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Mapping of a Protective Helper T Cell Epitope of Human Influenza A Virus Hemagglutinin

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The synthetic peptide comprising the 317–341 region of human influenza A virus (H1N1 subtype) hemagglutinin elicits peptide-specific antibody and helper T cell responses and confers protection against lethal virus infection. Molecular mapping of the 317–329 region, which encompasses the epitope recognized by peptide-specific T cells, revealed that the minimal size required for T cell activation was the 317–326 segment. The most likely peptide alignment, which placed 320Leu to pocket 1 of the I-E^d peptide binding groove, was predicted by molecular mechanics calculations performed with the parental and with the Ala-substituted analogs. In line with the prediction data, the results of the peptide binding assay, where the relative binding efficiency to I-E^d molecules expressed on the surface of antigen-presenting cells was monitored, identified the 320–326 core sequence interacting with the major histocompatibility class II peptide binding groove. Functional analysis of Ala-substituted variants by functional assays and by calculating the surface-accessible areas of the single peptidic amino acids in the I-E^d–peptide complexes demonstrated that 324Pro is a primary contact residue for the T cell receptor. Our results show that this type of analysis offers a suitable tool for molecular mapping of helper T cell epitopes and thus provides valuable data for subunit vaccine design.

Virus-specific cellular immunity is focused to few immunodominant epitopes the generation of which is governed by the rules of antigen processing and that are selected by major histocompatibility gene complex (MHC) products and by the available T cell repertoire. CD8$^+$ cytotoxic T cells recognize octa- to decapeptides in the context of MHC class I molecules and are the major effectors against virus infected cells (1). Recent data highlighted the importance of their collaboration with virus-specific CD4$^+$ T cells in the development and maintenance of efficient anti-viral immunological memory and thus in therapeutic vaccination (2). CD4$^+$ T cells recognize MHC class II-bound peptide fragments of 15–25 amino acid length (3, reviewed in 4). Identification of potentially active peptides of minimal size, that would fit into a given MHC class II allele, is more difficult than in the case of MHC class I molecules because of the ambiguity in the alignment of peptide sequences of different lengths (5–7).

The C-terminal 306–329 region of human influenza A virus hemagglutinin 1 subunit (HA1) is relatively conserved within subtype sequences and is not affected by antigenic drift (reviewed in 8, 9). In our previous studies we identified the 317–329 region of the A/PR/8/34 (H1N1) influenza virus hemagglutinin (HA) as a subdominant but protective epitope which is the target of both antibody and CD4$^+$ helper T cell recognition (10–12). The synthetic peptide covering the 317–329 sequence induced full activation of peptide-specific T cell hybridomas in an MHC class II-restricted manner in the presence of different antigen presenting cells (APC) as monitored by IL-2 and tumor necrosis factor (TNF) secretion, increase of intracellular free calcium levels and expression of T cell activation markers (13, 14). The 317–329 sequence, elongated by the HA2$_{1–12}$ fusion peptide, acquired immmunogenicity and elicited both peptide-specific T helper and antibody responses (10, 12).

In this study a detailed mapping of the 317–329 HA1 region was undertaken to determine the minimal peptide size required for full T cell activation and to identify the critical residues which are essential for the

Abbreviations used: APC, antigen presenting cell; CDR, complementary determining region; DCC, dicyclohexyl-carbodiimide; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HA, hemagglutinin; HA0, immature hemagglutinin; HA1, subunit 1 of hemagglutinin; HA2, subunit 2 of hemagglutinin; HPLC, high-performance liquid chromatography; NHSB, *N*-hydroxysuccinimide biotin, TCR, T cell receptor.

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contact with the I-E^d molecule and with the antigen specific receptor of the IP12-7 T cell hybridoma, which represents the typical specificity pattern of the polyclonal anti-peptide T cell response (9). MHC class II-peptide interaction is attained by evenly distributed H-bonds along the peptide binding groove ensured through broadly conserved MHC residues and by polymorphic allele-specific peptide binding pockets (4, 15). The most likely alignment of the 317–329 peptide in the I-E^d peptide binding groove was predicted by molecular mechanics calculations following energy minimization of the MHC-peptide complexes as described previously (16, 17). It was also shown, that a peptide with most residues substituted to alanine retained binding to HLA-DR1 (5), a human MHC class II allele closely related to murine I-E^d. These data suggested that Ala substitution might not influence significantly the efficiency of peptide binding to MHC class II, but could alter the interaction with the T cell receptor (TCR). Therefore the analysis of systematic Ala substitutions along the peptide was applied for the mapping of primary contact residues with the TCR.

Screening of truncated and site-specific Ala substituted synthetic peptides in functional assays, which measured the magnitude of T cell activation, and relative peptide binding efficiency to the murine I-E^d molecule, revealed that the minimal size of the T cell activating peptide was the 317–326 decapeptide. This analysis also marked those residues which were essential for T cell recognition. Comparison of the surface accessible areas of the peptide amino acids and the number of their atomic contacts with amino acids of the MHC binding pockets enabled us to identify the exposed residues which might contact the IP12-7 TCR. Our results demonstrate that such analysis of structure/function relationships between MHC class II binding and T cell activation may be a useful tool for design of subunit vaccines.

MATERIALS AND METHODS

Peptide synthesis. The 317–329 peptide and its truncated analogs were synthesized as described before (9, 11). The alanine-substituted variants were synthesized by the solid-phase technique utilizing ^{t}Boc chemistry (18). The peptide chain was elongated on a *p*-methylbenzhydrylamine resin (0.48 mmol/g) (19) and the syntheses were carried out using an ABI 430A automatic machine with certain minor modifications of the standard protocol. Side chain protecting groups were as follows: Arg(Tos), Thr(Bzl), and Ser(Bzl). Couplings were performed with DCC, with the exception of Gln and Arg, which were incorporated as their HOBt-esters. The completed peptide resins were treated with liquid HF/dimethyl sulfide/*p*-cresol/*p*-thiocresol (43:3:2:1, v/v), on 0°C, 1 h (20). HF was removed and the resulted free peptides were solubilized in 10% aqueous acetic acid, than filtered and lyophilized. The crude peptides were purified by reverse-phase HPLC. The appropriate fractions were pooled and lyophilized. The purified peptides were characterized by mass spectrometry using a Finnigan TSQ 7000 tandem quadrupole mass spectrometer equipped with electrospray ion source. Peptide purities were above 97% (HPLC) and the measured Mw values were in good agreement with the calculated values in all cases (Table 1).

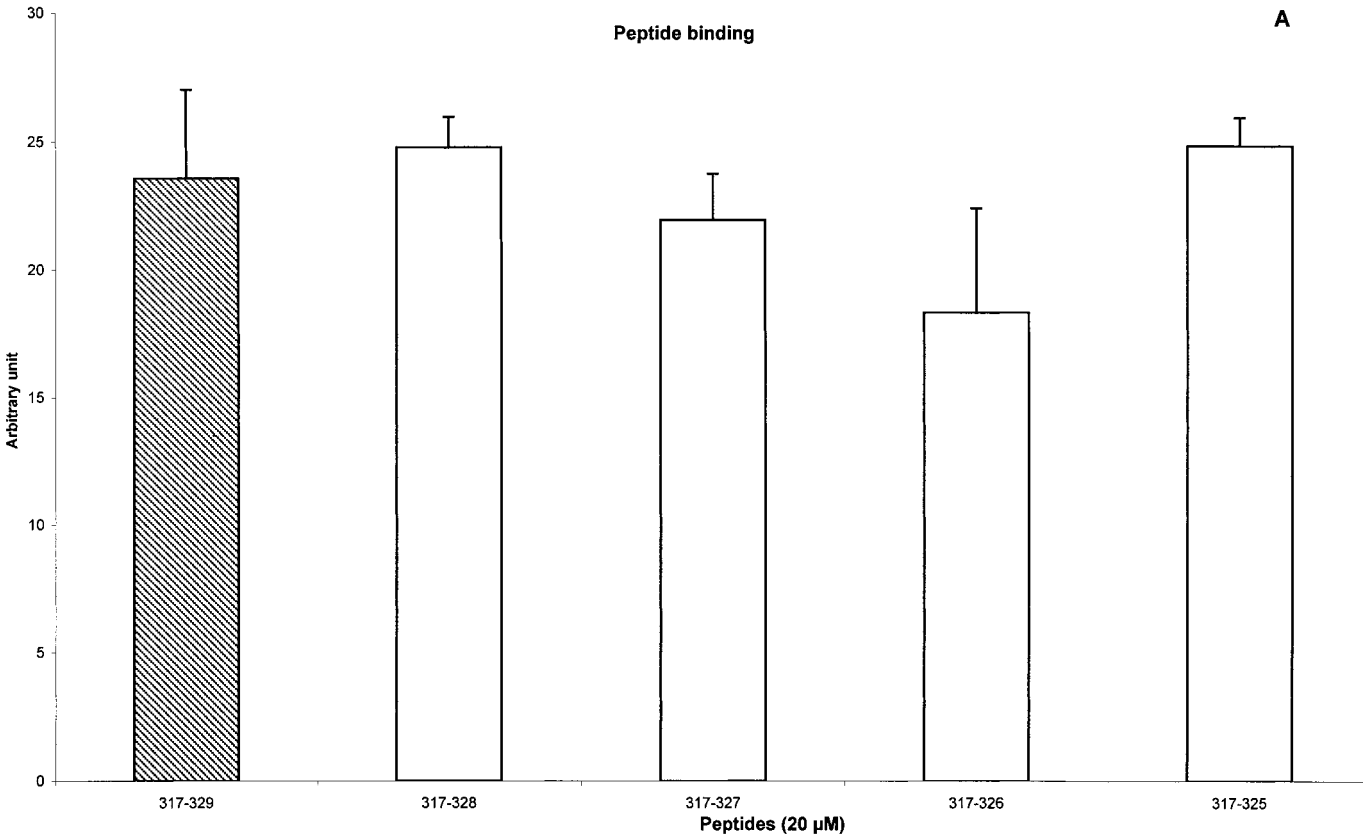
N-terminal biotinylation of peptides. The peptides listed in Table 1 were biotinylated similarly to described earlier (11). Briefly, 2 mM peptide solution was prepared in 0.1 M $NaHCO_3$ and cooled in ice. Two milligrams per microliter of biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, Hungary) dissolved in dimethyl sulfoxide (DMSO) was added to 20% molar excess. The reaction was allowed to proceed for 2 h at 0°C.

Cell lines and monoclonal antibodies. The murine B lymphoma line A20 (ATCC TIB 208) and 2PK3 (ATCC TIB 203) was used as APC in the T cell activation and in the binding assays. The IP12-7 T cell hybridoma was developed from the spleen of BALB/c mice immunized with the 317–329 (H1) peptide and subsequently infected with the A/PR/8/34 influenza A virus (9). For IL-2 quantitation the IL-2-dependent CTLL-2 (ATCC TIB 214) cell line was used. All cells were cultured in RPMI supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 5% FCS (complete RPMI).

Peptide binding assay and cytofluorometry. The peptide binding assay for the truncated peptides was performed as described earlier (11). This procedure was slightly modified for Ala-scanned peptides namely, 5×10^5 2PK3 cells, suspended in 100 μl RPMI supplemented with 2 mM L-glutamine and 0.1% FCS, were incubated in standard FACScan tubes (Falcon) with biotinylated peptides at 20 μM concentrations at 37°C for 2 h. Cells were washed twice with PBS containing 0.1% FCS and cooled in ice. Extravidin R–phycoerythrin conjugate (Sigma, Hungary) was added at 3 μl/tube and incubated for 30 min at 0°C. Cells were washed twice with cold PBS–0.1% FCS, resuspended in 0.5 ml washing solution and fluorescence intensity was measured by FACScan (Fig. 1) or FACScalibur (Fig. 2) equipment. Flow cytometric data were analyzed by using Lysis II or CellQuest software, respectively (Becton–Dickinson). Viable cells were gated out on the basis of their forward and side direction light scatter. Relative peptide binding was given as increase of mean fluorescence in arbitrary units as described previously (11).

Monitoring T cell activation by IL-2 production. T hybridoma cells (2×10^4) were cultured in 96-well flat bottom tissue culture plates (Nunc) in complete RPMI in the presence of different concentrations of peptides and 5×10^4 A20 cells. Seventy-five microliters of culture supernatants was removed at 24 h of culture and transferred to secondary cultures where the amount of secreted IL-2 was measured by the proliferation of CTLL-2 detector cells. In this assay, the indicator cells were used at 5×10^3 cells/well starting density and cell proliferation was measured by addition of [^{3}H]thymidine (9). The amount of secreted IL-2 was given as arbitrary units which were calculated from the cpm values of the titration curves. One arbitrary unit corresponded to 50% of maximal IL-2 secretion.

Molecular mechanics calculations. Simulated 3D structure and the atomic coordinates of the I-E^d molecule were derived from the X-ray data of I-E^k complexed with the murine hemoglobin 64–76 peptide obtained from the PDB code 1iea (21), by replacing the appropriate I-E^d residues in the I-E^k structure. The linker segment B2L–B16L was removed and the peptide residues from B6N to B1L were replaced by the HA317–329 peptide and by the Ala-substituted tridecapeptide variants. The equilibrium conformation of these complexes was determined by conformational energy minimization. Hydrogen atoms were added to the heavy atoms and energy minimization was performed using CVFF force field. Dielectric constant was set to $\epsilon = r$ [Å]. The cut-off distance of 15 Å was used for unbound interactions and energy minimization with steepest descent and conjugate gradient algorithms went on until the maximal derivative of the energy function was less than 0.1 kcal/mol/Å. Conformational energy minimization were carried out using the INSIGHT II software package, containing DISCOVER (Biosym Technology, Inc. San Diego, CA) on a Silicon Graphics Indigo Workstation. Contact be-



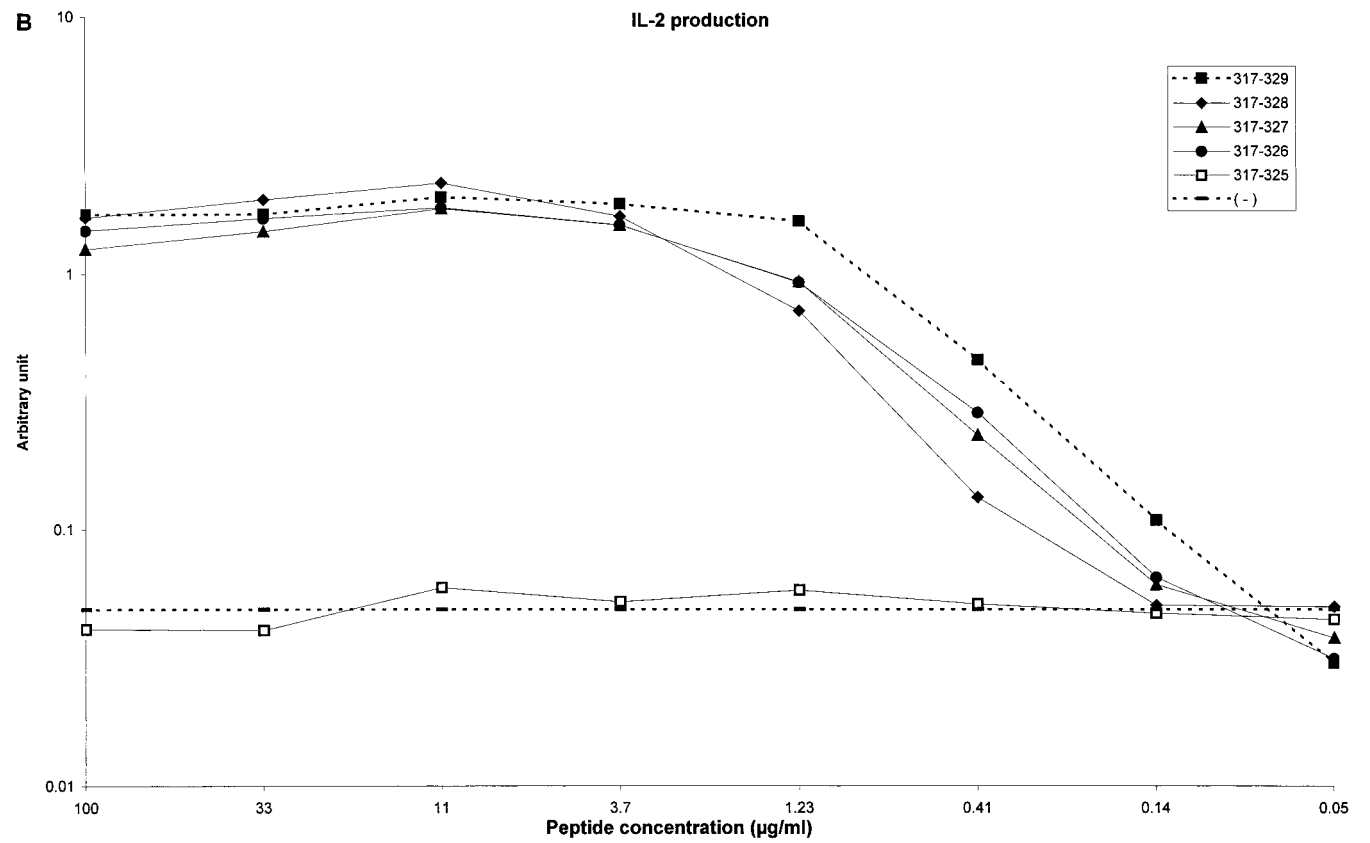
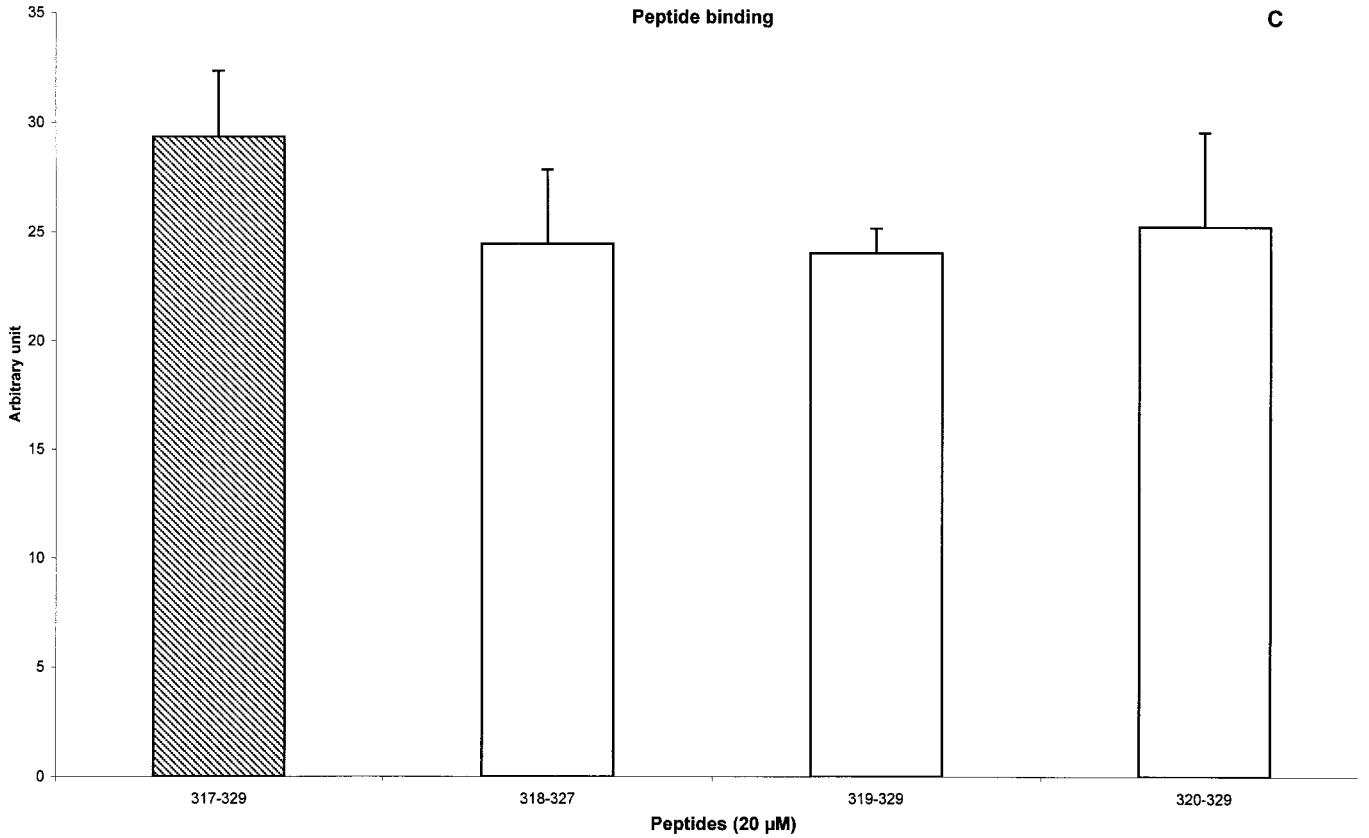


FIG. 1. The effect of C- and N-terminal truncation of the 317–329 peptide on I-E^d binding (A, C) and on the activation of IP-12-7 T cells (B, D). Measurement of T cell activation and the calculation of arbitrary units for the increase of fluorescence intensity (A, C) and for the amount of secreted IL-2 (B, D) was performed as described under Materials and Methods. Mean values of three measurements ± SD are given.



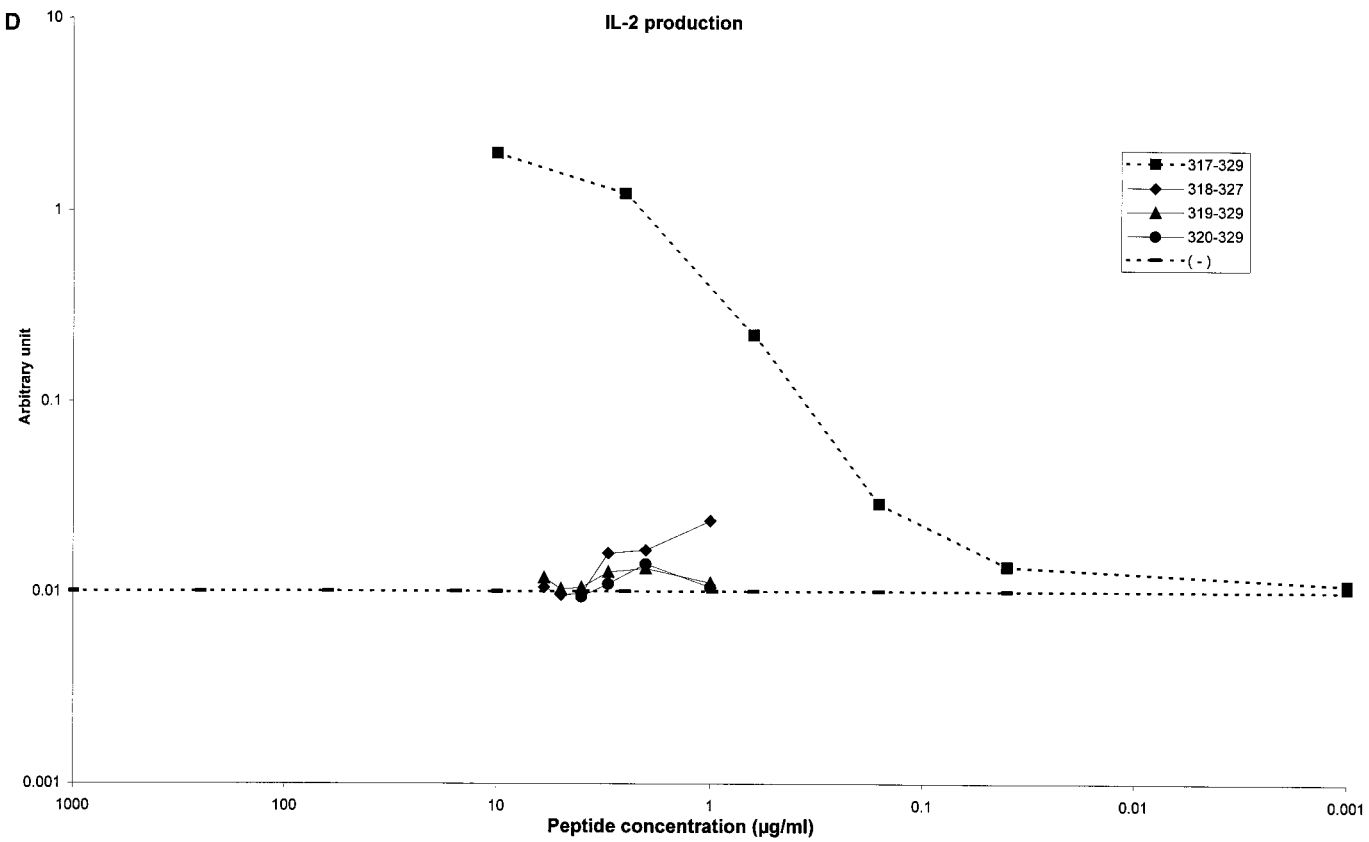


FIG. 1—*Continued*

TABLE 1

Structural and Functional Characteristics of the Alanine-Substituted 317–329 HA1 Peptides

Sequence	Code	MW calculated	MW found	Retention time	MHC binding[j]	T cell activation[k]
V**A**GLRNIPSIQSR	H1A318	1409.67	1409.3	6.82[a]	+	+++++
VT**A**LRNIPSIQSR	H1A319	1453.72	1453.0	8.49[b]	+	−
VTG**A**RNIPSIQSR	H1A320	1397.61	1398.2	6.26[c]	+	+
VTGL**A**NIPSIQSR	H1A321	1354.58	1354.4	7.86[d]	+	−
VTGLR**A**IPSIQSR	H1A322	1396.67	1397.2	4.94[e]	+	−
VTGLRN**A**PSIQSR	H1A323	1397.61	1398.0	5.07[f]	+	−
VTGLRNI**A**SIQSR	H1A324	1413.66	1413.2	10.62[g]	++	−
VTGLRNIP**A**IQSR	H1A325	1423.63	1423.3	11.05[h]	+	+++++
VTGLRNIPS**A**QSR	H1A326	1397.61	1397.2	6.66[i]	+	−

[a] 10 → 90% 15 min; [b] 10 → 90% 20 min; [c] 20 → 35% 15 min; [d] 21 → 36% 15 min; [e] 23 → 38% 15 min; [f] 18 → 33% 15 min; [g] 20 → 35% 15 min; [h] 18 → 33% 15 min; [i] 18 → 33% 15 min; [j] measured by flow cytometry with N-terminal biotinylated peptides; [k] measured by IL-2 secretion.

tween atoms was recognized when the distance between the centers of two atoms, belonging to different residues, was less than 4 Å. The solvent accessible area of peptide amino acids, which is indicative of the occupation of the corresponding pockets, was calculated by the MSRoll program, using a probe radius of 1.4 Å (22). The molecule mechanics calculations were performed on various peptide alignments that place different amino acids to pocket 1.

RESULTS

C- and N-Terminal Truncation of the 317–329 Peptide

The functional activity of truncated peptide analogs was monitored by IL-2 secretion of the IP12-7 $CD4^+$ T cell hybridoma. Figure 1 shows that elimination of the C-terminal 329–327 residues does not affect the binding to I-E^d (Fig. 1A) or the secretion of IL-2 when presented on A20 APC (Fig. 1B). Removal of the first Val residues from the N-terminus, however, almost completely abolished the T cell activating capacity of the peptide without substantial change in its peptide binding efficiency (Figs. 1C and 1D). Further truncations of the N-terminal up to 320Leu did not significantly reduced MHC binding but generated functionally inactive peptide analogs (Figs. 1C and 1D). These results show that the minimal size of the epitope, recognized by IP12-7 T cells, is the 317–326 decapeptide but efficient binding to I-E^d can be achieved with shorter overlapping peptides as well (Figs. 1A and 1C).

Ala Scanning of the 317–329 Peptide

Single amino acids of the 317–329 peptide were substituted by Ala starting from residue 317 to 326. Since residues 327, 328, and 329 were shown to be dispensable for T cell activation; therefore, their Ala modification was not tested. As summarized in Table 1 and Fig. 2 substitution of residues 319Gly, 321Arg, 322Asn, 323Ile, 324Pro, and 326Ile by Ala completely abolished T cell activation. The change of 317Val, 318Thr, or 325Ser to Ala did not affect the activating capacity of the peptide while substitution of 320 Leu to Ala resulted in substantial reduction of the T cell activating capacity compared to the parental 317–329 peptide (Figs. 2B and 2C). All these substituted analogs did bind to the I-E^d molecule albeit with slightly different efficiency (Fig. 2A). Therefore these results do not discriminate those amino acids which affect MHC binding, contact directly the TCR or alter the orientation of other TCR contact residues. Interestingly, the 324P → Ala substitution resulted in a functionally inactive peptide (Fig. 2C) despite of its increased relative binding efficiency to I-E^d (Fig. 2A). This suggests that 324Pro may be a primary contact residue for the TCR.

Prediction of Peptide Alignment and Estimation of Amino Acid Accessibility by Molecular Mechanics Calculations

Based on the known I-E^d peptide binding motif (17) several alignments could be predicted for the 317–329 peptide. Placing 320Leu to pocket 1 would define the 320–325 region as an I-E^d binding core sequence, where the surface accessible areas of 320Leu, 323Ile and 325Ser are small, while the number of atomic contacts is large (Table 2). This indicates that these residues accommodate pockets 1, 4, and 6, respectively. In line with the results obtained with the C-terminally truncated peptides none of the Ala substitutions affected the surface accessible areas or the number of atomic contacts of 326Ile, 327Gln, 328Ser, or 329Arg. The 324Pro → Ala change did not affect the surface accessible areas or the atomic contacts of other residues either but completely abolished the T cell activating capacity parallel to increasing binding to I-E^d (Table 2, Fig. 2). In contrast to the 324Pro → Ala changes, Ala modification of 318Thr, 321Arg, 323Ile, and 325Ser substantially altered the surface accessible areas and the atomic contacts of 324Pro (shaded boxes). These calculations suggest that their effect on T cell activa-

TABLE 2

The Effect of Ala Substitutions on the Solvent-Accessible Areas and Atomic Contacts of Single Amino Acids of the 317–329 Peptide

Solvent-accessible area (Å^2)

Position	−3	−2	−1	1	2	3	4	5	6	7	8	9	10
Sequence	V	T	G	**L**	**R**	**N**	**I**	**P**	**S**	I	Q	S	R
317–329	161.3	78.3	28.6	**8.7**	**63.1**	**34.6**	**6.2**	**64.9**	**10.0**	18.2	92.5	11.4	187.4
A317	127.4	82.3	28.9	10.8	65.1	36.7	8.3	63.0	8.4	19.1	93.6	10.7	193.3
A318	134.8	40.1	49.2	12.9	65.2	38.9	9.9	28.8	10.1	23.8	89.6	10.6	192.2
A319[a]	161.6	76.4	41.9	4.5	62.4	34.5	6.0	62.0	10.2	21.3	92.8	8.7	187.3
A320	162.3	72.2	31.9	11.7	66.0	32.6	7.5	69.5	9.1	20.4	92.4	9.4	188.8
A321[a]	157.4	81.4	27.0	13.6	17.7	48.1	5.5	37.2	11.6	12.6	96.5	14.4	187.4
A322[a]	156.3	77.3	33.7	9.2	67.6	27.8	7.9	56.6	8.4	20.4	93.2	9.7	186.5
A323[a]	163.2	78.0	25.7	6.9	67.4	39.4	8.3	31.6	13.4	25.6	90.7	10.8	193.0
A324[a]	160.7	76.7	26.0	10.5	63.2	41.9	11.2	73.4	10.4	16.4	92.5	13.0	185.2
A325	160.3	75.7	25.4	9.7	60.6	39.2	7.9	45.0	15.8	12.5	82.8	13.9	179.6
A326[a]	164.5	76.6	26.5	8.5	63.8	34.5	7.5	71.7	9.2	4.9	95.5	10.5	187.2

Interatomic contacts

Position	−3	−2	−1	**1**	**2**	**3**	**4**	**5**	**6**	7	8	9	10
Sequence	V	T	G	**L**	**R**	**N**	**I**	**P**	**S**	I	Q	S	R
317–329	8	20	18	**64**	**52**	**33**	**50**	**4**	**25**	44	28	37	30
A317	6	21	17	63	52	34	48	4	30	49	26	39	27
A318	24	23	14	56	53	36	51	18	25	50	29	40	28
A319[a]	8	20	20	61	53	35	50	5	28	44	27	37	29
A320	9	22	18	32	49	32	48	4	27	45	27	36	28
A321[a]	8	18	18	60	28	33	52	13	27	52	26	36	29
A322[a]	9	18	16	56	50	23	47	5	26	45	30	33	29
A323[a]	8	20	17	62	51	37	37	23	36	46	26	38	27
A324[a]	8	20	19	59	51	34	48	1	25	46	27	38	30
A325	9	20	19	58	52	35	50	8	20	50	29	34	30
A326[a]	8	20	17	57	52	32	48	4	28	33	29	34	27

[a] Residues of the Ala substitution which abolished T cell activation. Bold defines the I-E^d binding core sequence. Shaded boxes represent changed parameters compared to the parental 317–329 peptide.

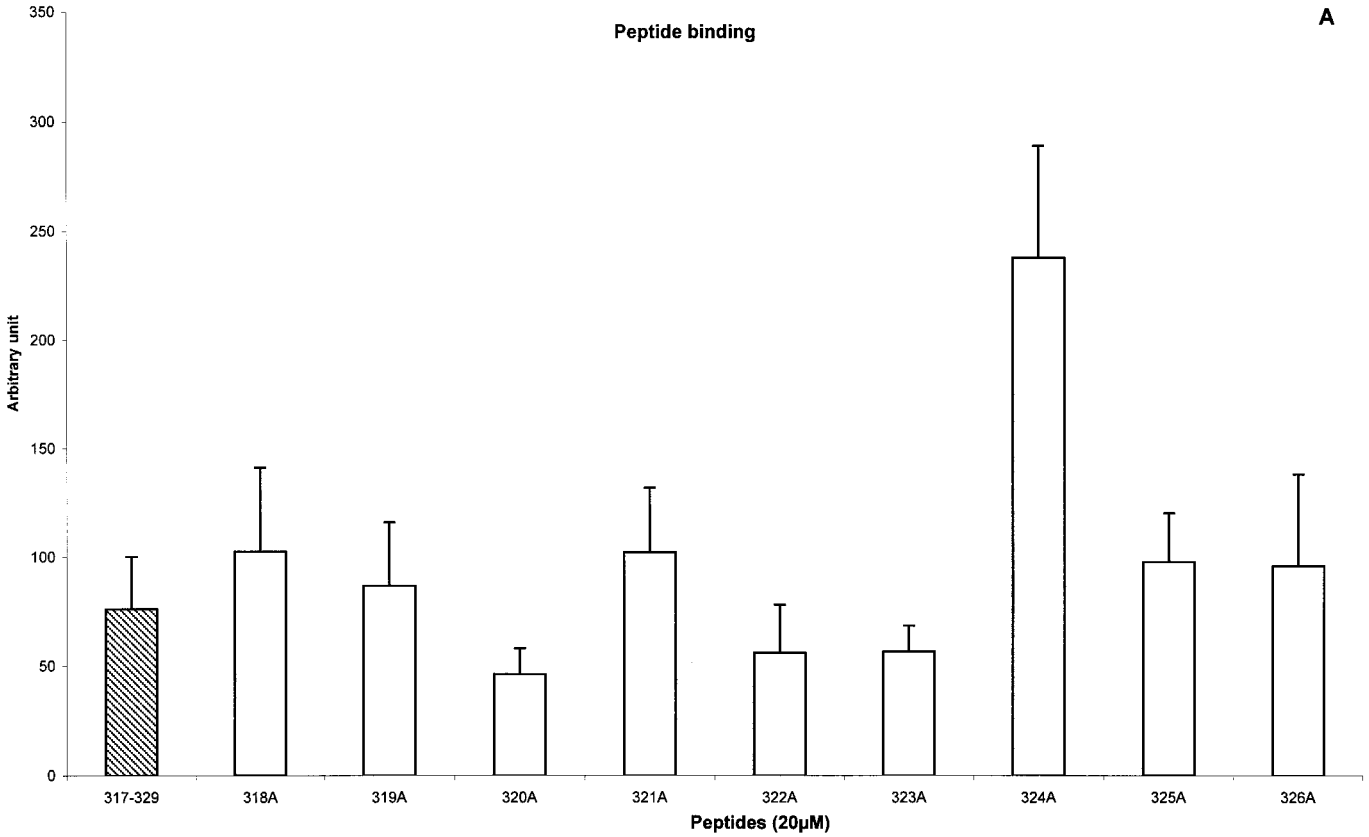
tion is rather indirect. The binding and the functional studies together combined with the theoretical approaches strongly suggested that the most probable fitting of the 317–329 peptide to I-E^d was the one that placed 320Leu to pocket 1 and marked 324Pro as a good candidate for a primary TCR contact residue. Molecular modeling studies of other alignments, where 317Val or 318Thr was placed to position 1, showed that the side chain of the residue located to relative position 5 is always pointed away from the MHC molecule. However, the Ala substitution data in these cases did not fit to the binding efficiency and functional activity data.

DISCUSSION

In this study the structural and functional mapping of a ternary complex, composed of the I-E^d MHC class II molecule, the 317–329 HA peptide and the TCR of the IP12-7 hybridoma was analyzed. Our approach applied functional assays performed with the 317–329 HA peptide and with its truncated and single Ala–substituted analogs to identify the most likely fitting of the parental tridecapeptide to the I-E^d peptide binding groove and to determine the residues which may be involved in the contact with the TCR. Our results revealed that the 320–325 HA sequence comprised the minimal core region which accommodated the peptide binding groove of I-E^d, and the 317–326 decapeptide represented the minimal T cell epitope for the IP12-7 hybridoma of the memory/effector Th1 like phenotype.

The overall similarity in the structure of various MHC molecules together with the canonical binding modes of peptides offered theoretical approaches for predicting the MHC class II–peptide interaction (7, 23, 24). Molecular mechanics calculations, combined with the results of the functional assays, predicted that the most likely alignment of the 317–329 peptide would be the one in which 320Leu accommodated pocket 1. In this case the N-terminal amino acids 317Val, 318Thr, and 319Gly, which are implicated in T cell recognition by the functional assays, reside out of the binding groove.

Previous results demonstrated that only a small proportion of the peptide is exposed for interaction with the TCR (24). It was also shown that bound water, the



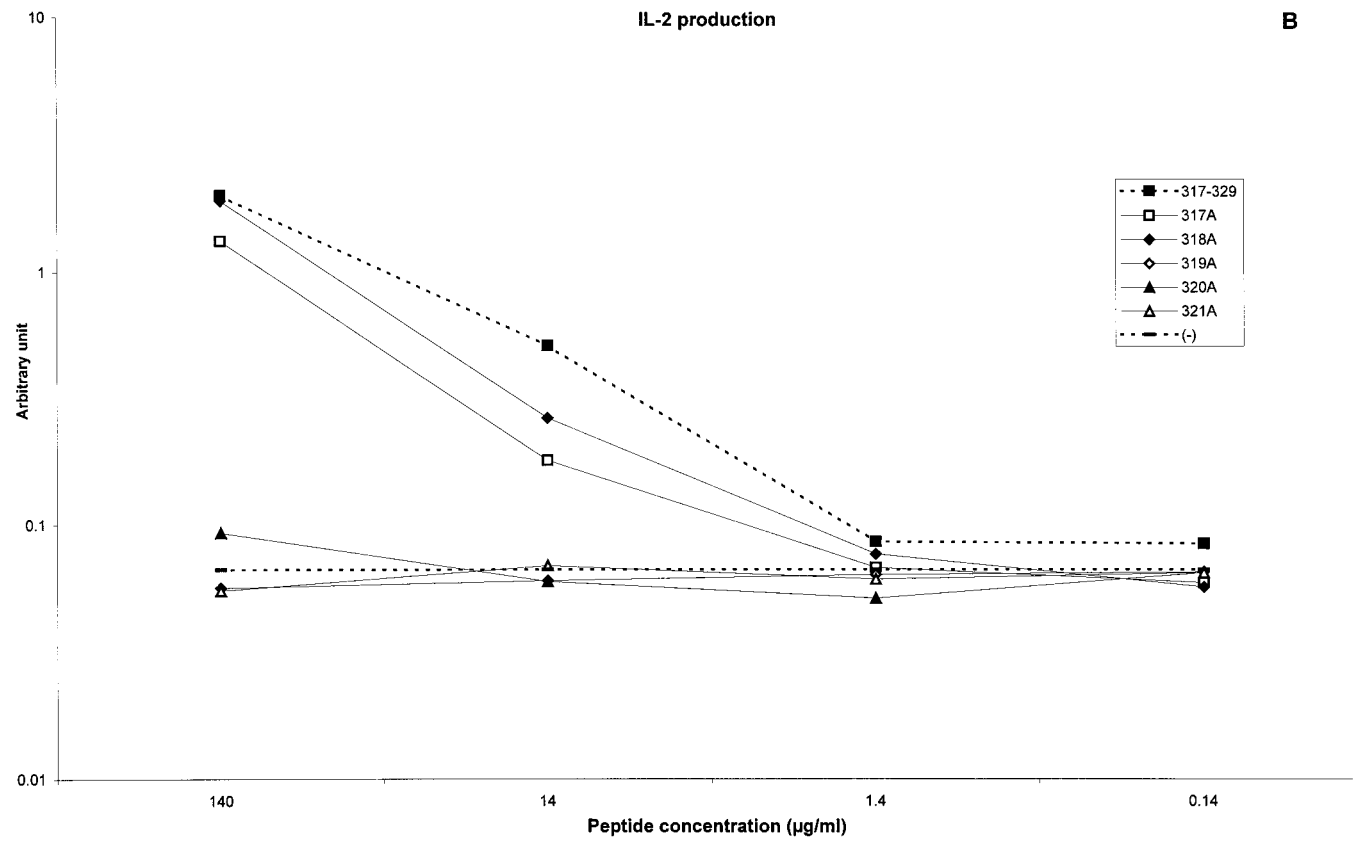


FIG. 2. The effect of Ala substitutions of the 317–329 peptide on I-E^d binding (A) and on the activation of IP-12-7 T cells (B, C). The measurement of peptide binding and of T cell activation was performed as described in the legend to Fig. 1.

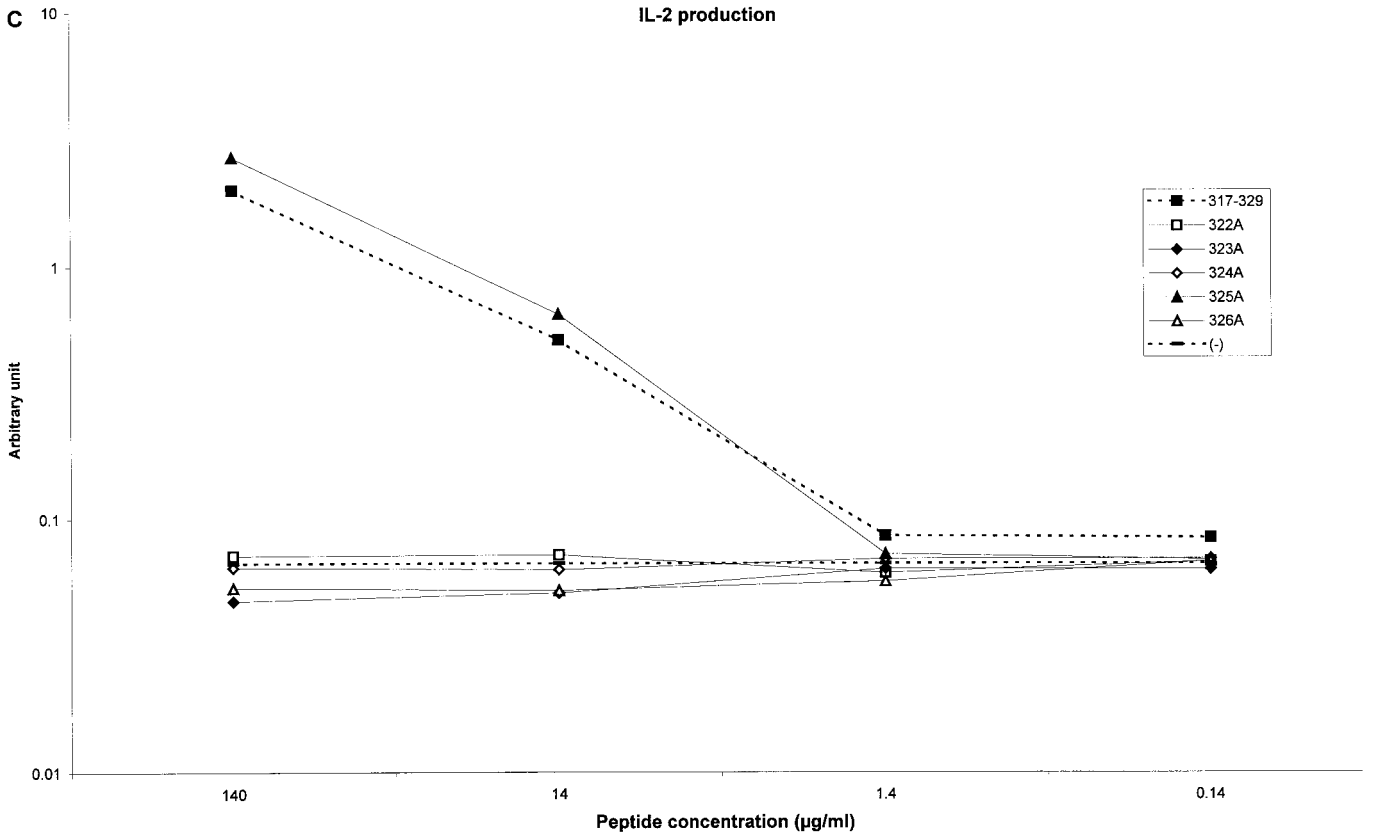


FIG. 2—*Continued*

flexibility of the central portion of the peptide and the dependence of peptidic and MHC side-chain conformations from each other could influence the mode how a given TCR interacts with a defined MHC–peptide complex (25, 26). Since the side-chain function of Ala is neutral, its space filling property offers a good tool to investigate the role of the side-chain functions of other amino acids in T-cell activation.

The TCR binds with low affinity to its ligand and only few complementary determining regions (CDR) contact with the peptide, while others of the flexible binding surface interact with the MHC protein itself (27, 28). The crystal structure of MHC–peptide–TCR complexes suggested a standard docking geometry where the CDR1 of the α- and β-chains face the N- and C-terminal of the peptide, respectively (27). The residue at relative position 5 was identified in several cases to contact with the CDR3 loops of both the α- and β-chains (29). In our system 324Pro at position 5 was identified as a critical residue which did not interact with I-E^d but was essential for T cell recognition. Analysis of the effect of Ala substitutions at positions 318Thr, 321Arg, 323Ile and 325Ser suggested that these residues were important for maintaining the original orientation of 324Pro. The functional inactivity of the peptides substituted by Ala on residues 321Arg and 323Ile could be due to their indirect effect on 324Pro thus identified as a primary TCR contact residue. 319Gly, which was not essential for the right orientation of 324Pro but was required for T cell recognition, might play a role in maintaining the MHC surface around the N-terminal of the peptide which may contact with the TCR. The other amino acids N-terminal to 320Leu may play a similar function.

The present study shows that the classical Ala scanning approach combined with computer assisted prediction methods is a useful tool for mapping MHC class II–peptide–TCR complexes and thus can be applied for subunit vaccine design.

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