILQINSR) binds strongly to the I-A^k class II MHC molecules (21–24). The specificity of the carbohydrate-specific T cells was diverse and was highly dependent on the site of attachment of the saccharide to the peptide. For example, one set of T cells was elicited to Gal2 conjugated to the amino terminus of the peptide (10). These T cells showed cross-reactivity with cellobiose (Glcβ1–4Glc) and also reacted with Gal2 containing an acetylated disaccharide moiety. They were specific for the 52–61 peptide since a different peptide, also conjugated to Gal2 at its amino terminal end, was not stimulatory. We interpreted these results to indicate that this set of T cells were reactive to a conformational change in the display of 52–61 brought about by the disaccharide attached to the amino terminus of the peptide.

A second set of T cells were elicited by 52–61 bearing the Gal2 moiety at its fifth residue of the peptide, i.e., at the center of it. Here, replacement of some of the residues of 52–61 (Gln57 and Ser60) with a serine that carried a Gal2 moiety resulted in loss of I-A^k binding. However, when Gal2 was attached in a similar way at position 53 or 56, of 52–61, binding of the glycopeptide to I-A^k was evident. These glycopeptides were immunogenic and elicited T cell responses. These T cells showed specificity to both the eliciting peptide and Gal2, as well as being restricted by the I-A^k molecule (11).

Our report, and those of others (9, 12–17), have brought evidence to support specific recognition of the sugar moiety. However, the analysis of the specificity of the carbohydrate has been quite limited. The present study represents an analysis of the set of T cells directed to Gal2 placed at residue 56 of 52–61, attempting to establish the extent of recognition of the carbohydrate and peptide components. We conclude that their TCR makes specific contact both with the Gal moiety, which is distal from 52–61, and with side chains of some amino acid residues.

Materials and Methods

The glycopeptides used in this study were synthesized by using glycosylated derivatives of serine as building blocks in the 9-fluorenylmethoxycarbonyl (F-moc) solid phase synthesis strategy (P. Sjölin, S. Roy, and J. Kihlberg,

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[4] Abbreviations used in this paper: Gal2, Galα1–4Galβ; RIC^{-1}, relative inhibitory capacity.

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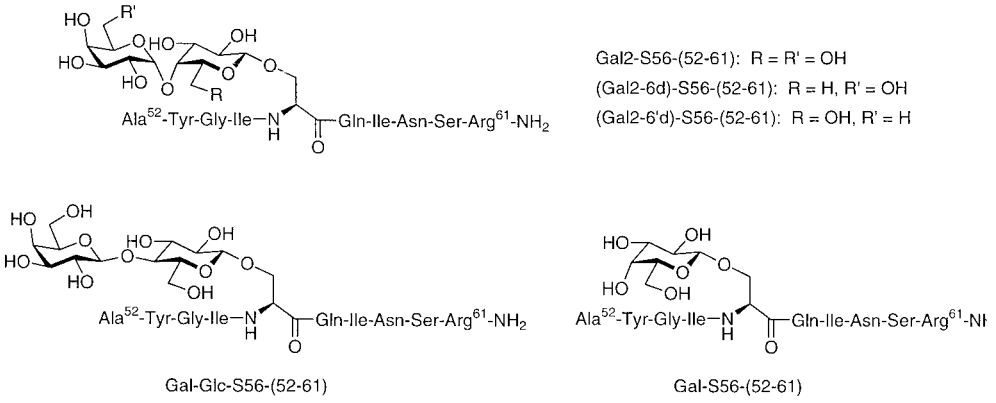

FIGURE 1. Diagrams of the glycopeptides used in this study.

manuscript in preparation). Thus, N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-[2,3-di-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-β-D-fucopyranosyl]-L-serine pentafluorophenyl ester was used to prepare (Gal2–6d)-S56-(52–61); N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4-tri-*O*-acetyl-α-D-fucopyranosyl)-β-D-galacto-pyranosyl]-L-serine pentafluorophenyl ester was used for (Gal2–6′d)-S56-(52–61); N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-[2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-β-D-glucopyranosyl]-L-serine pentafluorophenyl ester was used for Gal-Glc-S56-(52–61); and N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine pentafluorophenyl ester was used for synthesis of Gal-S56-(52–61). N^{α}-(9-fluorenylmethoxycarbonyl)-3-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-β-D-galactopyranosyl]-L-serine pentafluorophenyl ester was used for preparation of the members of the series of glycopeptides that had Gal2 linked to Ser56 of 52–61 and that contained single alanine substitutions at all of the peptide residues except Gly54. All glycopeptides were prepared as C-terminal amides. After completion of the solid-phase synthesis, the *O*-acetylated glycopeptides were cleaved from the resin and purified by reversed-phase HPLC. The *O*-acetyl groups were then removed using methanolic sodium methoxide, and fully deprotected glycopeptides were obtained after HPLC purification. Chemical structures were confirmed with amino acid analysis, 500 MHz ^{1}H nuclear magnetic resonance spectroscopy, and fast atom bombardment mass spectroscopy.

Fig. 1 indicates the structure of the different glycopeptides. Fig. 2 compares the structures of Gal2 with Gal-Glc.

Each glycopeptide was tested for its binding to purified I-A^k molecules by a competitive binding method previously described (10, 11, 23). Purified I-A^k molecules were incubated with an ^{125}I-radiolabeled peptide known to bind with high affinity (YEDYGILQINSR). The reaction was conducted in the absence or presence of the unlabeled glycopeptides. Each experiment had a reference reaction with unlabeled standard peptide; from the reference titration we determined the concentration that inhibited 50% of the standard labeled peptide. The amount of test glycopeptide was then compared with the reference value (RIC^{-1} = concentration test/concentration standard). All glycopeptides bound well to I-A^k molecules, in fact, slightly better than the unglycosylated 52–61 peptide. The binding results of Ala-substituted glycopeptides are included in Table I.

Each glycopeptide was tested for its stimulation of two T cell hybridomas (10, 11) (clones 56.15.2 and 56.15.16), elicited by immunizing CBA/J mice with the Gal2-S56-(52–61) glycopeptide in Freund's adjuvant. The lymph node CD4 T lymphocytes were stimulated in vitro and fused to produce hybridomas. These two are specific for Gal2-Ser56-(52–61) bound to I-A^k molecules (11). In the T cell assays, 5×10^4 C3.F6 cells (serving as APC) were incubated with 10^5 T cells, at various peptide concentrations, for 24 h in 96-well trays, in a total of 200 μl of media. Indicated in Figs. 3 and 4 is the release of IL-2 in the culture media, assayed by a bioassay on the CTLL-2 indicator cells.

Results and Discussion

The CD4 T cell hybridomas were elicited against a glycopeptide consisting of galabiose (Gal2) attached to serine at position 56 of the 52–61 peptide of HEL (Gal2-S56-(52–61)) (Fig. 1). The descriptions of these T cells were given in our previous paper (11).

Three issues were now examined. The first was the recognition by the TCR of the Gal2 moiety. We wanted to examine whether the T cells contacted either or both of the Gal residues directly. Alternatively, the Gal2 at the peptide center could distort the display of the peptide in such a way as to create a new set of the TCR contact amino acids of 52–61, as observed with Gal2 at the N terminus of 52–61 (10).

The specificity of the two T cell hybridomas was examined by engineering two Gal2 molecules in which the hydroxyl group at the C-6 position was removed (deoxygenated), either from the Gal distal to the anchoring Ser, or from the proximal Gal ((Gal2–6′d)-S56-(52–61), and (Gal2–6d)-S56-(52–61), in Fig. 1). Such modifications of Gal2 have previously been shown not to affect the conformation of Gal2 (25–27). That is, deoxygenation affects neither the conformation of each of the Gal residues nor the orientation of these with respect to each other, which is rather rigid. This

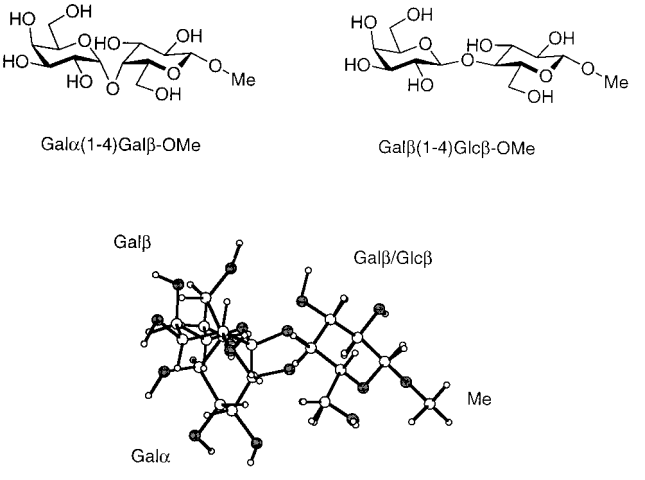

FIGURE 2. A comparison of the Gal2 (Galα(1–4)Galβ-OMe) and Gal-Glc (Galβ(1–4)Glcβ-OMe) moieties of glycopeptides Gal2-S56-(52–61) and Gal-Glc-S56-(52–61), revealing the different positions adopted in space by the distal Gal unit of the disaccharides. For clarity, the two disaccharides are shown as β-methyl glycosides, in which the methyl group represents the β-carbon of serine 56, which anchors the saccharides to the 52–61 peptide. In the comparison, the Galβ unit of Gal2 was superimposed on the Glcβ unit of Gal-Glc simultaneously with superimposition of the methyl (Me) groups. The Galβ and Glcβ units, as well as the Me groups representing 52–61, superimpose perfectly on each other. However, the Galα unit of Gal2 occupies a different position in space than Galβ of Gal-Glc. Minimum energy conformations, determined previously for the disaccharides in aqueous solution (31), were used in the superimpositions. Oxygen atoms are grey, whereas carbon atoms (large) and hydrogen atoms (small) are white (31).

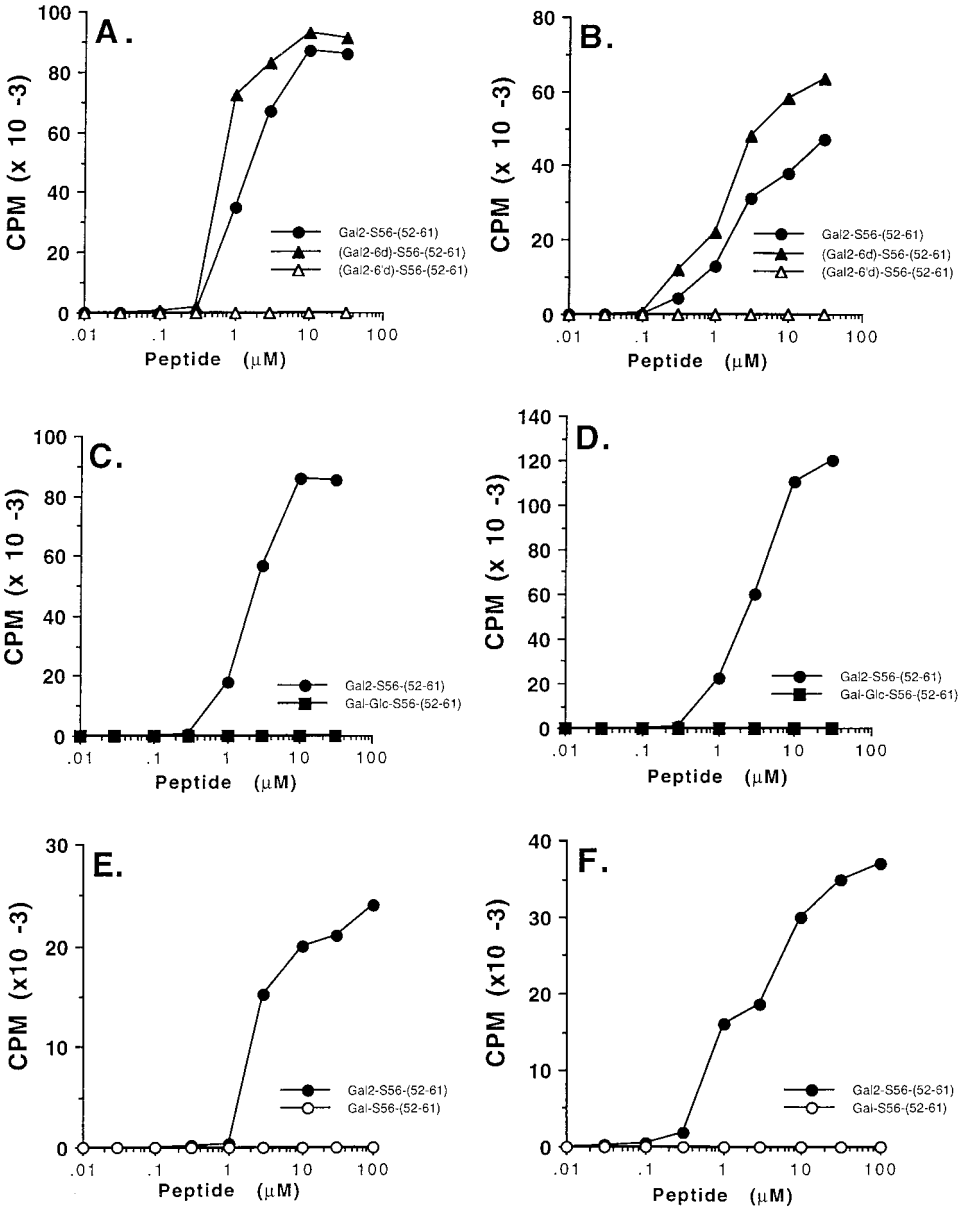

FIGURE 3. The response of two different T cell hybridomas to substituted Gal2 derivatives. One T cell hybridoma is shown in panels *A*, *C*, and *E* of this figure, as well as in the same labeled panels of Fig. 4. The second is represented in panels *B*, *D*, and *F* of each figure. *A* and *B* indicate the response to Gal2 bearing a deoxygenated C-6 in the outer (6′d) or inner (6d) Gal. The response is lost only on removal of the OH group of the distal Gal. *Middle* panels *C* and *D* show that the Gal-Glc-S56-(52–61) glycopeptide is not recognized. The same lack of recognition applies to a single Gal at Ser^{56} of 52–61 (*E* and *F*). All experiments were done two or three times, with identical results.

conclusion should apply also to the orientation of Gal2 with respect to Ser56 in the 52–61 peptide. Consequently, if removal of any of the hydroxyl groups in Gal2-S56-(52–61) significantly affects the T cell reactivity, this reveals a fine recognition of the sugar moiety.

Both modified glycopeptides bound equally to the I-A^k molecule (RIC^{-1} of 4.4 for each, compared with 3.8 for the unchanged glycopeptide, and 27 for 52–61), thus establishing that deoxygenation did not affect MHC binding. Fig. 3, *A* and *B*, shows that the glycopeptide with a distally deoxygenated Gal was not recognized by T cells. The one with the proximal substitution was recognized equally as well as the wild-type glycopeptide. Thus, there is indeed fine specificity of recognition, but it is apparent only to the portion of the disaccharide that is most distal to the peptide, that is to say, the one expected to contact the TCR. However, we do not rule out that TCR recognition may also involve the proximal Gal unit of the Gal2-S56-(52–61) peptide,

A second issue was the spatial relationship between the saccharides and the peptide. The spatial relationship of the distal T cell contact Gal, with the rest of the peptide and I-A^k molecule, may be critical. To do this, we engineered the presentation of the Gal moiety in two ways. One was by having a single Gal instead of two (Gal-S56-(52–61); Fig. 1). The second was by having the Gal linked to a proximal Glc by a β1–4 linkage (Gal-Glc-S56-(52–61)). A single Gal molecule was not seen by the T cells (Fig. 3, *E* and *F*). (We have not elicited T cells to a single Gal-S56-(52–61) but predict that such can be elicited.) The two T cell hybridomas did not recognize the Gal-Glc-S56-(52–61) peptide (Fig. 3, *C* and *D*). This may be explained by the fact that the distal Gal in Gal2 is positioned differently, compared with the Gal moiety of Gal-Glc, when linked to S56 of 52–61 (Fig. 2). The TCR may be sensitive to such differences. The inability of the hybridomas to recognize Gal-Glc-S56-(52–61) is in agreement with their lack of recognition of Glc2-S56-(52–61), which has a Glcβ1–4 Glcβ moiety linked to S56 in 52–61 (11).

A third issue that was examined referred to the engagement of amino acid residues of the glycopeptide by the TCR. To examine the contribution of amino acid side chains to the recognition of the glycopeptide, we proceeded to make an Ala scan of it. Gal2 peptides were produced having single alanine substitutions at each

Table I. *Summary of Ala scan of the Gal2-S56-(52-61) peptide*[a]

Residue	Role in 52–61	T Cell to 52–61 (Ala Scan; No. Affected/Total)	T cells to Gal2-S56-(52–61)	
			Binding (RIC^{-1})	T Cell Response
52: Asp	MHC contact - P1 pocket	7/7	>150	Markedly reduced
53: Tyr	TCR contact	7/7	12.8	No response
54: Gly	—	2/7	—	—
55: Ile	MHC contact - P4 pocket	1/7	25.0	No response
56: Ser-Gal2	TCR contact (*)	7/7	—	—
57: Gln	MHC contact - P6 pocket	1/7	13.9	Slightly reduced
58: Ile	MHC contact - P7 pocket	6/7	17.9	No response
59: Asn	TCR contact	4/7	4.0	No response
60: Ser	MHC contact - P9 pocket	0/7	5.7	Improved response
61: Arg	TCR contact	1/7	3.7	Improved response

[a] The functions of the different residues were reported in Refs. 22, 23, and 28. The summary of the responses of seven different T cell hybridomas to Ala scans of 52–61 represents mostly unpublished results of M.B.D. and E.R.U. ("T cells to 52–61"). In the 52–61 peptide, residue 56 is a leucine (*). Each T cell was titrated as in Fig. 4. Those showing a reduction of 75% or more in the amounts required to stimulate a half-maximal response were considered significant. The T cell response of the Ala scan of the Gal2-S56-(52–61) peptide was shown in Fig. 4 and summarized in the last two columns. Dashes indicate that no substitution was made. Binding of the glycopeptides is shown as RIC^{-1} and should be compared with the value determined for Gal2-S56-(52–61) of RIC^{-1} of 3.8.

amino acid residue of 52–61, except for Gly54 and Gal2-Ser56. Such studies have been done in the past to determine residues involved in contacting the TCR or the MHC molecule (22, 23). Each mutant peptide was tested for its binding to I-A^k and for its stimulation of the two T cell hybridomas (Table I). The results of the Ala scan of Gal2-S56-(52–61) are shown in Fig. 4 and summarized in Table I. Table I also compares the results of our two T cells with seven other T cells that were studied in the past (Ref. 22;

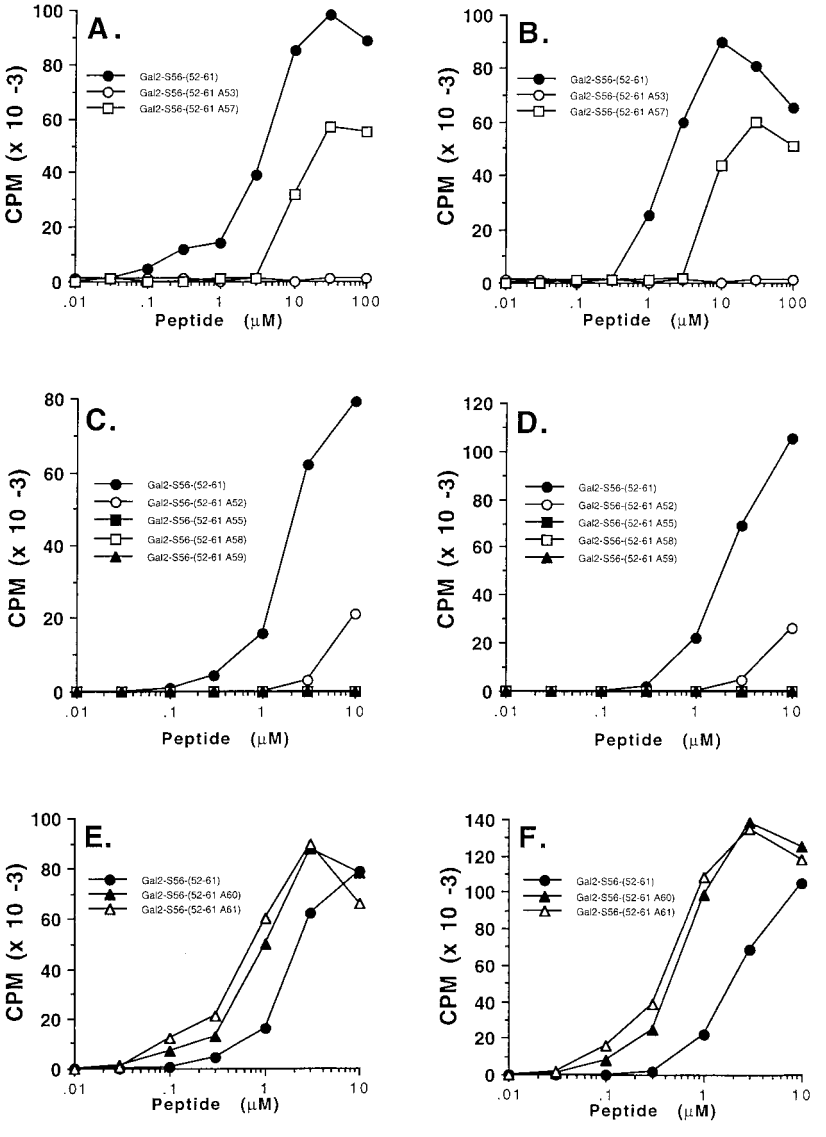

FIGURE 4. The response of the two hybridomas to Gal2-S56-(52–61) in which Ala substitutions were made. The residues with the changes are indicated as A, followed by the number of the residue. The explanation of the results is given in the text.

and unpublished results of M. B. Deck and E. R. Unanue). These seven T cells were specific for 52–61 bound to I-A^k.

The crystal structure of 52–61 bound to I-A^k has just been resolved (28). The peptide 52–61 anchors to I-A^k mainly by way of its Asp52 residue, which fits into the P1 pocket, or site, of I-A^k molecules. Most of the high affinity natural peptides that bind to I-A^k bear an Asp residue, usually at the fourth or fifth residue from the amino terminus (28, 29). In addition, Ile55, Gln57, Ile58, and Ser60 are less critical anchors for the P4, P6, P7, and P9 pockets, respectively. Of the five anchor residues of 52–61, only Asp52 has a major effect on binding to I-A^k. As expected, the substitution by alanine affects binding of the peptide and the T cell response. Of the other MHC anchor residues, alanine substitutions of residues 55 (anchoring to P4), 57 (anchoring to P6), or 60 (anchoring to P9) had no effect on most T cell responses. However, alanine substitution of residue 57 (anchoring to P7) did have a negative effect. (The P7 pocket is a shallow site located sideways on the β-chain α helix and could interact with TCR.) Finally, the solvent exposed residues in 52–61 are Tyr53 (~40%), Ile56 (~50%), Asn59 (~ 50%), and Arg61 (~40%). Except for Arg61, all of these residues affect the T cell response, as evidenced by Ala-substituted 52–61 peptides.

There were similarities and differences with alanine substitutions of the Gal2-S56-(52–61) peptide. Substitution of the strong Asp52 anchor affected binding and TCR recognition, as expected. Also similar to 52–61, the strong TCR contact of Tyr53, when substituted by alanine in the glycopeptide, resulted in a nonstimulatory peptide. The same result was found with the other TCR contact site, in Asn59. The main differences between the glycopeptide and the wild-type peptide were found with the Ile55, a P4 pocket anchor that affected the T cell response to the glycopeptide. We see this effect as another example of how some anchor residues affect the display of TCR contact sites (30). It may mean that the glycopeptide has a different conformation from 52–61 when bound to I-A^k molecules.

We conclude, first, that there is a fine specificity of recognition by the TCR of carbohydrate molecules anchored to MHC binding peptides. In our examples, the distal Gal of Gal2 is a contact residue for the TCR. Second, residues from the peptide also contribute directly to the binding by the TCR of our carbohydrate-specific hybridomas. Since neither the wild-type peptide nor the galabiose moiety, by themselves, trigger the T cells, we assume that each must not have sufficient binding energy to engage independently the TCR (11). Finally, the display of the distal Gal moiety with respect to the other elements within the complex is also important, as shown by the lack of response to Gal-Glc-S56-(52–61).

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