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Novel Immunodominant Peptide Presentation Strategy: a Featured HLA-A*2402-Restricted Cytotoxic T-Lymphocyte Epitope Stabilized by Intrachain Hydrogen Bonds from Severe Acute Respiratory Syndrome Coronavirus Nucleocapsid Protein▽†

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Antigenic peptides recognized by virus-specific cytotoxic T lymphocytes (CTLs) are presented by major histocompatibility complex (MHC; or human leukocyte antigen [HLA] in humans) molecules, and the peptide selection and presentation strategy of the host has been studied to guide our understanding of cellular immunity and vaccine development. Here, a severe acute respiratory syndrome coronavirus (SARS-CoV) nucleocapsid (N) protein-derived CTL epitope, N1 (QFKDNVILL), restricted by HLA-A*2402 was identified by a series of *in vitro* studies, including a computer-assisted algorithm for prediction, stabilization of the peptide by co-refolding with HLA-A*2402 heavy chain and β_2-microglobulin (β_2m), and T2-A24 cell binding. Consequently, the antigenicity of the peptide was confirmed by enzyme-linked immunospot (ELISPOT), proliferation assays, and HLA-peptide complex tetramer staining using peripheral blood mononuclear cells (PBMCs) from donors who had recovered from SARS donors. Furthermore, the crystal structure of HLA-A*2402 complexed with peptide N1 was determined, and the featured peptide was characterized with two unexpected intrachain hydrogen bonds which augment the central residues to bulge out of the binding groove. This may contribute to the T-cell receptor (TCR) interaction, showing a host immunodominant peptide presentation strategy. Meanwhile, a rapid and efficient strategy is presented for the determination of naturally presented CTL epitopes in the context of given HLA alleles of interest from long immunogenic overlapping peptides.

In 2003, severe and acute respiratory syndrome (SARS), emerging from China, caused a global outbreak, affecting 29 countries, with over 8,000 human cases and greater than 800 deaths (5, 9, 24, 33, 37). Thanks to the unprecedented global collaboration coordinated by the WHO, SARS coronavirus (SARS-CoV), a novel member of *Coronaviridae* family, was rapidly confirmed to be the etiological agent for the SARS epidemic (36). Soon after the identification of the causative agent, SARS was controlled and then quickly announced to be conquered through international cooperation on epidemiological processes (9). However, the role that human immunity played in the clearance of SARS-CoV and whether the memory immunity will persist for the potential reemergence of SARS are not yet well understood.

In viral infections, $CD8^+$ cytotoxic T lymphocytes (CTLs) are essential to the control of infectious disease. Virus-specific $CD8^+$ T cells recognize peptides which have 8 to 11 amino acids, in most cases presented by major histocompatibility complex (MHC) class I molecules. However, identification of virus-specific $CD8^+$ T-cell epitopes remains a complicated and time-consuming process. Various strategies have been developed to define CTL epitopes so far. One of the most common practices to determine immunodominant CTL epitopes on a large scale is based on screening and functional analysis of overlapping 15- to 20-mer peptides covering an entire viral proteome or a given set of immunogenic proteins (19, 23, 32). However, peptides identified through this method are too long to be naturally processed CTL epitopes, and the definition of MHC class I restriction of these peptides still requires further

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TABLE 1. Characteristics of the peptides used in this study

Name	Derived protein	Positions	Sequence[a]	Score[b]
N1	SARS-CoV N protein	346–354	QFKDNVILL	19
NC9585	SARS-CoV N protein	338–354	IKLDDKDP**QFKDNVILL**	NA[c]
NC9586	SARS-CoV N protein	346–363	**QFKDNVILL**NKHIDAYKT	NA
Nef138-10[d]	HIV Nef protein	138–147	RYPLTFGWCF	22
B35-18[e]	HIV Pol protein	587–596	EPIVGAETFY	2
VYG[f]	Human telomerase	461–469	VYGFVRACL	23

[a] Boldface letters indicate peptide N1 within the peptides NC9585 and NC9586 (21).
[b] Estimated binding affinity to HLA-A*2402 calculated through the website http://www.syfpeithi.de/ (35).
[c] NA, scoring for peptides other than the nonamer and decamer to bind to HLA-A*2402 is not available.
[d] Nef138-10 was used as a positive control in the T2-A24 cell binding assay (11).
[e] B35-18 was used as a negative control in the T2-A24 cell binding assay (42).
[f] VYG was the peptide from the first peptide/HLA-A24 structure (7).

analysis. Rapid and efficient strategies should be developed for the determination of naturally presented CTL epitopes in the context of any given HLA allele of interest. Furthermore, no other HLA alleles except HLA-A2-restricted CTL epitopes have been reported for SARS-CoV-derived proteins (16, 22, 31, 43, 46, 47, 49). This is primarily because of the limitation of the experimental methods for the other HLA alleles. HLA-A24 is one of the most common HLA-A alleles throughout the world, especially in East Asia, where SARS-CoV emerged, second only to HLA-A2 (30). The development of a fast and valid method to screen and identify HLA-A24-restricted epitopes would greatly contribute to the understanding of the specific CTL epitope-stimulated response and widen the application of the epitope-based vaccine among a more universal population (17). A genomewide scanning of HLA binding peptides from SARS-CoV has been performed by Sylvester-Hvid and colleagues, through which dozens of peptides with major HLA supertypes, including HLA-A24 binding capability, have been identified (41).

There are strong indications that different peptide ligands, such as peptides with distinct immunodominance, can elicit a diverse specific T-cell repertoire, and even subtle changes in the same peptide can have a profound effect on the response (25, 44). Furthermore, a broader T-cell receptor (TCR) repertoire to a virus-specific peptide–MHC complex can keep the host resistant to the virus and limit the emergence of virus immune-escape mutants (29, 34, 38). Recent studies have demonstrated that the diversity of the selected TCR repertoire (designated as T-cell receptor bias) is clearly influenced by the conformational characteristics of the bound peptide in the MHC groove. Peptides with a flat, featureless surface when presented by MHC generate only limited TCR diversity in a mature repertoire, while featured peptides with exposed residues (without extreme bulges) protruding outside the pMHC landscapes are rather associated with the more diverse T-cell repertoire (15, 28, 39, 44, 45). Therefore, being able to determine the binding features of a peptide to MHC and describe the peptide-MHC topology will help us understand the immunodominance of a given peptide and demonstrate the peptide presentation strategy of the host.

Structural proteins of SARS-CoV, such as spike, membrane, and nucleocapsid (N), have been demonstrated as factors of the antigenicity of the virus, as compared with the nonstructural proteins (12, 20). Coronavirus nucleocapsid (N) protein is a highly phosphorylated protein which not only is responsible for construction of the ribonucleoprotein complex by interacting with the viral genome and regulating the synthesis of viral RNA and protein, but also serves as a potent immunogen that induces humoral and cellular immunity (13, 14, 26, 48). The $CD8^+$ T-cell epitopes derived from SARS-CoV N protein defined so far mainly cluster in two major immunogenic regions (4, 21, 23, 31, 32, 43). One of them, residues 219 to 235, comprises most of the N protein-derived minimal CTL epitopes identified so far—N220-228, N223-231, N227-235, etc.—all of which are HLA-A*0201 restricted (4, 43). The other region, residues 331 to 365, also includes high-immunogenicity peptides that can induce memory T-lymphocyte responses against SARS-CoV (21, 23, 32). However, until now, no minimal CTL epitope with a given HLA allele restriction has been investigated in this region.

Here, based on previously defined immunogenic regions derived from SARS-CoV N protein (21), we identified an HLA-A*2402-restricted epitope, N1 (residues 346 to 354), in the region through a distinct strategy using structural and functional approaches. The binding affinity with HLA-A*2402 molecules and the cellular immunogenicity of the peptide were demonstrated in a series of assays. The X-ray crystal structure of HLA-A*2402 complexed with peptide N1 has shown a novel host strategy to present an immunodominant CTL epitope by intrachain hydrogen bond as a featured epitope.

MATERIALS AND METHODS

Peptide prediction and synthesis. To identify the potential HLA-A*2402-binding peptides within SARS-CoV N protein (GenBank accession no. AY278741), a computer-based program was applied by access through the website of SYFPEITHI (35). One peptide with the sequence positions N346 to 354 (QFKDNVILL) was predicted with high binding affinity to HLA-A*2402. This peptide, later termed N1, happened to be located in one of the highest-immunogenicity regions of the SARS-CoV N protein, which is between amino acids 330 and 354 (21). N1 was synthesized, and the purity was determined as ~95% by analytical high-performance liquid chromatography (HPLC) and mass spectrometry (Scilight-Peptide, Inc.). HLA-A*2402-binding peptide Nef138-10 (RYPLTFGWCF) (11), derived from HIV Nef protein, and HLA-B*3501-restricted peptide B35-18 (EPIVGAETFY) (42), derived from HIV Pol protein, were synthesized and used as control peptides. The peptides used in the following assays are listed in Table 1.

Refolding of peptides with HLA-A*2402 heavy chain and β_2m. HLA-A*2402 heavy chain and β_2-microglobulin (β_2m) were overexpressed as recombinant proteins in *Escherichia coli* and subsequently *in vitro* refolded and assembled in the presence of high concentration of peptide or without any peptide. Generally, the refolding buffer was 250 ml and the molar ratio of heavy chain, β_2m, and peptide was 1:1:2. After sufficient time for protein refolding, the buffer was

TABLE 2. Characteristics of the subjects used in this study

Patient group	ID	Age (yr)	Sex	HLA allele			
				HLA-A		HLA-B	
HLA-A24$^+$	Patient 1	28	Female	A24	A3	B60	B27
	Patient 2	35	Male	A24	A11	B62	B13
	Patient 3	43	Male	A24	A2	B46	B13
HLA-A24$^-$	Patient 4	33	Female	A2	A2	B60	B67
HLA-A24$^+$ healthy donors	Donor 1	30	Male	A24	A11	B60	B60
	Donor 2	41	Female	A24	A3	B7	B13

concentrated and analyzed by Superdex 200 10/300 GL gel-filtration chromatography (GE Healthcare).

MHC stabilization assay with T2-A24 cells. MHC stabilization assays were performed by previously described methods (11). Briefly, T2-A24 cells were incubated at 26°C for 16 h, and then 2×10^5 cells were incubated with peptides at concentrations from 10^{-8} to 10^{-4} mM for 1 h at 4°C. After incubation for 3 h at 37°C, the cells were stained with anti-HLA-A24 monoclonal antibody (MAb), A11.1 M (10), and an R-phycoerythrin (RPE)-conjugated F(ab′)$_2$ fragment of anti-mouse immunoglobulin (Dako). The mean fluorescence intensity was measured by FACSCalibur (Becton Dickinson).

X-ray crystallography, structure determination, and refinement. HLA-A*2402/peptide complexes were refolded by the gradual-dilution method as described above (6, 7, 40). Subsequently, the remaining soluble portion of the complex was concentrated and purified by Superdex 200 10/300 GL gel filtration chromatography and Resource-Q anion-exchange chromatography (GE Healthcare). Crystals were grown by the hanging-drop vapor diffusion method at 4°C. Single HLA- A*2402/N1 crystals were grown at a final concentration of 20 mg/ml in a mixture of 0.2 M ammonium sulfate, 0.1 M Tris (pH 8.9), and 25% (wt/vol) polyethylene glycol 3350. Over the course of 3 days by microseeding, the crystals grew to the maximal size of 400 by 80 by 80 μm. For cryoprotection, crystals were transferred to reservoir solutions containing 20% glycerol. Crystallographic data were collected at 100K in house on a Rigaku MicroMax007 rotating-anode X-ray generator operated at 40 kV and 20 mA (Cu Kα; λ = 1.5418 Å) equipped with an R-AXIS VII++ image-plate detector. Data were indexed and scaled using DENZO and the HKL2000 software package.

The structure was determined using molecular replacement with the program CNS (3). The search model was PDB (Protein Data Bank) code 2BCK with water coordinates omitted (7). Extensive model building was performed by hand using COOT (8) and with restrained refinement using REFMAC5. The further rounds of refinement were performed using the phenix refine program implemented in the PHENIX package with isotropic ADP refinement and bulk solvent modeling (1). The stereochemical quality of the final model was assessed with the program PROCHECK (18). Figure 3 and Fig. 4 were generated using PyMOL (http://www.pymol.org/).

PBMCs from donors. During the 2003 SARS epidemic in Beijing, China, we enrolled and sequentially followed up SARS patients who were diagnosed and recovered from SARS-CoV infection, according to the clinical criteria released by the World Health Organization (WHO; http://www.who.int/csr/sars/casedefinition/en). The purpose and performance of the study were fully explained to all participants from the Beijing Union Medical College Hospital, Beijing, China. Collection of peripheral blood mononuclear cell (PBMC) samples was authorized by the Hospital Ethics Review Committee. Standard serologic HLA typing was performed with peripheral blood from the donors. PBMC samples from three HLA-A24$^+$ patients and from one HLA-A24$^-$ patient who had recovered from SARS were selected. Two HLA-A24$^+$ healthy donors' PBMCs were used as a control (Table 2). The frequencies of HLA-A24 expression in patients who had recovered from SARS and healthy donors were 12.5% and 20%, respectively.

***In vitro* stimulation and culture of PBMCs.** The cells were thawed and incubated with 10 μM peptides in RPMI 1640 containing 10% fetal calf serum (FCS) at 37°C with 5% CO_2 at a cell density of 2×10^6/ml in a 24-well culture plate, and on day 3, 20 U/ml recombinant human IL-2 (rhIL-2) was added to the medium. Half of the medium was changed on day 4 and day 7 with supplementation with 20 U/ml rhIL-2. On day 9, cells were harvested and tested for the presence of peptide-specific CD8$^+$ T cells by enzyme-linked immunospot (ELISPOT) assay and tetramer staining.

ELISPOT assay. The antigen-specific response of T lymphocytes induced by peptides was measured by the use of a gamma interferon (IFN-γ) ELISPOT set (U-CyTech). Briefly, a 96-well ELISPOT plate membrane was preincubated with diluted anti-IFN-γ coating antibody overnight at 4°C. The next day, wells were washed with phosphate-buffered saline (PBS) and blocked with diluted blocking solution for 1 h at 37°C. PBMCs from donors were incubated in microwells (1×10^5 to 3×10^5/well) along with stimulating peptides (20 μM) or phytohemagglutinin (PHA) as a positive control of nonspecific stimulation for 24 h at 37°C with 5% CO_2. Cells incubated without a stimulator were employed as a negative control that produced less than five spots per well in 90% of the experiments. Subsequently the cells were removed, and the plate was processed in turn with biotinylated detection antibodies, streptavidin-horseradish peroxidase (HRP) conjugate, and substrate 3-amino-9-ethylcarbazole (AEC). Development of colored spots was stopped by thorough rinsing with demineralized water. The results were analyzed using an automatic ELISPOT reader.

Tetramer production and staining. HLA-peptide tetramers were produced as described previously (49). Briefly, expression of the HLA heavy chain was limited to the extracellular domain (residues 1 to 276), and the C terminus of the α3 domain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Large amounts of soluble pHLA complexes were generated by refolding. *In vitro* biotinylation of the pHLA complexes was achieved by incubating the sample with the biotin protein ligase BirA (recombinant expressed) and other D-biotin and ATP (Avidity). The samples were purified again through gel filtration before the multimerization by using streptavidin conjugated with phycoerythrin (PE) (Sigma). Cells from the subjects were stained with tetramer (0.05 μg/μl), PE-Cy5-labeled anti-CD3, and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 antibody. The cells were then resuspended in 400 μl staining buffer and analyzed by flow cytometry immediately.

Proliferation assay with CFSE staining. PBMCs were thawed and resuspended in RPMI 1640 medium at 2.5×10^6/ml. Cells were stained with carboxyfluorescein isothiocyanate (CFSE) at 1 μg/ml for 25 min at 37°C in the dark. Cells were washed three times with cold RPMI 1640 medium containing 10% FBS, stimulated with 10 μg/ml peptide and 25 ng/ml IL-7, and incubated in the dark. IL-2 (20 U/ml) was added on day 3. On day 7, cells were washed and fluorescence was detected by flow cytometry. p24 antigen from HIV was used as a negative control.

Protein structure accession number. The accession number of the structure of N1 pMHC in the Protein Data Bank is 3I6L.

RESULTS

Analysis of the potential HLA-A24-restricted epitopes in SARS N protein. Since no HLA allele-restricted epitopes other than for HLA-A2 have been identified throughout the proteome of SARS so far, we are interested in the definition of HLA-A24-restricted epitopes derived from the SARS-CoV N protein, which can play a critical role in cellular immunity in HLA-A24$^+$ patients against SARS-CoV. We predicted the potential HLA-A*2402-restricted epitopes derived from the sequence of N protein through computer analysis. It was found that the peptide with the highest score is nonamer peptide N1 (QFKDNVILL), covering the residues from positions 346 to 354 of the N protein. Further analysis indicated that peptide

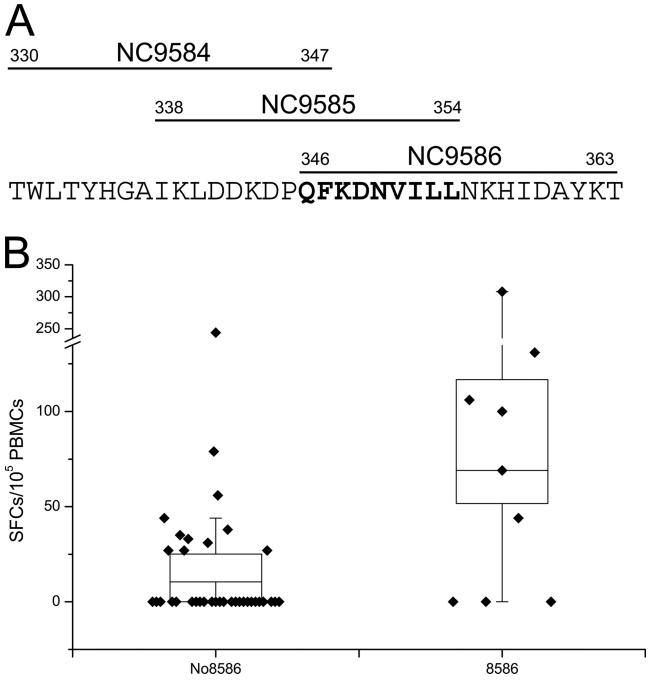

FIG. 1. HLA class I restriction of the immunogenic region within SARS N protein. (A). The sequence of amino acid residues 330 to 363 along the N protein. N330 to N354 was an immunogenic region within SARS N protein identified in a previous study (21). Peptide N1 (N346 to N354), which is shown in boldface, is included by both peptide NC9585 (N338 to N354) and NC9586 (N346 to N363). (B) Data reanalysis of the ELISPOT assays performed with three HLA-A24$^+$ donors recovered from SARS donors (patient 1, patient 2, and patient 3 in the ID [identification] column in Table 2). The entry 8586 represents the peptide pools NX6, NY7, and NY8, which contain peptides NC9585 and NC9586. Meanwhile, No8586 represents the other peptide pools that do not include these two peptides. The top and bottom of each rectangular box denote the standard error (SE), with the median shown inside the box. Horizontal bars extending from each box represent the 90th and 10th percentiles. Rhombus dots indicate the specific CD8$^+$ T-cell responses of the recovered individual donors stimulated by different peptide pools. One dot represents the average response of one individual donor reacting to one of the peptide pools from two independent tests. The total number of dots for entry 8586 is 9, which was calculated as follows: 3 peptide pools $\times$ 3 HLA-A24$^+$ donors. There are 12 pools which do not include these two peptides; therefore, the number of dots for No8586 is 36, which was calculated as follows: 12 peptide pools $\times$ 3 HLA-A24$^+$ donors. Details for peptide pools can be found in reference 21.

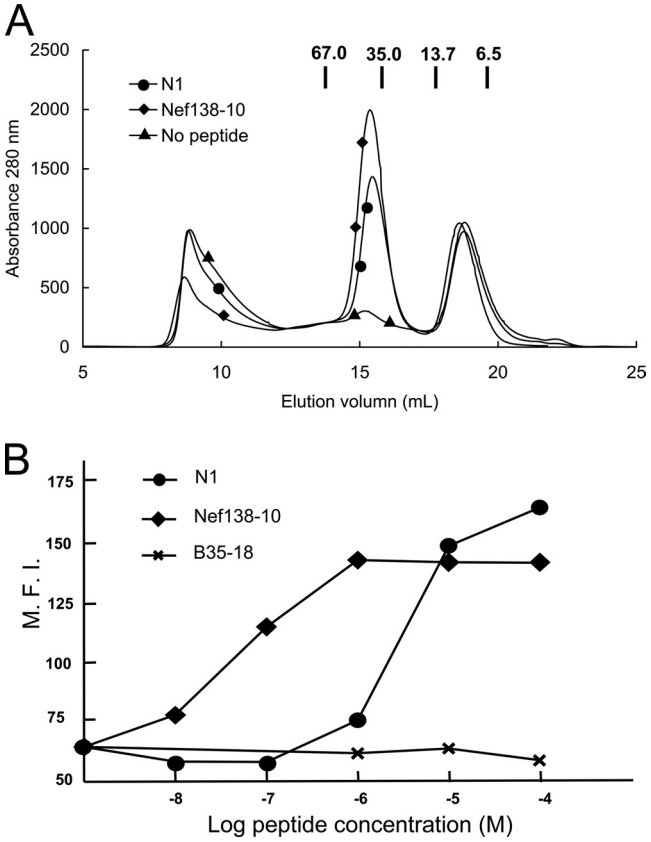

FIG. 2. Binding affinity of peptide N1 to the HLA-A*2402 molecule. (A) *In vitro* refolding of HLA-A*2402 heavy chain and β_2m with N1. As compared to the much lower peak of refolding without any peptide, N1 together with the positive control peptide Nef138-10 could help the HLA-A*2402 complexes refold. The peaks of the complexes with the expected molecular mass of 45 kDa were eluted at the estimated volume of 16 ml on a Superdex 200 column (GE Healthcare). The profile is marked with the approximate positions of the molecular mass standards of 67.0, 35.0, 13.7, and 6.5 kDa. (B) Peptide binding to HLA-A*2402 was quantified by using a T2-A24 stabilization assay. M.F.I., mean fluorescence intensity. These results are representative of three independent experiments.

N1 exists in one of the previously defined two immunodominant regions: positions 330 to 354 (21), which contains potential CTL epitopes in the N protein, as demonstrated in our previous study (Fig. 1A).

Peptide N1 is included in the overlapping peptides NC9585 (N338 to N354) and NC9586 (N346 to N363), and peptide pools containing these two overlapping peptides are NX6, NY7, and NY8 (21). A second analysis of the ELISPOT data shows that, with regard to HLA-A24$^+$ donors who had recovered from SARS, these three peptide pools stimulated distinct CTL epitope-stimulated responses in donors who had recovered from SARS compared to other peptide pools ($P < 0.01$) (Fig. 1B). Taking all these findings into consideration, region N338 to N363 may contain HLA-A24-restricted CTL epitopes, and peptide N1 acts as the candidate with the most potential among them.

Binding affinity to HLA-A*2402. Peptide N1 was selected and synthesized for further analysis. To evaluate the binding efficiency of the peptide to the HLA-A*2402 molecule, we performed a peptide-induced refolding assay of the HLA-A*2402 heavy chain and β_2m *in vitro* (Fig. 2A). Peptide N1 could conspicuously help HLA-A*2402 heavy chain and β_2m refold and form a stable complex. Peptide Nef138-10 acted as the positive control and presented a high binding capability to HLA-A*2402 molecule. HLA-A*2402 heavy chain and β_2m could not form the MHC complexes without the presence of any peptide.

To further determine the binding affinity of these two peptides to the HLA-A*2402 molecule at cellular level, we manipulated an MHC stabilization assay by using T2-A24 cells (Fig. 2B). Although, compared to the positive control peptide Nef138-10, peptide N1 had a lower binding avidity to HLA-A24, N1 distinctly increased HLA-A24 expression on the cell

TABLE 3. X-ray data and refinement statistics

Parameter	HLA-A*2402/N1 result(s)
Data collection statistics	
Space group	P212121
Dimensions, *a*, *b*, *c* (Å)	70.4, 85.8, 92.2
Angles, α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å)	2.4 (2.49–2.40)[a]
Total no. of reflections	149,410
No. of unique reflections	22,272
Completeness (%)	98.6 (97.5)
I/σ	40.7 (6.2)
R_{merge} (%)[b]	9.9 (38.7)
Refinement statistics	
Resolution (Å)	2.4
R_{work} (%)[c]	21.3
R_{free} (%)	28.4
Average B-factor (Å^2)	43.5
Root mean square deviation from the ideal	
Bond length (Å)	0.013
Bond angle (°)	1.443

[a] The numbers in parentheses refer to the number of structure factors used in the measurements.

[b] $R_{merge} = \Sigma_{hkl}\Sigma_i|I_i - \langle I\rangle|\Sigma_{hkl}\Sigma_i I_i$, where I_i is the observed intensity and $\langle I\rangle$ is the average intensity of multiple observations of symmetry-related reflections.

[c] $R = \Sigma_{hkl}||F_{obs}| - k|F_{cal}||/\Sigma_{hkl}|F_{obs}|$, where R_{free} is calculated for a randomly chosen 5% of reflections and R_{work} is calculated for the remaining 95% of reflections used for structure refinement.

surface, indicating that it bound and stabilized the HLA complexes on the cell surface. Negative control peptide B35-18 was determined to have no affinity of binding to HLA-A24, even at a high concentration in the assay.

Overview of HLA-A*2402/N1 structure. The crystal structure of the HLA-A*2402/N1 complex has been determined to a resolution of 2.4 Å (Table 3). The only HLA-A24 structure released in the Protein Data Bank to date is that of HLA-A*2402/VYG (2BCK) with a resolution of 2.8 Å. The higher resolution of HLA-A*2402/N1 complex allows for a more detailed interpretation of the peptide binding of HLA-A24 and rigorous incorporation of additional water molecules.

The HLA-A*2402 structure in the HLA-A*2402/N1 complex is very similar to the HLA-A*2402/VYG structure, with root mean square differences (RMSDs) of 0.771 Å and 0.272 Å for the heavy chain and β_2m, respectively. The unambiguous electron density for the peptide ligand N1 clearly shows the main chain conformation of the peptide and the orientations of the residue side chains. As seen in the previous HLA-A*2402 structure, peptide positions 2 and 9 are major anchors, with F2 deeply buried in the B pocket and L9 in the F pocket (Fig. 3). Comparison of the B pockets in the two structures indicates that the changing of primary anchor residue from Y to F does not induce position changes of the residues in peptide binding pocket B. The backbones of the residues forming the B pockets (Y7, S9, A24, V25, V34, M44, K65, V67, and Y99 of HLA-A24 heavy chain) of the two structures superimpose to an RMSD of 0.249 Å. However, the structure of HLA-A*2402/N1 contains phenylalanine, whereas HLA-A*2402/VYG contains a tyrosine and therefore lacks a hydrogen bond with His 70 of the heavy chain, the only observed discrimination between the two major anchor residues in position 2 of HLA-A24 binding peptides. The secondary anchor of N1 is quite different from that of peptide VYG. Position 3 of N1 is a secondary anchor, with K3 inserted into the D pocket, while V5 of peptide VYG at position 5 participates as the secondary anchor residue.

Conformational features of N1 presented by HLA-A*2402. The main chain of the central region of N1 exists in a unique conformation which can be described as "A" shaped. This is quite different from VYG, which adopts an "M"-shaped conformation. For VYG, the prominently exposed residues are F4 and R6, which form the two tops of the "M." The secondary anchor residue V5, which forms the dip in the "M," secures the peptide in the groove. However, for N1, the three adjacent residues D4, N5, and V6 of the central region bulge out of the

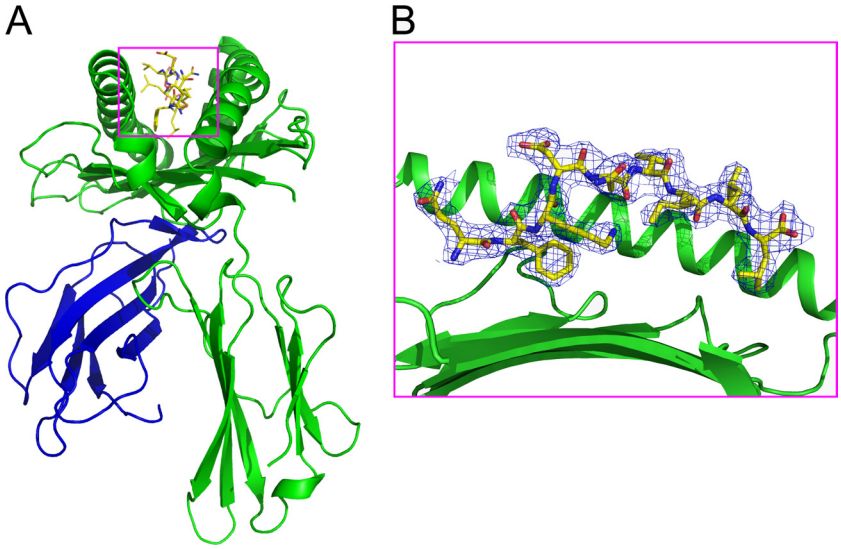

FIG. 3. Structure of the HLA-A*2402/N1 complex. (A) Overview of the structure of HLA-A*2402/N1. (B) The electron density of N1 shows the conformation of the peptide with the second residue of N-terminus F2 and the C-terminus residue L9 to anchor in the HLA groove. Shown here are final 2Fo-Fc-stimulated annealing omit maps contoured at 1.0 σ.

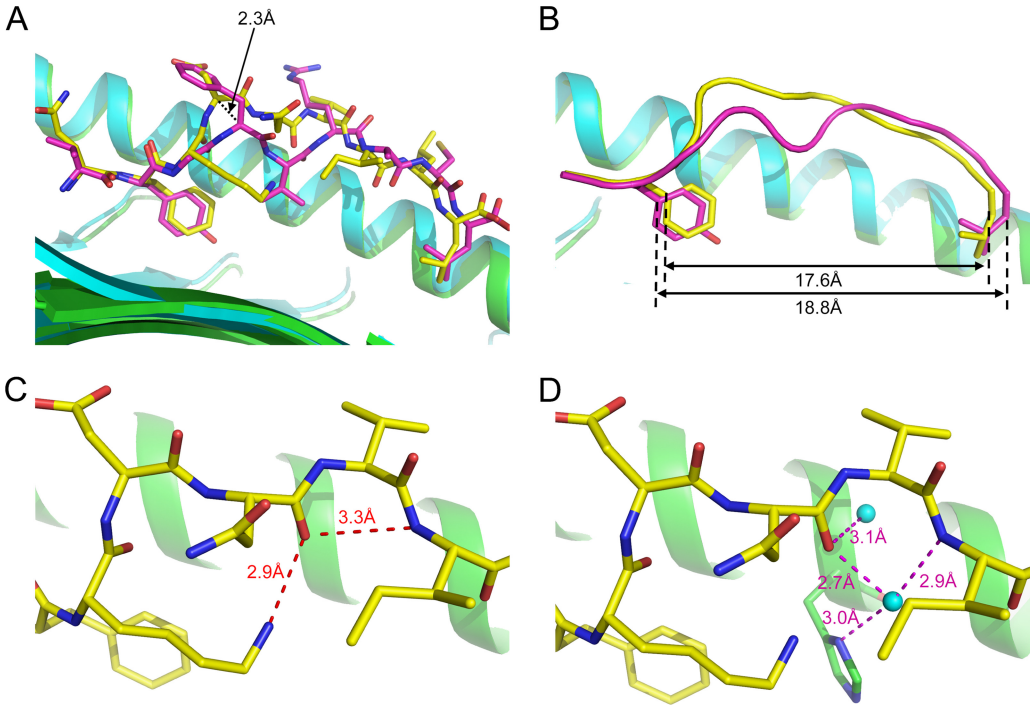

FIG. 4. Conformational features of peptide N1 in the binding groove of HLA-A*2402. (A) D4, N5, and V6 in the middle part of peptide N1 (yellow) bulge out of the peptide binding groove, with the backbone of the three residues rising about 2.3 Å compared to peptide VYG (pink; PDB ID no. 2BCK). (B) The distance between β-carbons of the residues at position 2 and position 9 of N1 (yellow) and VYG (pink) shows the whole length of N1 adopts a more bulged conformation. (C) Two intrachain hydrogen bonds are formed between K3 and N5 and also between N5 and I7, respectively. The side chains of residues D4 and V6, between the two hydrogen bonds, protrude out of the HLA groove and may play a dominant role in the TCR-MHC docking. (D) Central residues of N1 interact with α-helix of HLA-A*2402 through two water molecules.

HLA-A24 surface for potential TCR docking. The distinct "A"-shaped conformation raised the backbone of the central region residues of peptide N1 about 2.3 Å compared to VYG (the distance between α-C of D4 of N1 and F4 of VYG) (Fig. 4A). Although the side chains of D4 and V6 of N1 are quite shorter than the corresponding residues in VYG, F4 and R6, the main chain ascending from N1 enables the side chain ends of D4 and V6 to reach out to an incredible level from the peptide binding groove of HLA. Especially, the side chain of D4 of N1 is raised to the same level as F4 of VYG, which may be helpful for TCR docking. The distance between β-carbons of the residues at position 2 and position 9 of N1 is shorter than that of VYG. The distance between β-carbons of F2 and L9 for N1 is 17.6 Å, and for Y2 and L9 of VYG, it is 18.8 Å (Fig. 4B). This phenomenon demonstrates that, not only the central region, but also the overall main chain of N1 adopts a more bulged conformation, while that of peptide VYG is a relatively extended one. This may also contribute to the protruding extent of the residues at the central region of N1, which can be defined to have the featured characteristic when presented by HLA-A24.

Further analysis of the HLA-A*2402/N1 structure indicated a distinct structure of the N1 peptide in the groove may facilitate the formation of the exclusive conformation of peptide N1. First, the presence of two intrachain hydrogen bonds in the ligand peptide is rarely found among the HLA ligand peptides. The carbonyl oxygen atom of N5 shares the hydrogen atom with the amino group of the side chain of K3 and the amino nitrogen of I7, respectively, to form two intrachain hydrogen bonds (Fig. 4C). These two intrachain hydrogen bonds act as the transverse line in the "A"-shaped conformation of N1 to help the rigid conformation become more stable. Second, the vacuous space formed by the stretching of the two hydrogen bonds is occupied by two water molecules. Residues N5 and I7 interact with these water molecules and are fixed to the α1-helix of HLA-A24 (Fig. 4D). No water molecules are found under the main chain of VYG in the HLA-A*2402/VYG structure.

Investigation of the immunogenicity of N1 with PBMCs of HLA-A24$^+$ in donors recovered from SARS. To determine the immunogenicity of peptide N1, PBMCs of HLA-A24$^+$ donors recovered from SARS were stimulated for 9 days in the presence of peptide N1. The induction of IFN-γ was revealed by the ELISPOT assays with the peptide N1 and two overlapping peptides NC9585 and NC9586 as stimulators. As shown in Fig. 5, N1 significantly elicited specific IFN-γ-producing CD8$^+$ T cells from the PBMCs of HLA-A24$^+$ donors recovered from SARS in comparison to the HLA-A24$^-$ donors recovered from SARS and HLA-A24$^+$ healthy controls (60.5 ± 20.8 versus 6.9 ± 5.4 and 1.3 ± 0.9 spot-forming cells (SFC)/10^5 PBMCs; $P < 0.01$). The overlapping peptides, NC9585 and NC9586, also possessed the ability to stimulate specific IFN-γ production in the HLA-A24$^+$ donors recovered from SARS in comparison to the negative controls ($P < 0.01$). This indicated that these two peptides (which cover the N1 peptide) possessed cross-immunogenicity with peptide N1.

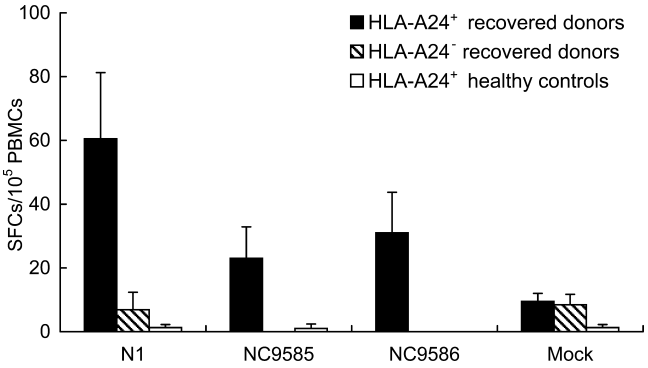

FIG. 5. Detection of peptide N1-specific $CD8^+$ T cells in PBMCs of $HLA\text{-}A24^+$ donors recovered from SARS by ELISPOT. The mean numbers of SFCs in 10^5 splenocytes are represented with bars as a measure of IFN-γ secretion from human PBMCs stimulated with peptides. The PBMC samples from two $HLA\text{-}A24^+$ donors recovered from SARS were thawed and manipulated in ELISPOT assays.

Consequently, the HLA-A*2402/N1 tetramer was prepared and used to confirm the frequency of N1-specific $CD8^+$ T cells. PBMCs from $HLA\text{-}A24^+$ donors recovered from SARS and $HLA\text{-}A24^+$ healthy donors were stained with HLA-A*2402/N1 tetramer after 9-day incubation with N1 and rhIL-2. An average of 0.2% of $CD8^+$ T cells were determined as N1-specific $CD8^+$ T cells from PBMCs of the $HLA\text{-}A24^+$ donors recovered from SARS. In contrast, no N1-specific T cells were detectable from the PBMCs of all tested $HLA\text{-}A24^+$ healthy controls (Fig. 6A).

In the proliferation assay, peptide N1 significantly induced proliferation responses among $HLA\text{-}A24^+$ donors recovered from SARS as measured by CFSE dilution (Fig. 6B), rather than $HLA\text{-}A24^+$ healthy controls (19% versus 4%). Furthermore, when PBMCs were stimulated with negative control HIV p24 protein, the proliferation rates showed no significant difference between $HLA\text{-}A24^+$ donors recovered from SARS and $HLA\text{-}A24^+$ healthy controls (1% versus 2%).

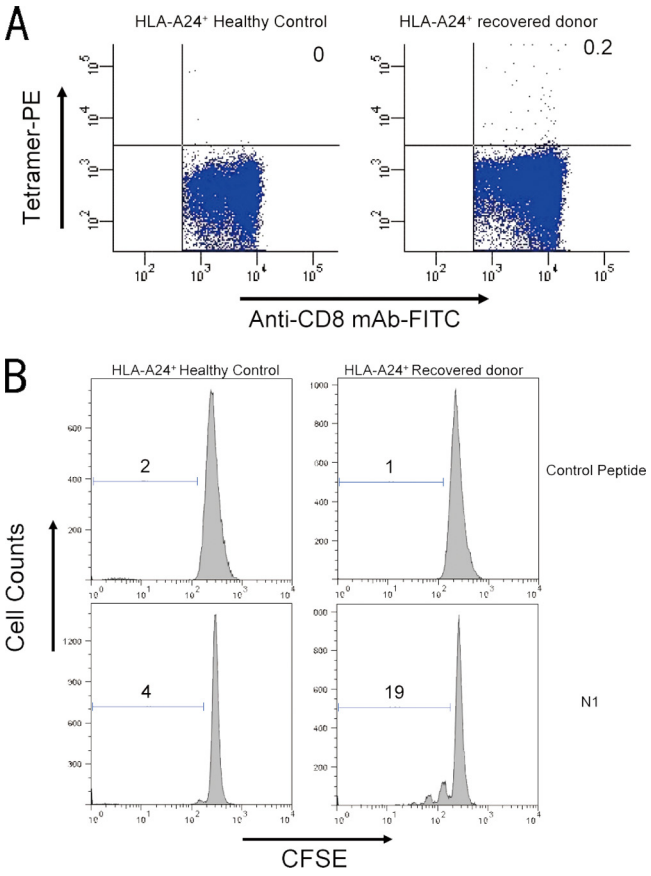

FIG. 6. Identification of the immunogenicity of N1 by fluorescence staining and flow cytometry. (A) In tetramer staining, N1-specific $CD8^+$ T cells were measured from N1-stimulated PBMCs of an $HLA\text{-}A24^+$ donor recovered from SARS (patient 1) and an $HLA\text{-}A24^+$ healthy donor (donor 1), using the PE-labeled HLA-A*2402/N1 tetramer along with PE-Cy5-labeled anti-CD3 and FITC-labeled anti-CD8 MAbs for cell staining. (B) PBMCs from an $HLA\text{-}A24^+$ donor recovered from SARS (patient 3) and an $HLA\text{-}A24^+$ healthy donor (donor 2) were stained with 1 μM CFSE and stimulated by peptides for 7 days. Panels represent percentages of cells that have undergone divisions.

DISCUSSION

Antigenic peptides recognized by virus-specific CTLs are not only useful tools for studying cellular immunity against virus, but also potential reagents for development of immunotherapy. However, the identification of novel CTL epitopes is generally time-consuming and labor-intensive. A large number of 15- to 20-mer peptides with determined immunogenicity for CTLs against viruses as SARS-CoV and influenza virus have been identified without awareness of the HLA allele (19, 21). The immunogenicities of the peptides are evaluated by immunological approaches like cytokine-specific ELISPOT or flow cytometry (23, 32). However, to identify naturally presented optimal epitopes within these long peptides and the HLA allele restriction of these peptides, a large amount of work is still required (2, 27). In a previous study, we have identified two long immunogenic domains within the sequence of SARS-CoV N protein, using the overlapping 15- to 18-mer peptides (21). In this study, we illustrate an efficient and rapid strategy to define minimal natural CTL epitopes presented by a specific HLA allele, HLA-A*2402, while targeting long overlapping peptides. Peptide N1 derived from SARS-CoV N protein was identified as an immunodominant epitope with a featured conformation when binding to HLA-A*2402.

As a nonameric peptide, N1 has no residues with long side chains but shows an immunodominant featured character (21). There are common indications that featured peptides with exposed side chains can generate a more diverse T-cell repertoire than flat, featureless peptides (15, 28, 39, 44, 45). However, how would the "featureless" amino acid contents of N1 help the peptide become a featured epitope? Our study in this report shows that N1 takes use of a featured "A"-shaped conformation with the side chain of N5 in position 5 projecting out of the peptide binding groove, instead of acting as a middle, secondary anchoring residue (7). The side chains of the central region of N1 protrude to the same level as peptide VYG, which has the characteristic featured residues (7). Taking advantage of this strategy, HLA-A*2402/N1 represents a typical structural landscape for a featured peptide which may help to generate a more diverse T-cell repertoire. This intrachain hydro-

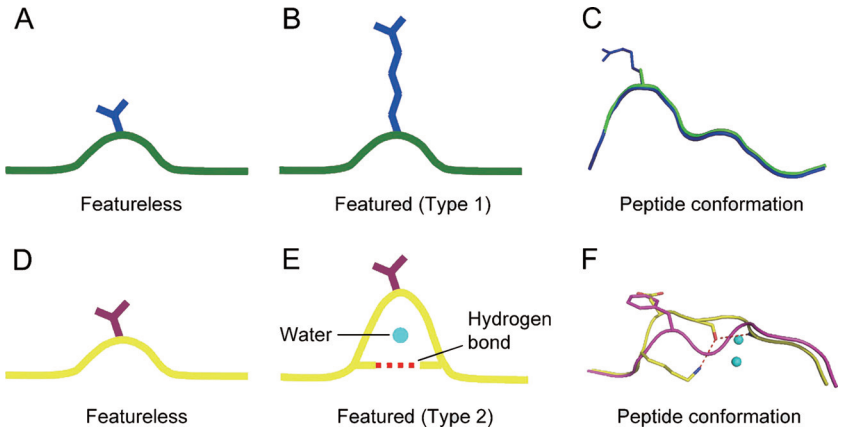

FIG. 7. Topology of the newly identified presentation strategy of featured peptides. Featureless peptides have a typical amino acid length (8 to 10 amino acids) but have few or no solvent-exposed residues with prominent side chains (A and D). The previously determined characteristic of featured peptide is defined as type 1 featured peptide (B). These peptides comprise solvent-exposed residues presenting prominent side chains for recognition by a diverse TCR repertoire (C). In this study, a new presentation strategy of featured peptides was identified (E). Peptide without solvent-exposed residue arches itself through intrachain hydrogen bonds and water molecules under the main chain of the peptide. Through this strategy, short side chains of peptide N1 (yellow) protrude to the level of solvent-exposed residues of peptide VYG (pink) and may be recognized by an immune T-cell repertoire of high diversity (F).

gen-bonding strategy of the host antigen presentation might represent a second type (type 2) of featured epitope in addition to the previously defined type with characteristic long-side chain residues (type 1) (44). This may help us to understand the peptide presentation strategy of the host: exposing the shorter side chain amino acid by making the middle region bulge through intrachain hydrogen bonds to make the peptide a featured epitope (Fig. 7).

The formation of the particular conformation of N1 may be partially due to the contrast of biochemical qualities between the residues in position 5 of peptide N1 and VYG. The D pocket of the HLA-A24 peptide binding groove is hydrophobic and can accommodate the side chain of valine from position 5 of VYG with higher hydrophobicity. In contrast, the side chain of asparagine in position 5 of N1 is repelled out of the D pocket because of the hydrophilicity of the asparagine. Exceptional intrachain hydrogen bonds and under-the-chain water molecules contribute to stabilize the conformation of the central region.

The newly identified peptide N1 (QFKDNVILL), which acts as a dominant epitope located in one of the immunogenic regions, residues 331 to 365 of N protein, could be used as a representative CTL antigen for detection of SARS-CoV-specific CTL epitope-stimulated response within the HLA-A24$^+$ donors recovered from SARS. In addition, it might be a candidate reagent for peptide vaccine development. The novel structure of HLA-A*2402 together with a pathogen-derived peptide in a higher resolution may expand our understanding of the peptide binding properties of HLA-A24 molecules and the strategy of the host to present immunodominant epitopes.

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