CIR6–8, and CIR7–9), there is a different preference at each position depending on which successive residues were randomized. It is conceivable that this phenomenon is affected by fixed residues flanking the randomized regions. Amino acids at relative position 6 in library CIR6–8 are not fixed, so that this position tolerates several amino acids with different chemical properties. In the isolated ligands from CIR6–8, if positively charged amino acids (arginine and histidine) located at relative position 6, hydrophobic amino acids (leucine, methionine, isoleucine, and valine) were followed at relative position 7. Subsequently, arginine, serine, or hydrophobic and aliphatic amino acids were followed at relative position 8 (see clones 6.8.S1–S11 in Fig. 5*B*). At the same time, it shapes the most dominant motif in CIR6–8, and 11 of 15 agonistic clones (73%) were positive for this motif. Also if glutamine located at relative position 6, neutral or hydrophobic amino acids were followed at relative position 7, and aliphatic amino acids were followed at relative position 8 successively (6.8.S12–S14). In the case of valine located at relative position 6, arginine at relative position 7 and proline at relative position 8 were followed successively (6.8.S15 and 7.9.S1–S8). Valine at relative position 6 was fixed in the CIR7–9 library, so that it preferentially made up the most dominant V-R-P (relative position 6–8) motif (62%) and excluded other motifs. If methionine was located at relative position 7, proline or arginine followed at relative position 8 (7.9.S10 and -S11). In case of valine at relative position 7, positively charged arginine or lysine followed at relative position 8 (7.9.S12 and -S13). These observations indicate that fixed residues sometimes limit adjacent residues and exclude other recognition motifs. In this way, peptide libraries with three successive randomized residues and their overlapping system yielded different information related to TCR recognition motifs depending on which successive ones are randomized.

In summary, SA32.5 exhibited a preference for proline at relative position 3 of peptides in screening of two separate libraries; CIR2–4 and CIR3–5. This residue does not correspond to the native sequence. In addition, this TCR tolerated phenylalanine, tryptophan, and histidine with similar structural side chains at relative position 5 when combined with some successive residues different from native ones. In the C-terminal side of peptide, three overlapping libraries, CIR5–7, CIR6–8, and CIR7–9, presented distinct recognition profiles. In this case, linear combinations of residues formed by the chemical properties of each were significantly influenced. With these promiscuous interactions, all of the TCR contacts may be altered by substitutions of other residues.

*Analysis of stimulatory ligands for MK20.2 TCR: CIR2–4 to CIR7–9 libraries*

MK20.2 did not respond to most of the agonistic sequences among many peptides identified with SA32.5 (Fig. 5*B*). At relative position 2, MK20.2 TCR has a preference for methionine, leucine, and tyrosine with hydrophobic side chains and for histidine and arginine with positively charged side chains in CIR2.4 (Fig. 5*C*). It seems that this position tolerates bulky side chains, because there is no relationship among chemical properties of the amino acid side chains identified. At relative position 3, this TCR indicates a strong preference for methionine and glutamine in two overlapped libraries, CIR2–4 and CIR3–5, that do not correspond to the native sequence. In addition, isoleucine, cysteine, and threonine in CIR2–4 and leucine and isoleucine in CIR3–5 are also tolerated. At relative position 4, this TCR has a preference for leucine, methionine, valine, glutamine, and serine in two overlapping libraries (CIR2–4 and CIR3–5). In addition, phenylalanine in CIR2–4 and alanine and threonine in CIR3–5 were also tolerated. In contrast, with the screening of CIR4–6, this TCR has a preference for glutamine and serine at this position, and leucine and alanine were also tolerated at this position. At relative position 5, this TCR showed almost exclusive specificity for tyrosine in three consecutive libraries, CIR3–5, CIR4–6, and CIR5–7, which is the same amino acid as the native sequence. In addition, when serine is at relative position 6, tryptophan was tolerated at position 5. At relative position 6, several amino acids with different chemical properties were tolerated. In CIR4–6, MK20.2 TCR tolerated serine, threonine, arginine, valine, and isoleucine. In CIR5–7 and CIR6–8, small amino acids such as alanine, valine, glycine, and serine were preferred. At relative position 7, valine, serine, threonine, arginine, and aspartic acid in CIR5–7 and valine, cysteine, serine, and alanine, in CIR6–8 were permitted. However, this TCR has a preference for alanine, serine, asparagine, and aspartic acid, at this position in CIR7–9. At relative position 8, MK20.2 TCR was highly specific to lysine in two overlapping libraries, CIR6–8 and CIR7–9, as in the native $GAD65_{115-127}$ sequence. Several amino acids with different chemical properties were tolerated at relative position 9. SA32.5 responded to a limited fraction of the agonistic sequences identified with MK20.2.

In summary, MK20.2 TCR was specific for tyrosine at relative position 5 and lysine at relative position 8 of the antigenic peptide, as judged by screening data from most of the separate libraries. However, several combinations of other residues were observed depending on which successive residues were randomized.

*Combinatorial effects of multiple residues in exhibition of antigenicity*

Several investigators reported that single amino acid modifications in a given peptide exert positive or negative effects when combined in one peptide species containing multiple substitutions (16, 17, 35). These phenomena were also observed in a series of agonistic sequences we identified using the CLIP-substituted Ii library, as shown in Fig. 6. As shown in Fig. 6, *A* and *B*, SA32.5 shows decreased or totally abrogated T cell reactivity against Y5W (standing for a peptide analog having Y to W substitution at the relative position 5), Y5F, Y5H, V7R, V7 M, K8P, and S9T. However, when these residues in each position were combined with other adjacent residues, these peptides had a completely restored T cell reactivity. For instance, when QWH (4.6.S6 clone in Fig. 5; substituted residues are underlined), QFR (4.6.S4), MHH (4.6.S9), at relative positions 4–6, and RPT (7.9.S5), and MPG (7.9.S10), at relative positions 7–9, were introduced into the peptide in combination, SA32.5 exhibited marked IFN-γ production and proliferation. Although the MK20.2 T cell clone exhibited strong specificity for lysine at relative position 8 (Fig. 5*C*), combinatorial

**FIGURE 5.** Identification of diverse cross-reactive epitopes recognized by GAD65-autoreactive T cell clones using a T cell epitope expression cloning strategy. *A*, Agonistic sequences isolated with SA32.5 TCR from library CIR−1–2 and their capacities to stimulate IFN-γ production by SA32.5 and MK20.2. *B*, Agonistic sequences isolated with SA32.5 TCR from CIR2–4, CIR3–5, CIR4–6, CIR5–7, CIR6–8, and CIR7–9 libraries and responses of SA32.5 and MK20.2. *C*, Agonistic sequences isolated with MK20.2 TCR from CIR2–4, CIR3–5, CIR4–6, CIR5–7, CIR6–8, and CIR7–9 libraries and the response of SA32.5 and MK20.2. The sequences of CLIP-substituted peptides and relative stimulatory activity of IFN-γ production measured in culture with a mixture of relevant: irrelevant DNA ratios at 1:50 are summarized. Data are given as percent wild-type (wt) response. All data are expressed as the mean value of duplicate determinations ± SD.

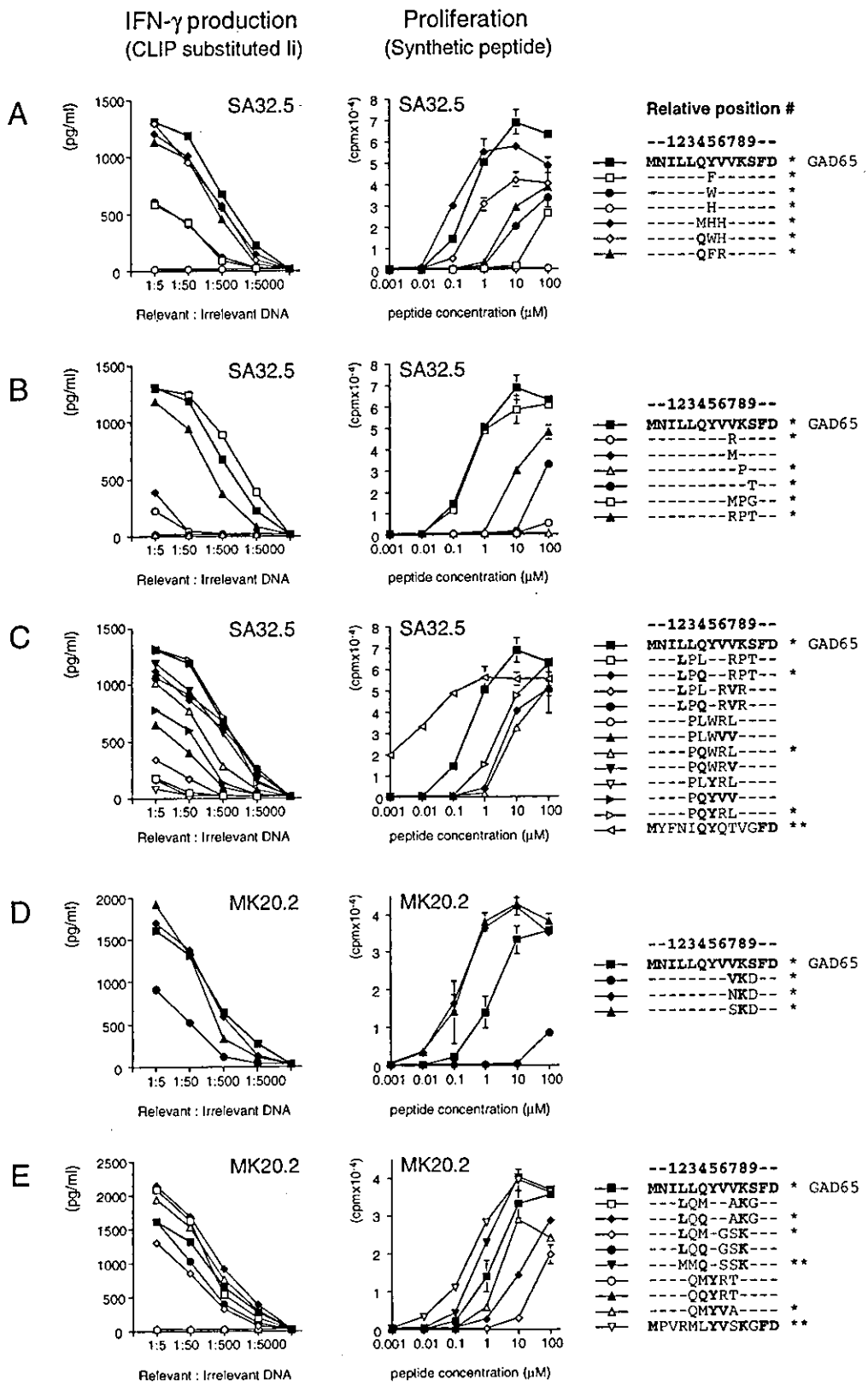



**FIGURE 6.** Combinatorial effects on TCR-recognition of multiple residues in antigenic peptide. *Left panels*, IFN-γ production stimulated by the CLIP substituted Ii chain epitope expression system in $GAD65_{116-127}$-specific T cell clones. All data are expressed as the mean value of duplicate determinations. *Right panels*, Proliferative responses of $GAD65_{116-127}$-specific T cell clones to the synthetic peptides at indicated concentrations. Medium control response without peptide were <200 cpm. All data are expressed as the mean value of triplicate determinations ± SD. Sequences of CLIP-substituted peptides encoded for by mutated Ii genes and synthetic peptides were indicated. Residues identical with $GAD65_{115-127}$ are represented in boldface. *, Peptides tested in both transfection and synthetic peptide assay; **, peptides tested only in proliferation assay to synthetic peptides.

effects were also observed for this TCR (Fig. 6*D*). For example, substitution of a single residue S9D decreased T cell reactivity but in combination with asparagine (7.9.M9) or serine (7.9.M5) at relative position 7 in combination exhibited an increased response. These data indicate that this strategy allows one to identify linear combinations of residues in antigenic peptide triggering TCRs.

To determine whether combinations of two stimulatory sequences consisting of three successive amino acids would affect TCR recognition, we constructed CLIP-substituted Ii expression vectors that encode peptides that incorporated two identified stimulatory sequences. These peptides did not always exhibit strong agonism for these TCRs (Fig. 6, *C* and *E*). It seems that substitution of multiple residues have a substantial effect on the recognition of SA32.5 TCR. It is conceivable that the overall linear combination of residues in antigenic peptide significantly affect recognition by SA32.5 TCR.

We asked whether the combinatorial effects we found in the analysis using the CLIP-substituted vector system were reproducible when epitopes were added as synthetic peptides. We synthesized 13-mer peptides and tested then for proliferative response of T cell clones to the peptides at various doses. As shown in Fig. 6, the results of proliferation assay using synthetic peptides were almost in parallel with those obtained in experiments using epitope presenting vector and quantification of IFN-γ produced by the T cell clones (Fig. 6). Therefore, we could verify the combinatorial effect also in the experiments using synthetic peptides. Thereafter, 13-mer peptides incorporating three successive amino acids which have been proven to stimulate T cell response. MYFNIQYQTVGFD for SA32.5 and MPVRMLYVSKGFD for MK20.2, were synthesized. As shown in the right panels of Fig. 6, *C* and *E*, these peptides with no resemblance to $GAD65_{115-127}$ activated T cell clones, respectively, at lower concentrations than did the native sequence, thereby indicating that simultaneous multiple residue modification deduced from the results obtained using epitope expression cloning strategy could generate superagonists.

These data strongly suggest that linear combinations of residues on antigenic peptides affect recognition by TCRs. Therefore, amino acid combinatorial effects should be considered when searching for cross-reactive epitopes. We also suggest that T cell epitope expression cloning can provide the strategy for efficient identification of optimal sequences.

### *Identification of microbial and self mimics for $GAD65_{115-127}$-autoreactive TCRs*

The results obtained from T cell epitope expression library were used to establish recognition motifs for SA32.5 and MK20.2 TCRs. As shown in Fig. 7, amino acids with similarities in chemical characteristics and with successive stimulatory combination with residues obtained from library screening that may induce mimicry were considered to provide search criteria for peptide mimics of $GAD65_{115-127}$. As for the SA32.5 TCR, motifs consisting of amino acids leucine, isoleucine, and proline at relative position 3, and tyrosine, tryptophan, and phenylalanine at relative position 5 were considered because these residues were critical for recognition by SA32.5 TCR (Fig. 5). With regard to the peptide C-terminal side (relative positions 6–8), three kinds of search criteria were considered (Fig. 7*A*), because a successive combination of residues in the antigenic peptide may significantly affect the recognition by SA32.5 TCR. As for MK20.2 TCR, amino acids methionine, glutamine, isoleucine, and leucine at relative position 3 with strong preference were considered. Amino acids tyrosine at relative position 5 and lysine at relative position 8 were fixed on the basis of exquisite specificity of MK20.2 TCR (Fig. 7*B*).

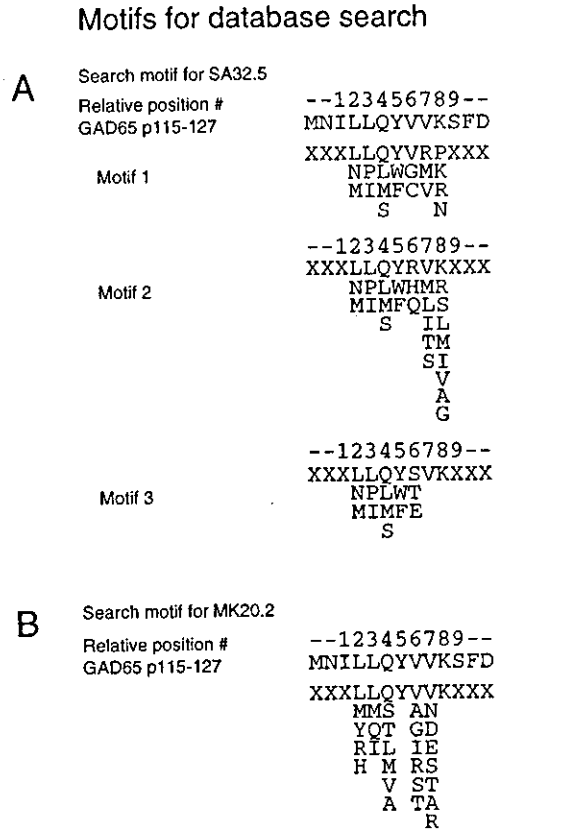



**FIGURE 7.** Summary of motifs recognized by TCRs for a database search of peptide mimics. The search motifs were considered by amino acid preference in TCR recognition. With regard to SA32.5 TCR, three kinds of search motifs were considered depending on specific recognition properties. Search motifs are given in single-letter amino acid code; X indicates all amino acids.

To identify potential mimicry epitopes from natural proteins, a pattern match search for microbial and self proteins was conducted using the SWISS-PROT (104,559 protein entries) database, the TrEMBL (560,376 protein entries) database, and the ScanProsite program (http://www.expasy.ch/tools/scnpsit2.html; January 2002). Among the candidates conforming to the criteria, we selected 47 sequences with successive stimulatory combinations recognized by TCRs. In addition, with respect to the selection of microbial peptide, proteins derived from possible infectious pathogens were considered and listed (Table II). To determine whether these candidate peptides would activate T cell clones, we constructed CLIP-substituted Ii expression vectors encoding these peptide candidates. These candidates were examined on their potential to stimulate production of IFN-γ by T cell clones using COS-7 cells expressing the CLIP-substituted Ii and HLA-DR53. Among the 47 candidates selected, SA32.5 responded to 7 candidates, *L. lactis*, *S. pyogenes*, *N. meningitidis*, *Chlamydia pneumoniae*, *Homo sapiens*/ claudin-17, tafazzin, and tafazzin-like protein-derived peptides. T cell clone MK20.2 responded to five candidates, *Legionella pneumophila*, *S. pneumoniae*, *Staphylococcus aureus*, *Rickettsia prowazekii*, and human herpesvirus 6-derived peptides.

On the basis of the data obtained, we synthesized 12 candidate peptides and tested their capacity to stimulate T cell clones (Fig. 8). These peptides stimulated each T cell clone respectively and with different activity. Peptides derived from *N. meningitidis*; putative dihydrolipoamide dehydrogenase (147–159) and self protein tafazzin (207–219) activated SA32.5 at concentrations of <1 nM

Table II. *Candidate selection for mimicry peptides*[a]

| | Mimicry Candidates for T Cell Clone SA32.5 | | | Mimicry Candidates for T Cell Clone MK20.2 | | |
|---|---|---|---|---|---|---|
| | No. | Sequence | Source | No. | Sequence | Source |
| | | Relative position no. —123456789— | | | Relative position no. —123456789— | |
| | | **MNILLQYVVKSFD** | *H. sapiens*/GAD65$_{115-127}$ | | **MNILLQYVVKSFD** | *H. sapiens*/GAD65$_{115-127}$ |
| Motif 1 | Y519 | KNLLIQFVRNHYG | *Haemophilus influenzae* | M307 | **KMSLLQYIVKNNKI** | *Legionella pneumophila* |
| | Y520 | DKNLIQFVVKGSN | *Borrelia burgdorferi* | M308 | KTSLQQYGSKDQL | *Mycoplasma pneumoniae* |
| | Y521 | TRLNPLWVRPVGS | Hepatitis G virus | M309 | **DDIMMSYAAKGFV** | *S. pneumoniae* |
| Motif 2 | Y531 | LLSLPLYHVSGQG | *H. influenzae* | M310 | **PYVLLAYIVKSVG** | *Staphylococcus aureus* |
| | Y532 | LIVNPLYRLKGYG | *Bacillus subtilis* | M311 | DAILQMYVSKFLF | *Mycoplasma capricolum* |
| | Y533 | LATLLLWHVVGAT | *Mycobacterium avium* | M312 | DTVLQMYGEKHAG | *S. typhimurium* |
| | Y534 | LGLMPLYHVVGFF | *Pseudomonas* sp. | M313 | SIILMLYATKFFE | *Mycoplasma pulmonis* |
| | Y535 | LLSLPLFHVSGQG | *Salmonella typhimurium* | M314 | RKLLQAYGAKLVL | *Listeria innocua* |
| | Y536 | **LVILPMFHVSGLS** | *L. lactis* | M315 | **ELVLMSYRDKLVL** | *Rickettsia prowazekii* |
| | Y537 | YNIMLQYRVKVES | *Vibrio cholerae* | M316 | KYLRLVYGNKILS | *Prevotella ruminicola* |
| | Y538 | AVLLPLYRLRQYA | *Bacillus halodurans* | M317 | AQNRMSYSNKDYD | *Listeria monocytogenes* |
| | Y539 | VQFNPQWQLALVA | *Pasteurella multocida* | M318 | GESMMAYAVKGHR | *P. aeruginosa* |
| | Y540 | **REILPQYQLVILA** | *S. pyogenes* | M319 | RDVMIAYATKAHV | *S. pneumoniae* |
| | Y541 | KKNLLQWQTSADS | *Clostridium acetobutylicum* | M320 | IAVRMAYSSKTPT | *Bacillus subtilis* |
| | Y542 | QEFLISYRLKIVD | *Helicobacter pylori* | M321 | TPMRLSYIEKKKG | *S. typhimurium* |
| | Y543 | **PVILPQWQSLGNR** | *N. meningitidis* | M322 | VNVMQTYTVKPGT | *Staphylococcus aureus* |
| | Y544 | PKVLPQYQSLQNW | *Chlamydia pneumoniae* | M323 | **KELLQSYVSKNNN** | Human herpesvirus 6 |
| | Y545 | **QAMNILYQTVQAF** | *C. pneumoniae* | M324 | MRNMLQYVSKNLD | Orf virus |
| | Y546 | WLMNPLFRLISKA | *H. influenzae* | M325 | IADLQQYRNKLET | Human rotavirus |
| | Y547 | SFVLPLFRVAALL | *Pseudomonas aeruginosa* | M326 | GEVRQAYGAKGFS | *H. sapiens*/Glypican-6 precursor 31–43 |
| | Y548 | RPINPLFHILVET | *S. pneumoniae* | | | |
| | Y549 | ILMLLQFRVLDRR | *Brucella melitensis* | | | |
| | Y550 | YLINPMFRIIANT | *B. melitensis* | | | |
| | Y551 | **TTLLPQWRVSAFV** | *H. sapiens*/claudin-17 24–36 | | | |
| | Y552 | **PIILPLWHVGMND** | *H. sapiens*/tafazzin 207–219 | | | |
| | Y553 | **PIILPLWHVGEPG** | *H. sapiens*/tafazzin-like protein 138–150 | | | |
| Motif 3 | Y554 | QYVMLQFTVKERP | *Treponema paraluiscuniculi* | | | |

[a] Of the peptide mimicry candidates we obtained from a pattern match search, sequences with stimulatory combinations of three successive amino acids for recognition by TCRs were selected. Candidates for mimicry peptide sequences are given in a single-letter amino acid code aligned with GAD65$_{115-127}$. Sequences stimulating significant IFN-γ production (>200 pg/ml) determined using the CLIP-substituted epitope expression system are represented in boldface.

despite a limited sequence homology to the native GAD65 sequence (Fig. 8, *A* and *B*). Peptide mimics derived from *Legionella pneumophila*, pilus assembly protein PilB (39–51) and *Staphylococcus aureus*, and hypothetical protein SAV0107 (227–239) activated MK20.2 at concentrations of <100 nM (Fig. 8*C*). In total, seven agonistic peptides derived from microbial or self protein (21.6% of the peptide mimic candidates conforming to the search criteria) were identified for SA32.5, and five agonistic peptides derived from microbial protein (25% of the peptide mimic candidates) were identified for MK20.2.

### *Cross-reactivity of T cell clones SA32.5 and MK20.2 to naturally processed microbial Ags*

To investigate whether these microbial peptide mimics identified by our library scanning can be naturally processed and presented, five recombinant proteins carrying mimicry epitopes were generated as GST-fusion proteins and tested for their capacity to induce proliferative responses of these T cell clones. As APCs, we used DCs generated from CD14$^+$ monocytes. As shown in Fig. 9, T cell clone SA32.5 responded to fusion proteins of putative dihydrolipoamide dehydrogenase (101–205) derived from *N. meningitidis* and *O*-succinylbenzoic acid-CoA ligase (101–201) derived from *L. lactis*. T cell clone MK20.2 responded to a fusion protein of glutamine amidotransferase, class I (1–104) derived from *S. pneumoniae*. However, fusion proteins derived from *S. pyogenes* and *Legionella pneumophila* did not activate either T cell clone. Furthermore, these responses were markedly inhibited by HLA-DR mAb L243 (data not shown). Cross-reactivity of the SA32.5 T cell clone to fusion proteins carrying mimicry epitopes for MK20.2 were not observed, and vice versa. These data suggest that some of these microbial peptide mimics identified by our strategy can be naturally processed in DCs and presented to GAD65-specific CD4$^+$ T cell clones.

## Discussion

In this study, we developed a novel strategy to analyze the combinatorial effects of residues in the antigenic peptides on recognition by TCRs. It allows for systematic separation and identification of diverse T cell epitopes from a mixture of randomized peptides. We showed that the combinatorial effects can be classified by analyzing the epitope sequences with agonistic properties. We searched protein databases with the defined search criteria incorporating combinatorial effect, and we identified mimicry epitopes of microbial origin, which stimulate GAD65-autoreactive T cell clones established from type I diabetes patients.

Because of the importance of CD4$^+$ T cells in autoimmunity, much effort has been directed toward identifying cross-reactive epitopes of microbial Ags recognized by autoreactive CD4$^+$ T cells. Thus far, cross-reactive epitopes have been predicted and identified by primary sequence homology, the data obtained from single-residue-substituted peptide analogs, or PS-SCLs (2, 7, 8, 36, 37). The majority of these approaches using synthetic peptides have been fundamentally based on the concept that Ag recognition surface of TCRs is relatively flat and that each amino acid on each

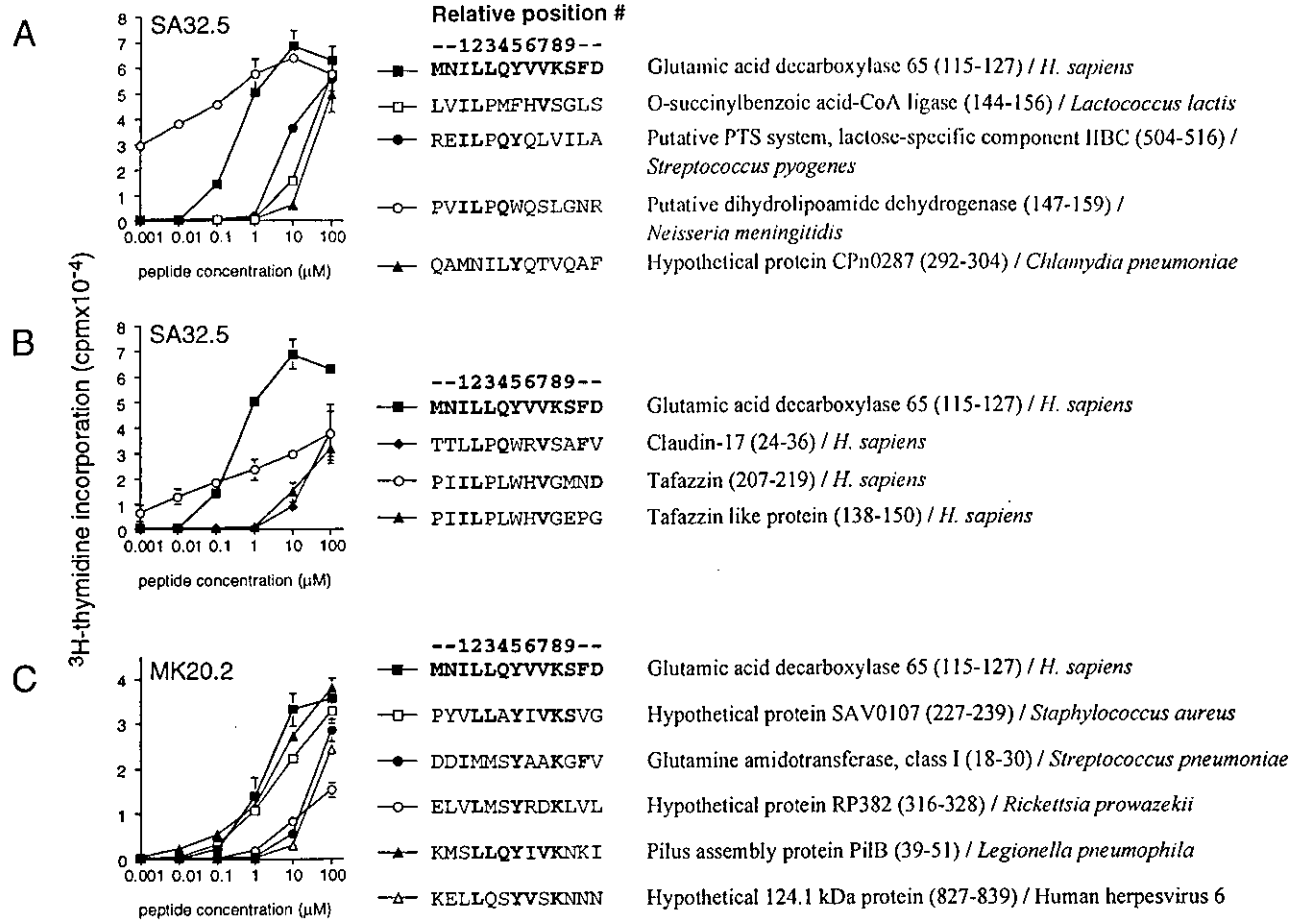



**FIGURE 8.** Proliferative response of $GAD65_{116-127}$-specific T cell clones to the mimicry peptides in comparison with $GAD65_{115-127}$. *A*, Response of SA32.5 to microbial mimicry peptides. *B*, Response of SA32.5 to self mimicry peptides. *C*, Response of MK20.2 to microbial mimicry peptides. Sequences of mimicry peptides are indicated, and residues identical with $GAD65_{115-127}$ are represented in boldface. Medium control responses without peptide were <200 cpm. All data are expressed as the mean value of triplicate determinations ± SD.

position of the peptide independently contributes to recognition by TCR (7, 38). On the basis of this assumption, mimicry epitopes have been searched in protein databases using pattern match searching with information on suitable amino acids for each position of the peptide. The use of PS-SCLs for analyses of T cell recognition has yielded the many useful concepts. However, some T cell clones did not respond to PS-SCLs (39) or responded to PS-SCLs ambiguously (40). In addition, artificial peptides composed of the optimal residue for each position selected based on analysis with PS-SCLs do not necessarily show agonistic activity, as we previously described (39). The major drawback of this method is that it identifies only the relative importance of each amino acid for each position of the peptide, and one could not directly analyze the agonistic activity induced by combinations of residues in antigenic peptides.

To address these problems, we used a totally different approach. The novelty of this system is that it enables one to directly identify sequences of T cell epitopes from plasmid-based epitope libraries composed of thousands of randomized sequences. Based on the accumulated information on sequences of agonistic peptides, one can determine the combinations of residues with agonistic properties. We used two different GAD65-specific T cell clones, SA32.5 and MK20.2, expressing distinct TCR but recognizing the same epitope with the same restriction element (25). At first, we verified that two T cell clones expressed single TCR, then we identified epitopes agonistic to each of these two TCRs from the epitope libraries and compared their sequences.

It is clear that TCRs of SA32.5 and MK20.2 represent distinct cross-reactivity and different recognition profiles (Fig. 5, *B* and *C*). It was observed that SA32.5 TCR tolerates structurally related amino acids at position 5 only when combined with specific adjacent residues (Figs. 5*B* and 6*B*). The patterns of the recognition profiles significantly differ depending on where randomized residues had been inserted. These findings were observed mainly on the peptide C-terminal side (Figs. 5*B* and 6*B*). Also, overall combinations of residues in the antigenic peptide affect the recognition by SA32.5 TCR more significantly than that by MK20.2 TCR (Figs. 5*B* and 6, *A–C*). Notably SA32.5 TCR permits the exchange of residues at all of the positions, as reported (41). In contrast, MK20.2 TCR permits specifically tyrosine and lysine for relative positions 5 and 8, respectively, even when residues around them are randomly exchanged (Fig. 5*C*). These residues are also conserved in the original $GAD65_{115-127}$. However, even in the case of this TCR, significant amino acid combinatorial effects were observed in some positions (Figs. 5*B* and 6*D*).

As shown in Table III, MK20.2 had the same V$\beta$3.1 usage as did HA1.7, and it was reported that V$\beta$3.1 was predominantly expressed in $HA_{306-318}$-specific TCRs which preferred P8K of $HA_{306-318}$ (42). Molecular modeling (Fig. 10*A*) of the fit of MK20.2 TCR to the recent crystal structure of HA1.7 TCR-HA peptide/HLA-DR1 complex (42) was done. As shown in Fig. 10, the model predicted that the D28$\beta$ and E30$\beta$ of the CDR1 loop of TCRV$\beta$3.1 made a charged interaction with lysine at position 8 of

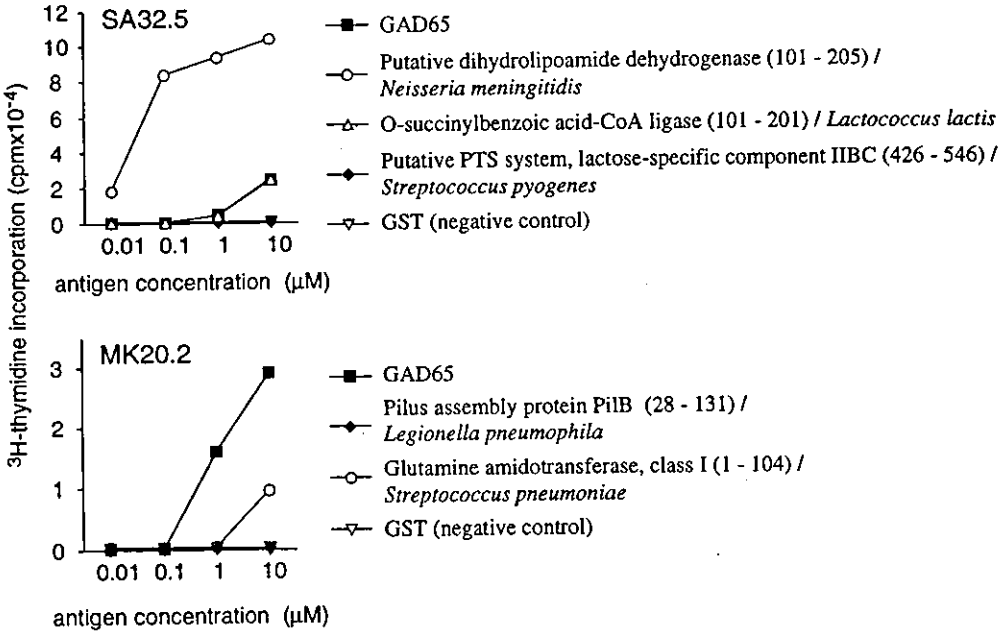



**FIGURE 9.** Proliferative response of $GAD65_{116-127}$-specific T cell clones to recombinant proteins carrying the mimicry peptides in comparison with GAD65 protein. *A*, Response of SA32.5 to microbial proteins: putative dihydrolipoamide dehydrogenase (101–205)/*N. meningitidis*, *O*-succinylbenzoic acid-CoA ligase (101–201)/*L. lactis*, and putative PTS system, lactose-specific component IIBC (426–546)/*S. pyogenes*. *B*, Response of MK20.2 to microbial proteins: glutamine amidotransferase, class I (1–104)/*S. pneumoniae* and pilus assembly protein PilB (28–131)/*Legionella pneumophila*. Medium control responses without recombinant protein were <150 cpm. Data are expressed as the mean value of duplicate determinations ± SD.

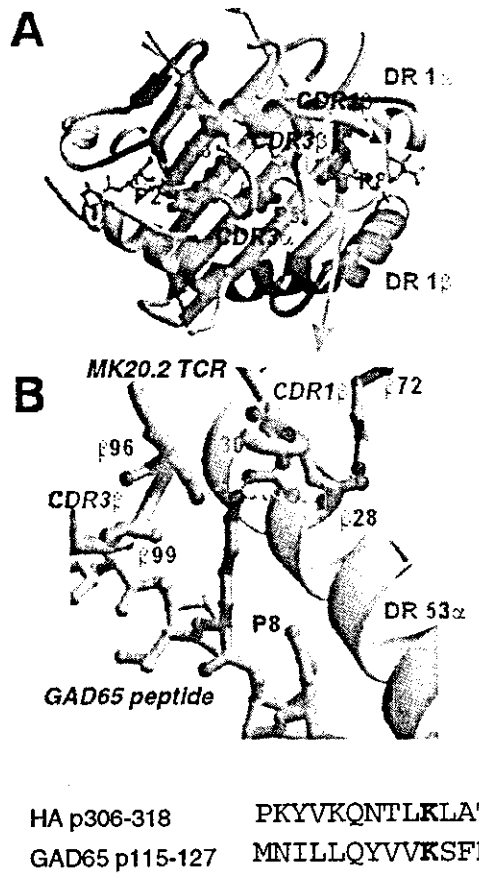
HLA-DR53-bound $GAD65_{115-127}$ peptide (Fig. 10*B*), thus implying the exclusive specificity for lysine at relative position 8. In contrast with BV3S1, BV9S1 of SA32.5 TCR uses the small amino acid glycine in 28β and the negatively charged amino acid aspartic acid in 30β (Table III), which would weaken electrostatic interactions toward lysine at position 8. This observation seems consistent with the finding that SA32.5 TCR did not have a strong preference for lysine at position 8 (Fig. 5*B*).

The HLA-DR53 binding motif has not yet been determined, and only a few peptides with binding affinity to HLA-DR53 molecule have been reported. However, the data obtained in our study led us to speculate on the peptide-binding properties to HLA-DR53 molecules. In a recent study on HLA-DR53-binding peptides, the molecule did not show any amino acid preference in P1 position (45). In fact, although HLA-DR53 possess pocket-1 consisting of valine at DRβ86, which commonly accommodates aliphatic residues, it permits lysine and tyrosine (45). These results are consistent with our finding that relative position 1 permits several residues with different chemical properties (Fig. 1). However, the peptide sequences isolated from the epitope expression library indicate that relative position 1 preferred aliphatic residues. A more comprehensive scan may yield the information of this issue. In celiac disease, the disease-susceptible HLA-DR53 molecule selectively binds a large number of gliadin-derived peptides that are extremely rich in glutamine and proline (46). Accordingly, it was suggested that one or more peptide binding pockets of HLA-DR53 molecules preferentially engaged the side chain of glutamine or proline. In the TCR recognition motif of the T cell clone SA32.5, proline at relative position 3 and glutamine at relative positions 4 and 6 are the most important residues for TCR agonism (Fig. 5*B*). Although the two TCRs represented a distinct specificity at relative positions 3, 5, 7, or 8 of the peptides, SA32.5 TCR and MK20.2 TCR represented similar preferences for certain amino acids at some positions. For example, at relative position 1, both TCR tolerate phenylalanine, methionine, leucine, isoleucine, valine, and cysteine; at relative position 4 they tolerate leucine, methionine, glutamine,

Table III. *Comparison of CDR sequences of TCRβ chains of $GAD65_{115-127}$-specific MK20.2, SA32.5, and $HA_{306-318}$-specific HA1.7*[a]

| T Cell Clone | Vβ usage | CDR1β | CDR2β | CDR3β |
|---|---|---|---|---|
| | | 28 30 | 48 51 54 56 | 96 99 |
| HA1.7 | TCRBV3S1 | ECVQD MDHENMFW | IYFSYDVKMKEKGD | CASSSTGLP--YGYTF |
| | | K(P8) | DR67α K DR39α K | K(P8) |
| MK20.2 | TCRBV3S1 | ECVQD MDHENMFW | IYFSYDVKMKEKGD | CASSSTGVS--PGELF |
| SA32.5 | TCRBV9S1 | KCEQN LGHDTMYW | MFSYNNKELIINET | CASSPTGQGAHTGELF |

[a] As determined in a crystallographic analysis (42), interactions between residues that provide TCR-DR binding sites between α-helical structure of the DRα chain and the CDR2 region of HA1.7 TCR are lined with dotted doublet lines. Electrostatic interactions between P8K of HA (peptides 306–318) and the CDR region of HA1.7 are indicated by solid doublet lines. Predicted interactions between residues of CDR region of MK20.2 or SA32.5 TCR and those of the $GAD65_{115-127}$/DR53 complex are indicated and lined.




HA p306-318 PKYVKQNTLKLAT
GAD65 p115-127 MNILLQYVVKSFD

**FIGURE 10.** Structure of HA1.7 TCR-HA/DR1 complex (*A*) and molecular modeling of MK20.2 TCR-GAD65/DR53 complex (*B*). *A*, Structure of the HA1.7 TCR-HA/DR1 complex (42) and relative orientation of the CDR loops of HA1.7 TCR on top of HA/DR1 complex. CDR loops of TCR V$\alpha$ and V$\beta$ chains are displayed in tubes and colored yellow and orange, respectively. $HA_{306-318}$ peptide is shown in a ball-and-stick model; its TCR binding residues are red and the others are gray. DR $\alpha$-chain is cyan, and the $\beta$-chain is blue. *B*, Molecular modeling of predicted electrostatic interactions between P8 lysine of $GAD65_{115-127}$ and acidic residues of MK20.2 TCR. Here the homology model of MK20.2 TCR-GAD65/DR53 complex was based on the above HA1.7 TCR-HA/DR1 complex. The residues on CDR1 and three of MK20.2 TCR V$\beta$ chain are orange. The GAD65 peptide is shown in a ball-and-stick model, and P8K is green. The DR53 $\alpha$-chain is displayed in cyan. Electrostatically active atoms are red and blue (negatively and positively charged, respectively). Numbers represent the position of relevant side chain residues. Hydrogen bonding and electrostatic interactions are dotted yellow lines. This figure was produced by BOBSCRIPT (43) and Raster 3D (44). The peptide sequences of $HA_{306-308}$ and $GAD65_{115-127}$ are given in single-letter amino acid code.

and serine; and at the relative position 6 neutral or positively charged residues and small aliphatic amino acids are allowed (Fig. 5). These residues may be HLA-DR53 anchor residues; this remains to be elucidated. It is conceivable that findings observed in this study also reflect peptide-binding characteristics in which anchor combinations are important for HLA binding, as described (47). Not only requisition for direct TCR contact but also constraints of HLA-DR53 binding could explain the combinatorial effect observed on the peptide C-terminal side, i.e. a positively charged amino acid is required at positions 122–124 in combination with a bulky amino acid at position 121 for the recognition by SA32.5 TCR.

Several groups of investigators have reported epidemiologic correlations between infection and type I diabetes (48, 49). Among the cross-reactive ligands identified in this study, epitopes derived from *N. meningitidis*, *S. pneumoniae*, and *L. lactis* can actually be produced from proteins by APCs to stimulate the T cells (Fig. 9). *N. meningitidis* is a Gram-negative and pathogenic bacterium. The outcome of meningococcal infection ranges from asymptomatic carriage to meningitis and fulminant meningococcemia in children and young people (50). *S. pneumoniae* is a Gram-positive and pathogenic bacterium, which causes bacterial sepsis, pneumonia, meningitis, and otitis media in young children (51). *N. meningitidis* and *S. pneumoniae*, which are also human respiratory commensals, in common can spread in the bloodstream under an immunocompromised state, etc. It is conceivable that these bacteria normally residing as harmless commensals may spread and participate in priming of autoreactive T cells and increase memory pools in the periphery. Also, asymptomatic carriages may predispose genetically susceptible children and initiate the autoimmune process. *L. lactis* is a nonpathogenic Gram-positive bacterium, used to produce fermented foods and dairy goods. When taken orally with food, this bacterium is metabolically active in all compartments of the intestinal tract (52). It is presumed to be taken up by M cells in intestinal lymphoid organs and to sensitize T cells. Although its relationship to autoimmune process has yet to be reported, it is intriguing in consideration of a recent report that short term breast-feeding and the early introduction of cow's milk-based infant formula can predispose genetically susceptible children to type I diabetes (53).

In conclusion, we established a novel system to analyze the combinatorial effects of residues in the antigenic peptide on recognition by HLA class II-restricted TCR. The degree of combinatorial effects differs depending on part of the antigenic peptide and structure of TCRs, even when the epitope and restriction molecule are identical. Importantly, the defined TCR recognition motif incorporating combinatorial effects proved useful in identifying mimicry epitopes of autoreactive TCRs. The findings demonstrate the importance of combinatorial effects of each amino acid residue in the antigenic peptide on TCR recognition and propose a new direction for examining cross-reactive epitopes of TCR in investigating autoimmunity.

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# Unique T cell proliferation associated with PKCμ activation and impaired ZAP-70 phosphorylation in recognition of overexpressed HLA/partially agonistic peptide complexes

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Altered peptide ligands (APL) induce T cell responses different from those induced by the original agonistic peptide. As shown for CD4⁺ T cells, partial agonists induce partial T cell activation without proliferation because of lower affinities and higher off rates to TCR than those of agonists. To determine whether overexpression of partially agonistic TCR ligands on antigen-presenting cells provides high-avidity TCR ligands, we generated L cell transfectants expressing various numbers of HLA-DR4 covalently linked with APL derived from a streptococcal peptide and observed responses of the cognate T cells. Some overexpressed HLA-DR4/partially agonistic APL complexes induced T cell proliferation in a density-dependent manner. However, tyrosine phosphorylation of zeta-associated protein-70 (ZAP-70) and linker for activation of T cells and kinase activity of ZAP-70 were not detectable. T cell proliferation stimulated with L cell transfectants was sensitive to the PKC inhibitor Gö6976, but to a lesser extent to Gö6983, suggesting the involvement of μ isotype of PKC (PKCμ). *In vitro* kinase assays revealed that PKCμ activity was up-regulated only in T cells stimulated with L cell transfectants that induced T cell proliferation. Our data suggest the presence of a unique signaling pathway coupling TCR ligation with T cell proliferation associated with PKCμ activation and impaired ZAP-70 activation.

**Key words:** Altered peptide ligand / Protein kinase Cμ / Protein kinase D / T cell activation / ZAP-70

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## 1 Introduction

CD4⁺ T cells recognize antigenic peptides in the context of self-MHC class II and are activated to secrete cytokines, to up-regulate expression of various cell surface molecules and finally to proliferate. Analogues of the antigenic peptides (or altered peptide ligands, APL) carrying single amino acid substitutions induce T cell responses different from those induced by the original peptide [1–3]. There are four known types of T cell responses to APL; full and partial agonism, simple antagonism and null. Partial agonists induce partial activation of T cells such as IL-4 production [1], $H^+$ release [4], increase in intracellular $Ca^{2+}$ concentrations [4–6], cell volume increase and up-regulation of T cell activation markers [7, 8] without causing proliferation, and cell survival [9].

Partial agonists cannot induce full CD4⁺ T cell activation, as explained by the so-called "kinetic model". MHC complexed with partially agonistic or simply antagonistic peptides have a lower binding affinity to TCR and a larger off rate, as compared with bindings with agonistic peptides [10–13]. Hence, there may be insufficient time and/or number of recruited interactions between TCR and their ligands so as not to provoke full activation signals in T cells. The question raised is whether the partial agonists and simple antagonists can overcome their lower binding affinity to TCR if present in excessive amounts and become agonistic to induce T cell proliferation. To address this question, we took advantage of (1) the well-characterized human CD4⁺ T cell clone YN5–32, which recognizes a streptococcal peptide M12p54–68 in the

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**Abbreviations: APL:** Altered peptide ligand **GST:** Glutathione S-transferase **HVS:** *Herpesvirus saimiri* **RPA:** Ribonuclease protection assay **PLC:** Phospholipase C **pY:** Phosphotyrosine **ZAP-70:** Zeta-associated protein-70 **LAT:** Linker for activation of T cells **ERK:** Extracellular signal-regulated kinases



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context of HLA-DR4 [6, 8, 14], and (2) mouse L cells expressing various amounts of surface HLA-DR4 molecules with covalently linked M12p54–68 or its single-amino-acid-substituted APL. Using the T cell clone and the L cell transfectants, we investigated the relationship among T cell responses, activation of signaling molecules and density of various TCR ligands in this study.

## 2 Results

### 2.1 Expression of HLA-DR4 molecules with covalently linked APL on the surface of L cell transfectants

We investigated detailed responses of the human CD4+ T cell clone YN5–32 to 156 single-amino-acid-substituted APL derived from streptococcal M12p54–68 [8]. We found that a number of APL induced partial activation of YN5–32 cells and stimulated increase in cell volume and up-regulation of CD markers, without inducing proliferation. We then asked whether these partially agonistic TCR ligands could induce T cell proliferation if overexpressed on APC. We generated a series of mouse L cell transfectants expressing various HLA-DR4 and M12p54–68-derived APL complexes and investigated the proliferative responses of YN5–32 cells. The APL used and their antigenicities are summarized in Table 1. Fig. 1 shows flow cytometric profiles of HLA-DR expression in each L cell transfectant. M12DR4 cells are L cells expressing HLA-DR4 with covalently linked wild-type M12p54–68. E58N, Q59G, Y61V, E63V, E63S and E63R cells are L cells expressing HLA-DR4 with covalently linked respective APL. L-DR4 cells and CD20DR4 cells express HLA-DR4 alone and HLA-DR4 with covalently linked irrelevant human CD20p26–45 with binding capacity to HLA-DR4 [15], respectively. The estimated numbers multiplied by $10^{-4}$ of HLA-DR4/peptide complexes per L cell are indicated in Fig. 1. The number of HLA-DR4/peptide complexes on the surface of L mock cells transfected with empty vectors, was assigned to be zero.

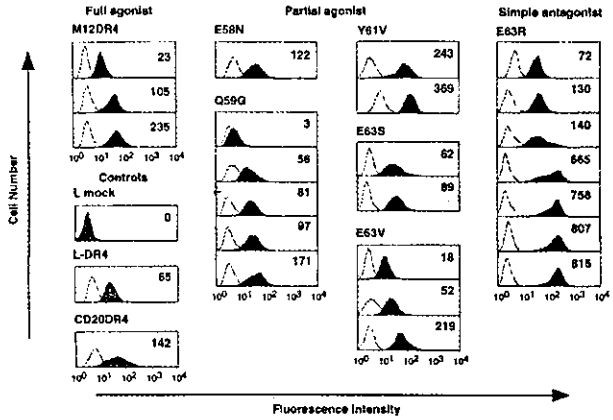



*Fig. 1.* Expression profiles of HLA-DR in L cell transfectants. M12DR4 cells express HLA-DR4 with covalently linked wild-type M12p54–68. E58N, Q59G, Y61V, E63S, E63V and E63R cells express HLA-DR4 with covalently linked respective APL. L mock cells were transfected with empty vectors. L-DR4 and CD20DR4 cells express HLA-DR4 alone and HLA-DR4 with covalently linked irrelevant CD20p26–45, respectively. Each transfectant was cloned using a cell sorter. Filled histograms indicate cells stained with FITC-conjugated anti-HLA-DR mAb and the broken lines indicate those stained with FITC-conjugated control antibody. The cell surface numbers of HLA-DR4 complexes $\times 10^{-4}$ are shown.

**Table 1.** Sequences of M12p54–68 and its analogue peptides used in this study

| peptide | sequence[a] | antigenicity[b] |
|---|---|---|
| M12p54-68 | NRDLEQAYNELSGEA | full agonist |
| E58N | NRDLNQAYNELSGEA | partial agonist |
| Q59G | NRDLEGAYNELSGEA | partial agonist |
| Y61V | NRDLEQAVNELSGEA | partial agonist |
| E63S | NRDLEQAYNSLSGEA | partial agonist |
| E63V | NRDLEQAYNVLSGEA | partial agonist |
| E63R | NRDLEQAYNRLSGEA | simple antagonist |

a) Substituted amino acid residues of analogue peptides are underlined.
b) According to results on stimulatory activities of peptide-pulsed APC reported in [8].

### 2.2 HLA-DR4 molecules with covalently linked partially agonistic APL induced T cell proliferation that depended on expression levels

Using L cell transfectants, we examined T cell proliferative responses to HLA-DR4/peptide complexes in the absence of exogenously added peptide (Fig. 2). L-DR4 cells pulsed with these APL up to 200 μM induced no T cell proliferation [8]. In Fig. 2 and hereafter, numbers in parentheses following the clone names indicate the numbers of the HLA-DR4/peptide complex $\times 10^{-4}$/cell.

L-DR4(65) cells prepulsed with 50 μM of M12p54–68 induced a strong T cell proliferative response, while L-DR4(65) cells alone did not. M12DR4 cells, which express HLA-DR4 covalently linked with fully agonistic M12p54–68, stimulated the plateau level T cell responses even at $2.3\times10^5$ /HLA-DR4/peptide complexes/cell. Interestingly, the HLA-DR4 complexed with the partially agonistic Q59G peptide induced T cell proliferation in a density-dependent manner. This ligand stimulated detectable T cell responses even at $3\times10^4$/cell, and Q59G(171) cells induced full response comparable to M12DR4 cells and L-DR4(65) cells prepulsed with 50 μM of M12p54–68. The HLA-DR4 complexed with partially agonistic E63S or E63V peptide induced no T cell proliferation at a relatively lower number, $2\times10^5$–$6\times10^5$/cell. However, when increasing the surface number ($9\times10^5$–$2\times10^6$/cell), these cells induced a weak but definite T cell proliferation (Fig. 2). HLA-DR4 complexed with other partially agonistic ligands, E58N and Y61V, did not induce T cell proliferation, even at $1\times10^6$–$3\times10^6$/cell. Therefore, not all but some partially agonistic TCR ligands can induce T cell proliferation if present at high density, and it seems likely that each partially agonistic APL has a different potential to become agonistic. On the other hand, HLA-DR4 complexed with the simply antagonistic E63R peptide did not stimulate T cell proliferation even at $8\times10^6$/cell (Fig. 2). CD20DR4 cells, which express a relatively large number ($1.4\times10^6$/cell) of HLA-DR4/CD20p26–45 complexes, had no apparent effect.

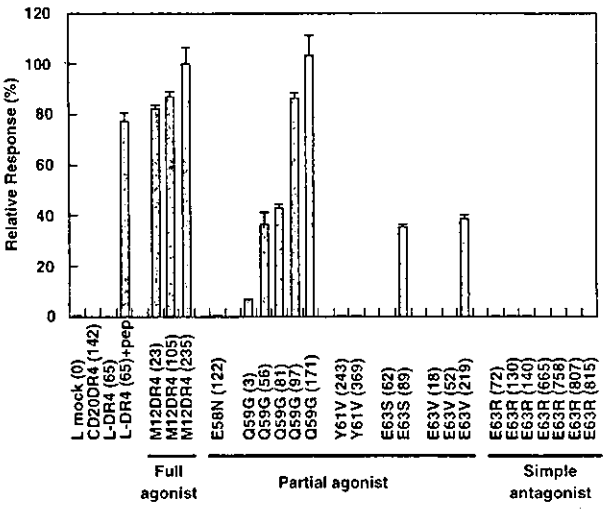
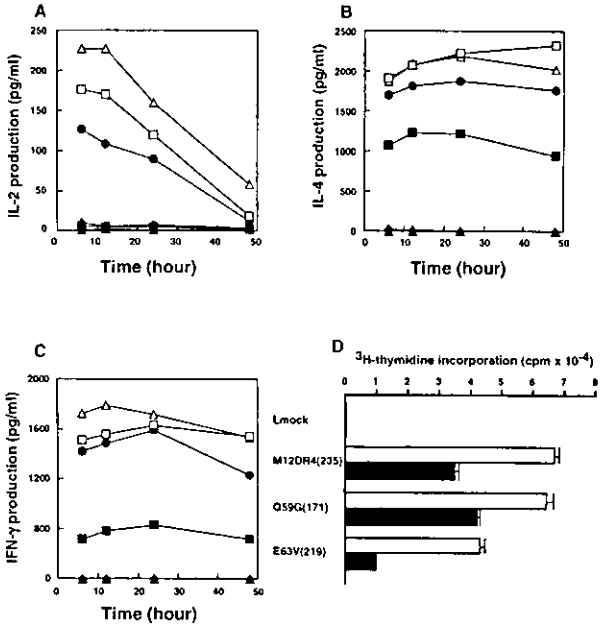


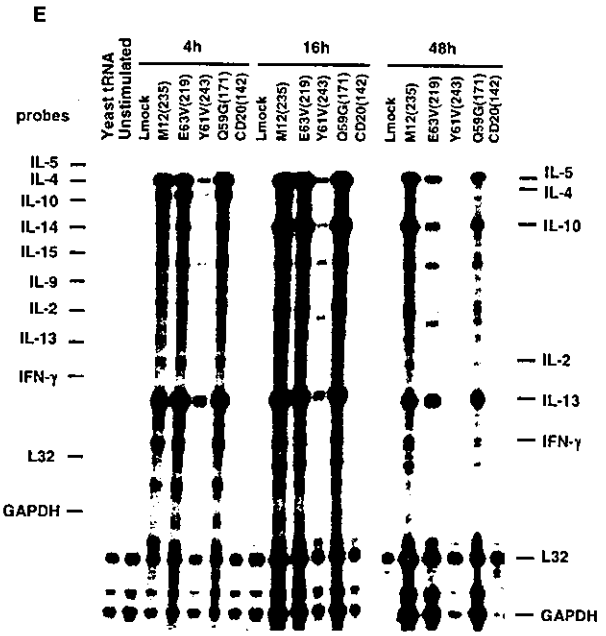

*Fig. 2.* Proliferative T cell response stimulated by L cell transfectants expressing various numbers of HLA-DR4/APL complexes. T cell proliferation was quantified by measuring [$^3$H]-thymidine incorporation. The average of triplicate T cell responses to M12DR4(235) (80,000–100,000 cpm) was assigned to be 100% and T cell response to each HLA-DR4/APL complexes was normalized. Numbers in parentheses indicate the number of the HLA-DR4/peptide complexes $\times 10^{-4}$/cell. L-DR4(65)+pep indicates L-DR4(65) cells prepulsed for 12 h with 50 μM M12p54–68. Mean ± SD of triplicate assay is indicated for each response.

### 2.3 Cytokine production by T cells co-cultured with L cell transfectants

We next checked the cytokine production by YN5–32 cells stimulated with the L cells using ELISA. Full IL-2, IL-4 and IFN-γ production was observed in T cells stimulated with L-DR4(65) cells prepulsed with 50 μM M12p54–68 and with M12DR4(235) cells (Fig. 3). The Q59G(171) cells induced production of these cytokines to a comparable or a slightly lesser extent. The E63V(219) cells stimulated lower levels of IL-4 and IFN-γ production, which was consistent with the weaker proliferative T cell responses (Fig. 2). Although IL-2 production was not detected in T cells stimulated with E63V(219) cells, the proliferative response seemed to be in part IL-2-dependent, since saturating amounts of neutralizing anti-IL-2 (50 μg/ml) mAb partially inhibited the proliferative response to E63V(219) cells (Fig. 3D). In addition, E63V(219) cells, as well as M12DR4(235) and Q59G(171) cells, stimulated the up-regulation of IL-2 mRNA in T cells as revealed by ribonuclease protection assay (RPA). The cytokines were not detectable in supernatants from co-culture with E58N(122) or L-DR4(65) cells.

Induction of cytokine mRNA in YN5–32 cells stimulated with the L cell transfectants were analyzed using RPA. Up-regulation of IL-4, IL-5, IL-10 and IL-13 mRNA was detected in T cells co-cultured with M12DR4(235), Q59G(171) and E63V(219) cells at either 4 or 16 h after co-culture and, except for IL-4 mRNA, the up-regulation persisted for up to 48 h (Fig. 3E). Y61V(243) cells, which did not induce T cell proliferation, stimulated a slight up-regulation of cytokine mRNA while Lmock and CD20DR4(142) cells did not. These data indicate that stimulation with overexpressed HLA-DR4/partially ago-




nistic APL complexes that induce T cell proliferation also induced gene expression of at least IL-4, IFN-γ and IL-2, as did the M12DR4(235) cells.

### 2.4 Phosphorylation of zeta-associated protein-70 and linker for activation of T cells was impaired in T cells stimulated with Q59G(171) or E63V(219) cells

Some overexpressed HLA-DR4/partially agonistic APL complexes stimulated both T cell proliferation and cytokine production. However, they did not induce tyrosine phosphorylation of zeta-associated protein-70 (ZAP-70) and linker for activation of T cells (LAT) at detectable levels in the T cells. Hereafter, we used *Herpesvirus saimiri* (HVS)-transformed YN5–32 cells to prepare a relatively large number of clonal human T cells for biochemical experiments. As shown in Fig. 4A, phospho-ZAP-70 was only detected in T cells stimulated with M12DR4(235) cells, but not in T cells stimulated with Q59G(171) or with E63V(219) cells. Phospho-ZAP-70 was also not detected in T cells stimulated with Lmock, Y61V(243) or CD20DR4(142) cells. LAT, the immediate substrate for ZAP-70, was also tyrosine-phosphorylated only in T cells stimulated with M12DR4(235) cells (Fig. 4B). Phospho-ZAP-70 was also observed in normal YN5–32 cells co-cultured with M12DR4(235) cells or L-DR4(65) cells pulsed with M12p54–68 (200 μM) for 30 min, but neither with Q59G(171) nor with E63V(219) cells (Fig. 4C); thus indicating that the ZAP-70 phosphorylation shown in Fig. 4A was neither a specific event for the HVS-transformed YN5–32 cells nor for the artificial HLA-DR4 molecules with covalently linked peptide. In parallel experiments, Q59G(171) cells induced comparable proliferative responses of YN5–32 cells with M12p54–68-pulsed L-DR4(65) and M12DR4(235) cells (Fig. 4C).

Undetectable phosphorylation of ZAP-70 did not seem to be due to retarded kinetics since phospho-ZAP-70 was not detected for up to 2.5 h after a co-culture with Q59G(171) cells in HVS-transformed YN5–32 cells (Fig. 4D). On the other hand, phospho-ZAP-70 was detected as early as 5 min of co-culture with

◀ *Fig. 3.* Profiles of cytokine produced by T cells stimulated with L cell transfectants. After starting T cell and L cell co-culture, IL-2 (A), IL-4 (B) and IFN-γ (C) concentrations in the culture supernatant at the indicated period were determined, using ELISA. L cells used were: L-DR4(65) only (closed triangles), M12DR4(235) (open squares), Q59G(171) (closed circles), E63V(217) (closed squares), E58N(122) (closed diamonds) and L-DR4(65) prepulsed with 50 μM M12p54–68 (open triangles). (D) Inhibitory effects of neutralizing anti-IL-2 mAb (50 μg/ml) on the T cell proliferation. (E) Three micrograms of total RNA from YN5–32 cells ($1\times10^6$) co-cultured with each L cell transfectant for the indicated period was subjected to RPA. Ribosomal L32 and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal controls. Yeast tRNA was used for the background control. M12DR4(235), E63V(219) and Q59G(171) cells, which stimulated T cell proliferation, induced similar up-regulation profiles of the indicated cytokine mRNA in the T cells over the stimulation period. Although Y61V(243) cells did not stimulate T cell proliferation, they induced a definite up-regulation of IL-4, IL-5, IL-10 and IL-13 mRNA in the T cells.

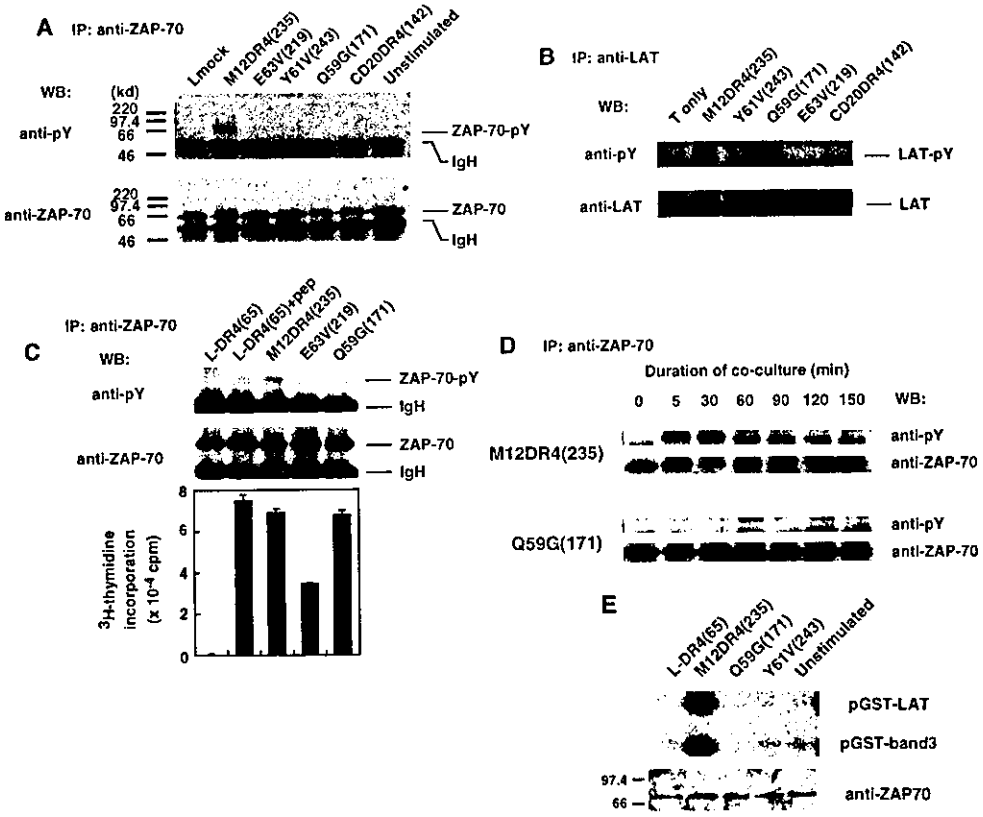



*Fig. 4.* Phosphorylation of ZAP-70 and LAT was impaired in T cells stimulated with Q59G(171) or E63V(219) cells that induce proliferative T cell responses. ZAP-70 (A) or LAT (B) immunoprecipitated from HVS-transformed YN5–32 cells co-cultured with each L cell transfectant for 5 min were subjected to SDS-PAGE and Western blots. The membranes were probed with an anti-pY mAb (A, B; upper panels) and reprobed with an anti-ZAP-70 mAb (A, lower panel) or with an anti-LAT antibody (B, lower panel). (C) Normal YN5–32 cells were co-cultured with L-DR4 cells alone or prepulsed with M12p54–68 (200 μM), M12DR4(235), E63V(219) or Q59G(171) cells for 30 min. Each T cell lysate was subjected to ZAP-70 immunoprecipitation and Western blots probed with an anti-pY mAb (top) and reprobed with an anti-ZAP-70 mAb (middle). The results of parallel experiments for T cell proliferation are shown (bottom). Phospho-ZAP-70 was detected only in T cells stimulated with M12DR4(235) cells or with L-DR4 cells prepulsed with M12p54–68. (D) T cells were co-cultured with M12DR4(235) or Q59G(171) cells for the indicated period and subjected to ZAP-70 immunoprecipitation and Western blots probed with an anti-pY mAb (upper panels) and reprobed with an anti-ZAP-70 mAb (lower panels). (E) Lysates from T cells co-cultured with respective L cell transfectants for 10 min were immunoprecipitated with an anti-ZAP-70 antibody. The kinase activity in the immunocomplexes was determined in *in vitro* kinase assays using GST-LAT and GST-band 3 as substrates (top). Equal amounts of ZAP-70 in the immunocomplexes were confirmed by Western blot analyses (bottom). ZAP-70-pY: tyrosine-phosphorylated ZAP-70, LAT-pY: tyrosine-phosphorylated LAT, pGST-LAT: phosphorylated GST-LAT, pGST-band 3: phosphorylated GST-band 3.

M12DR4(235) cells and persisted for up to 2.5 h. The total amount of ZAP-70 was down-regulated in T cells stimulated with M12DR4(235) cells, in relation to the co-culture period, while such down-regulation was not observed in T cells stimulated with Q59G(171) cells. In *in vitro* kinase assays, only ZAP-70 immunoprecipitates from M12DR4(235) cell-stimulated T cells phosphorylated the substrates glutathione S-transferase (GST)-LAT and GST-band 3 (Fig. 4E). On the other hand, none of ZAP-70 immunoprecipitates from Q59G(171), L-DR4(65) and Y61V(243) cell-stimulated or unstimulated T cells did phosphorylate the substrates. These observations indicate that the magnitude of cytokine productions and proliferative responses, which was comparable between T cells stimulated with Q59G(171) cells and with M12DR4(235), was irrespective of ZAP-70 kinase activity.

### 2.5 Inhibition of PKCμ abrogated both T cell proliferation and cytokine production

Since ZAP-70-independent but PKC-dependent extracellular signal-regulated kinases (ERK) activation pathway in the ZAP-70-deficient Jurkat T cell line was

reported [16], we asked whether the responses of T cells stimulated with L cells were PKC-dependent. IFN-γ production by HVS-transformed YN5–32 cells co-cultured with M12DR4(235) or Q59G(171) cells for 4 h was inhibited by pretreatment and presence of the PKC inhibitors Gö6976, Ro318220 (bisindolylmaleimide IX) and Gö6850 (GF109203X, bisindolylmaleimide I) (Fig. 5A). Gö6976 is a specific inhibitor of conventional PKC, while Ro318220 and Gö6850 are the inhibitors of three groups (conventional, non-conventional and atypical) of PKC [17]. Among these PKC inhibitors, the conventional PKC inhibitor Gö6976 most potently inhibited the T cell responses, while Ro318220 was less effective and Gö6850 showed only moderate inhibition. Pretreatment and presence of these PKC inhibitors also inhibited T cell proliferation stimulated with M12DR4(235) or Q59G(171) cells (Fig. 5A). Again, Gö6976 was most potent, while Ro318220 and Gö6850 were less effective.

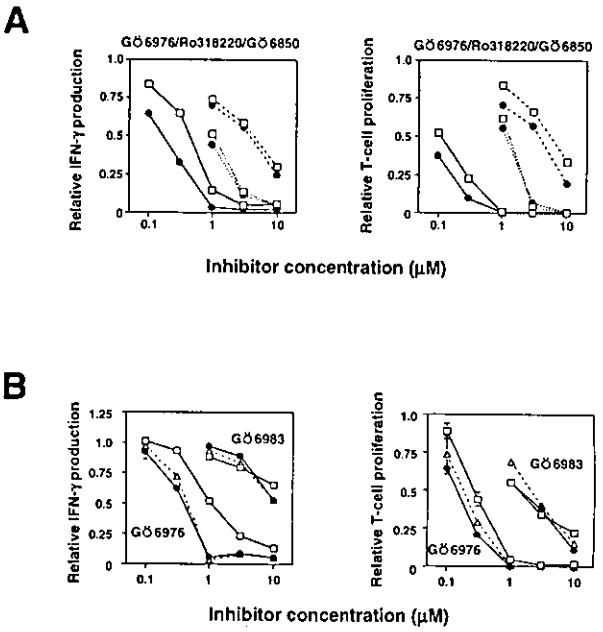



*Fig. 5.* PKC inhibitors blocked IFN-γ production and T cell proliferation. (A) IFN-γ production (left) and proliferation (right) of HVS-transformed YN5–32 cells. T cells were pretreated with 100 μl of the indicated concentrations of Gö6976 (solid lines), Ro318220 (dotted lines) or Gö6850 (dashed lines), and co-cultured with 100 μl of M12DR4(235) cells (open squares) or Q59G(171) cells (closed circles). (B) IFN-γ production (left) and proliferation (right) of HVS-transformed YN5–32 cells pretreated with the indicated concentrations of Gö6976 or Gö6983. T cells were co-cultured with M12DR4(235) cells (open squares), Q59G(171) cells (closed circles) or L-DR4(65) cells prepulsed with 50 μM M12p54–68 (open triangles with dashed lines). T cell responses without inhibitors were assigned to be 1.0.

These contradictory observations together with recent reports that kinase activity of the μ subtype of PKC (or protein kinase D, PKD) could be inhibited by Gö6976 but was resistant to Ro318220 and Gö6850 [18] suggested that activation of PKCμ was involved in the T cell activation pathways. PKCμ was classified into the fourth group of PKC [19] or into the independent PKD family [20] because of its fewest structural similarities with other PKC. Another PKC inhibitor Gö6983, which is a potent PKC inhibitor except for PKCμ [18], exhibited effects on T cell responses similar to those of Gö6850 and much less effective than those of Gö6976 (Fig. 5B), thus suggesting the association of PKCμ activation in the T cell activation pathway. It must be noted that IFN-γ production and proliferation of T cells stimulated with Q59G(171) cells were more susceptible to treatment with Gö6976 than in case of stimulation with M12DR4(235) or L-DR4(65) cells pulsed with M12p54–68.

Since PKCμ are activated by diacylglycerol produced by phospholipase C (PLC) [21], effects of the PLC-specific inhibitor U73122 on the T cell responses were examined. Pretreatment and the presence of U73122 inhibited both IFN-γ production and T cell proliferation in a dose-dependent manner; however, T cells stimulated with Q59G(171) cells were more susceptible than those stimulated with M12DR4(235) cells or with L-DR4(65) cells pulsed with 50 μM M12p54–68 (Fig. 6A). Phosphorylation of tyrosine residue 783 of PLCγ1, which is essential for the activation of PLCγ1 [22], was observed in M12DR4(235) and Q59G(171) cell-stimulated T cells, suggesting PLCγ1 activation in those T cells (Fig. 6B). On the other hand, there was no such phosphorylation in the T cells unstimulated or co-cultured with L-DR4(65) cells.

The Src inhibitor PP2 also inhibited both IFN-γ production and T cell proliferation stimulated with the L cell transfectants, suggesting the involvement of Src kinases in the T cell activation pathways (Fig. 6A). Compared with stimulation with M12DR4(235) cells, Q59G(171) cell-stimulated T cell responses were selectively inhibited by PP2 at the concentration of 3 μM, and those stimulated with L-DR4(65) cells prepulsed with 50 μM M12p54–68 showed intermediate susceptibility. The MAPK/ERK kinase (MEK)-specific inhibitor PD98059 suppressed T cell proliferation in a dose dependent manner, while effects on IFN-γ production were slight (Fig. 6A).

### 2.6 PKCμ activity was up-regulated only by stimulation with L cell transfectants that induced T cell proliferation

*In vitro* kinase assays using an anti-human PKCμ polyclonal antibody revealed that co-culture with M12DR4(235) cells induced a marked incorporation of

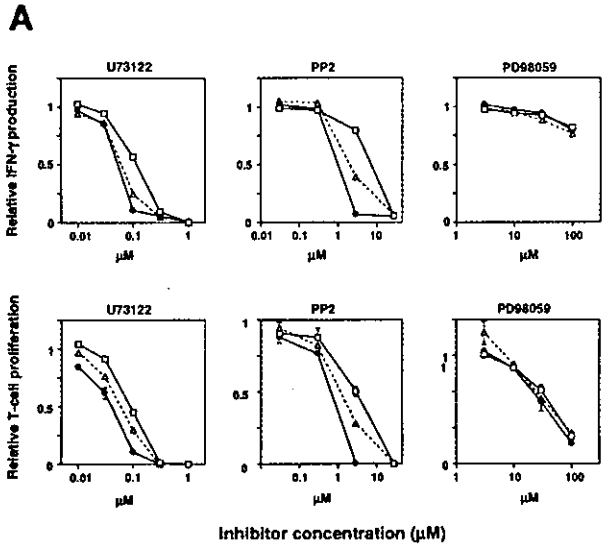



*Fig. 6.* Activation of PLC and Src were involved in the T cell responses. (A) HVS-transformed YN5–32 cells were pretreated with PLC inhibitor U73122, Src inhibitor PP2, or MEK inhibitor PD98059 as described in the legend of Fig. 5. T cells were then co-cultured with 100 μl of M12DR4(235) cells (open squares), Q59G(171) cells (closed circles) or L-DR4(65) cells prepulsed with 50 μM M12p54–68 (triangles with dashed lines). IFN-γ production (upper panels) and proliferation (lower panels) of the T cells were quantified as described in Sect. 4. T cell responses without inhibitors were assigned to be 1.0. (B) Lysate from T cells co-cultured with respective L cell transfectants for 10 min were subjected to PLCγ1 immunoprecipitation and Western blots probed with an anti-phospho-PLCγ1 (tyrosine 783) antibody. Phosphorylation of tyrosine residue 783 of PLCγ1 was observed in T cells co-cultured with M12DR4(235) and Q59G(171) cells but not in those unstimulated or co-cultured with L-DR4(65) cells (top panels). Equal amounts of PLCγ1 were confirmed by reprobing the membrane with an anti-PLCγ1 antibody (bottom panels).

radioactivity into PKCμ of the T cells, indicating strong up-regulation of the kinase activity (Fig. 7, upper left panel) [23]. Co-culture with Q59G(171) cells also up-regulated the incorporated radioactivity into PKCμ, while no such up-regulation was observed in T cells unstimulated or co-cultured with Y61V(243), Lmock and L-DR4(65) cells. The radioactivity was reduced to baseline level by the presence of 3 μM Gö6976 in the kinase buffer while the same concentration of Gö6983 had no effect (Fig. 7, upper right panel), thus confirming that up-regulation of the PKCμ phosphorylation was due to PKCμ activation.

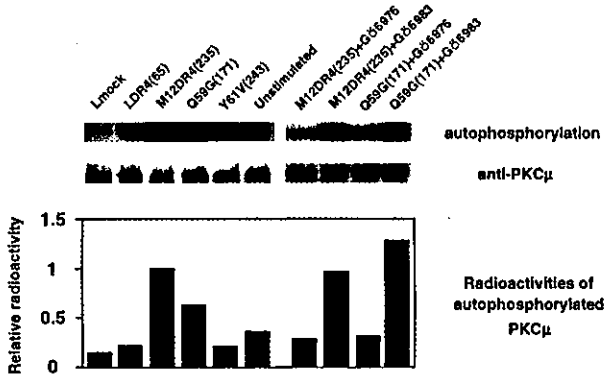



*Fig. 7.* Stimulation with L cell transfectants that induce T cell proliferation up-regulated PKCμ activity. PKCμ was immunoprecipitated from HVS-transformed YN5–32 cells co-cultured with respective L cell transfectants, and the kinase activity in the immunocomplexes was measured as autophosphorylation by quantifying incorporated $^{32}PO_4^{3-}$ (upper panels). The radioactivity was reduced to baseline level in the presence of 3 μM of Gö6976 but not in the presence of Gö6983 (upper right panel). The relative radioactivity of each PKCμ band normalized by the relative amount of each PKCμ protein is shown in the bottom graph.

## 3 Discussion

We found that T cell proliferative responses stimulated with overexpressed HLA-DR4/partially agonistic APL complexes differed depending on the partial agonist used and on the density of the complexes: E63S and E63V cells required a relatively high cell surface number ($9\times10^5$–$2\times10^6$) to stimulate T cell proliferation, Q59G cells induced a detectable T cell proliferation at only $3\times10^4$/cell and induced full responses at $>9\times10^5$/cell, whereas E58N and Y61V cells did not stimulate T cell proliferation even at $1\times10^6$–$3\times10^6$/cell.

At the interface between T cell and APC, MHC/peptide/TCR clusters are surrounded by complexes of costimulatory and adhesion molecules, and the three-dimensional supramolecular structure formed is called "immunological synapse" [24–27]. Regarding the density of MHC/peptide/TCR complexes in the cluster, a weak agonist and an antagonist form MHC/peptide complexes of lower densities (34–193 molecules/μm$^2$) at the central

part of the cluster than does the agonist (132–352 molecules/$\mu m^2$), and the size of the cluster relative to the threshold value determines whether or not T cells begin to proliferate [25]. If the cell surface MHC/APL density becomes much higher than the MHC/peptide density observed in the physiological immunological synapse by forced overexpression, the density of TCR complexes bound to the MHC/APL complexes might be high enough to transduce full T cell activation signals. Indeed, in our experiment, a steep increase in T cell proliferative responses was observed at a ligand number of $9\times10^3$/cell for Q59G and E63S (Fig. 2), which corresponds to 1,300 molecules/$\mu m^2$ on average on the surface of L cells, and is several times higher than densities of MHC/agonistic peptide complexes accumulating at the T cell contact area that stimulate T cell proliferation in the planar bilayer [25].

However, the Q59G(171) cell stimuli were not simply agonistic because they did not induce any detectable amount of tyrosine-phsophorylated ZAP-70 and LAT, such being characteristic observations for T cells stimulated with partial agonists or simple antagonists [5, 11, 28, 29]. Therefore, the Q59G(171) cell stimuli retained some potency of a partial agonist. Since the impaired phosphorylation of TCR proximal signaling molecules is considered to cause a lack of downstream signal transduction, resulting in the failure of T cell proliferation, the question is which signaling pathways are needed to induce full proliferative responses in T cells stimulated with Q59G(171) cells. ZAP-70-independent T cell activation pathways have been reported [16, 30, 31] and these observations indicate that there might be TCR-mediated T cell activation pathways that skip phospho-ZAP-70. Using P116 ZAP-70-negative Jurkat cell lines, Shan et al. [16] reported alternative ERK activation pathways stimulated with a high dose of anti-CD3 mAb. Since ERK phosphorylation in P116 cells was susceptible to the PKC inhibitors Gö6850 and Ro318220 but not to the conventional PKC inhibitor Gö6976, they proposed that non-conventional PKC mediate ZAP-70-independent ERK activation pathways, and this ruled out the involvement of PKCμ. On the contrary, the conventional PKC inhibitor Gö6976, but not Ro318220, Gö6850 and Gö6983, potently inhibited both IFN-γ production and T cell proliferation in our system. The apparent contradiction may be due to differences in the readouts, ERK1/2 phosphorylation or the proliferation and IFN-γ production.

PKCμ is present in various tissues [19, 32], including lymphocytes [23, 33, 34]. In T cells, PKCμ was activated by cross-linking of TCR complexes using an anti-CD3ε antibody and cognate TCR ligands (present study); however, the target molecule for the activated PKCμ is unknown. The MEK/ERK pathway is apparently involved in T cell activation pathways leading to proliferation since it was suppressed by treatment with PD98059. PKCμ did not seem to activate this pathway since Gö6976 had almost no effect on the ERK1/2 phosphorylation (data not shown) [16]. Therefore, the PKCμ-mediated downstream signaling pathway seems to be distinct from the MEK/ERK pathway.

Although the upstream molecules responsible for activation of PKCμ remain to be clarified, several non-conventional PKC such as PKCη [34–36], PKCε [34] and PKCθ [37] have been reported to activate PKCμ *in vivo*. Actually, pretreatment of A20 mouse B lymphoma and human peripheral T lymphocytes with 2.5 μM Ro318220 and 3.5 μM Gö6850, respectively, abrogated activation of PKCμ, and transfection of constitutively active forms of PKCη or PKCε induced almost maximal PKCμ activity in RBL 2H3 mast cells, suggesting that PKCμ activation was controlled by those non-conventional PKC [34]. PKCμ might have been also activated by certain non-conventional PKC in our system, since the T cell responses were abrogated by treatment with 3 μM Ro318220. Both non-conventional PKC and PKCμ as well as conventional PKC have cysteine-rich zinc finger-like motifs to which diacylglycerol binds and activates the kinases [17, 20, 21]. Inhibition of T cell responses by U73122 and phosphorylation of PLCγ1 tyrosine 783 observed in the T cells stimulated with M12DR4(235) and Q59G(171) cells indicated PLCγ1 activation. This may induce PKC activation in those T cells; however, the absence of ZAP-70 kinase activity in Q59G(171) cell-stimulated T cells raise the question as to which kinase is responsible for the PLCγ1 activation. One possibility is that Src kinases may directly phosphorylate PLCγ1 [16].

It is evident that Src kinases are involved in the Q59G(171) cell-stimulated T cell proliferation since the Src inhibitor PP2 effectively inhibited the T cell responses. Compared with stimulation with M12DR4(235) cells, Q59G(171) cell-stimulated T cell responses were selectively inhibited by PP2 at the concentration of 3 μM. These observations suggest that a smaller fraction of Src kinases is activated in the T cells, which in turn activate lesser amounts of PLC and then PKC. We were not able to directly evaluate Lck and Fyn kinase activities; however, to clarify whether the up-regulation of PKCμ is indispensable for the full T cell response, one needs to determine the active Src kinases needed for PKCμ activation and its downstream pathways. The correlation between PKCμ activation and phospho-ZAP-70 less-dependent T cell proliferation we observed also remains to be investigated.

## 4 Materials and methods

### 4.1 Materials

The CD4+ T cell clone YN5–32, which recognizes streptococcal peptide M12p54–68 (NRDLEQAYNELSGEA) in the context of HLA-DR4 (DRA/DRB1*0406), was established as described [8]. In some experiments, HVS-transformed YN5–32 cells were used. The transformed YN5–32 cells exhibited the same magnitude of reactivity to antigens as did the normal YN5–32 cells (unpublished observations). The mouse L cell transfectant L-DR4 cell clone expressing DRA/DRB1*0406 was distributed in the 11th International Histocompatibility Workshop.

Human LAT cDNA encoding the amino acid residues 118–223 and human erythrocyte band 3 protein cDNA encoding amino acid residues 1–14 [38] were ligated into pGEX4T vectors. The GST fusion proteins (GST-LAT and GST-band 3) were expressed in *Escherichia coli* DH5α and were purified with glutathione-agarose beads (Pharmacia).

### 4.2 Generation of transfectants and quantification of the expressed peptide/HLA-DR4 complexes

Synthesized sense and antisense DNA fragments encoding amino acid residues of M12p54–68 plus linker were annealed and inserted into DRB1*0406 cDNA as described [39]. The cDNA fragment was subcloned into pBJ1neo vector (provided by Dr. M. M. Davis). To generate HLA-DR4 with covalently linked APL (Table 1), mutations were introduced into the DNA sequence encoding M12p54–68. As an HLA-DR4-binding irrelevant peptide control, synthetic DNA fragments encoding CD20p26–45 (GPKPLFR RMSSLVGPTQSFF) [15] were used.

The expression vectors for HLA-DRA and peptide/linker/HLA-DRB1*0406 were co-transfected into L cells. Cells stably expressing the HLA-DR4/peptide complex were stained with FITC-conjugated anti-HLA-DR mAb (Becton Dickinson), and their expression levels were analyzed using FACScan (Becton Dickinson). Transfectants expressing various amounts of HLA-DR were cloned using FACSVantage (Becton Dickinson). The surface number of each HLA-DR4/peptide complex on the L cell was quantified using Quantum Simply Cellular™ microbeads (Flow Cytometry Standards).

### 4.3 Assays for T cell responses

Mitomycin C (Sigma)-treated (20 μg/ml for 30 min at 37°C) L cell transfectants ($3.7\times10^4$/well) were incubated in 96-well plates for 1 day and YN5–32 cells ($3\times10^4$/well) were added to each well. In some experiments neutralizing mouse anti-human-IL-2 mAb (50 μg/ml), rabbit anti-human-IL-4 antibody (25 μg/ml) or isotype-matched control IgG were added throughout the culture period. T cell proliferative responses were measured as reported [8]. IL-2, IL-4 and IFN-γ contents in the culture supernatant were determined using ELISA kits (BioSource, R&D Systems and Endogen, respectively). For RPA, mitomycin C-treated L cells were plated in 10-cm culture dishes ($2\times10^6$/plate), and T cells ($1\times10^6$) were added. After the indicated co-culture periods, T cell RNA were extracted using TRIZOL™ reagent (Life Technologies) and subjected to a commercial RPA system (PharMingen).

In some experiments, the HVS-transformed YN5–32 cells ($3\times10^4$/100 μl) were pretreated with indicated concentrations of PKC inhibitors (Gö6976, Gö6850, Gö6983 and Ro318220; Calbiochem), PLC inhibitor (U73122, Calbiochem) and Src inhibitor (PP2, Sigma) for 30 min at 37°C. Then the T cells and L cells were co-cultured in 96-well plates and T cell responses were assayed as mentioned above.

### 4.4 Immunoprecipitation and Western blot analyses

T cells ($1\times10^7$) were plated on the monolayer of L cells confluently grown in 15-cm culture dishes and co-cultured for indicated periods. T cells were lysed using lysis buffer I [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet-P40 (NP40), 1 mM $Na_3VO_4$, 10% glycerol and a proteinase inhibitor cocktail (Boehringer Mannheim)]. ZAP-70, LAT and PLCγ1 were immunoprecipitated with anti-ZAP-70 mAb (Santa Cruz Biotechnologies), rabbit anti-LAT polyclonal IgG and mouse anti-PLCγ1 mixed mAb (Upstate Biotechnology), respectively, together with protein A beads (Pierce). The proteins were analyzed using 10% SDS-PAGE and transferred onto nitrocellulose membrane. Phospho-ZAP-70 and LAT were blotted with anti- phosphotyrosine (pY) mAb (4G10, Upstate Biotechnology) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Amersham). Phospho-PLCγ1 was blotted with anti-phospho-PLCγ1 (tyrosine 783) antibody (Cell Signaling) and HRP-conjugated anti-rabbit IgG antibody (Amersham). The membrane was reprobed with either anti-ZAP-70 mAb, anti-LAT antibody or anti-PLCγ1 antibody together with HRP-conjugated second antibodies. The bands were visualized by enhanced chemiluminescence (ECL, Amersham).

### 4.5 *In vitro* ZAP-70 kinase assay

The HVS-transformed YN5–32 cells co-cultured with L cell transfectants for 10 min were lysed with lysis buffer II [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM $Na_3VO_4$, 10 mM NaF and a proteinase inhibitor cocktail]. ZAP-70 was immunoprecipitated using an anti-human ZAP-70 antibody (Santa Cruz Biotechnology) and protein A beads at 4°C for 2 h. The immunocomplexes were washed twice with lysis buffer II and once with kinase buffer [30 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 1 mM DTT] and then incubated with 20 μl kinase buffer containing 2.4 μg each of

GST-LAT and GST-band 3 and 0.6 MBq of [$\gamma$-$^{32}$P]ATP (Amersham) at 30°C for 10 min. The supernatants were analyzed with 12% SDS-PAGE followed by autoradiography. ZAP-70 in the immunocomplex was subjected to 10% SDS-PAGE and total amounts of ZAP-70 were estimated on Western blots.

### 4.6 *In vitro* PKCμ kinase assay

The HVS-transformed YN5–32 cells co-cultured with L cells for 1 h were lysed with lysis buffer II. PKCμ was immunoprecipitated using an anti-human PKCμ antibody (D-20, Santa Cruz Biotechnology) and protein A beads at 4°C for 2 h. The immunocomplexes were successively washed with lysis buffer II, RIPA buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM DTT, 5 mM $Na_3VO_4$, 10 mM NaF, 10% glycerol and a proteinase inhibitor cocktail] and kinase buffer and then incubated with 12.5 μl kinase buffer containing 0.25 MBq of [$\gamma$-$^{32}$P]ATP at 30°C for 10 min. PKCμ in the immunocomplex was subjected to 10% SDS-PAGE and transferred onto nitrocellulose membrane. The kinase activities were determined by measuring the incorporated radioactivity using FLA-2000 (Fuji Photo Film). Total PKCμ protein was detected on Western blots and quantified using NIH image software (http://rsb.info.nih.gov/nih-image/).

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REVIEW ARTICLE

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# Specificity, degeneracy, and molecular mimicry in antigen recognition by HLA-Class II restricted T cell receptors: implications for clinical medicine

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**Abstract** In humans, increased susceptibility to specific autoimmune diseases is closely associated with specific HLA-class II alleles. $CD4^+$ T cells that recognize short self-peptides in the context of HLA-class II molecules via their T cell receptor (TCR) are considered to mediate the central role of pathogenesis in autoimmunity. Although both self- and nonself-peptides are presented on HLA-class II molecules under physiological conditions, several mechanisms exist to avoid the T cell response to the self-peptide/HLA-class II complex. One of the mechanisms that account for the breakdown in immune tolerance is cross-recognition by TCR between a pathogen-derived antigen and a host antigen (molecular mimicry theory). Epidemiological studies have indicated that a number of autoimmune diseases are developed or exacerbated after infections. Therefore, elucidating the recognition nature of HLA-class II restricted TCR in detail is necessary in order to understand disease processes. A large body of evidence indicates that T cell recognition is highly degenerate, and many different peptides can activate an individual T cell. Degeneracy of TCR recognition also can appear in various physiological outcomes, ranging from full activation to strong antagonism. Here, we review the clinical implications of our findings on T cell recognition, as well as a new direction of future applications for analyses in molecular mimicry. We also describe the latest developments in methods of mapping TCR epitopes for $CD4^+$ T cells using a peptide epitope expression library generated in the class II-associated invariant chain peptide substituted invariant chain gene format.

**Key words** Autoimmunity · Epitopes · HLA · Molecular mimicry · T cell receptors (TCR)

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## Introduction

Particular class-II human histocompatibility leukocyte antigen (HLA-class II) alleles are associated with susceptibility to particular autoimmune diseases.[1] $CD4^+$ T cells recognize 10–20 amino acid long peptides in the context of class II molecules expressed on antigen-presenting cells through their T cell receptor (TCR). Autoreactive $CD4^+$ T cells are considered to have a central role in development of autoimmune diseases. Even in the presence of exogenous nonself antigens, the majority of HLA-class II molecules bind self-peptides processed mainly from self-membrane or secretory proteins. If the density of self-peptides/HLA-class II complexes expressed on the surface of cells is large enough to ensure a high avidity engagement of TCR, most autoreactive $CD4^+$ T cells are deleted in the thymus or become anergic in the periphery. If the density of self-peptide/HLA-class II complexes is small enough not to activate T cells in the periphery, T cells do not need to acquire tolerance to such complexes and ignore them.[2] Thereby, $CD4^+$ T cells do not respond to these self-peptides in the context of self HLA-class II molecules, except in autoimmune states.

Epidemiological studies have indicated that a number of autoimmune diseases either develop or are exacerbated after infections. Several possible mechanisms can account