recognized with overlapping these two antigenic sites 5 and 1 [7], suggesting that the antigenic determinants in H5 HA may differ from those observed for H1 and H3 HAs.

To better understand the antigenic epitopes, especially the neutralization epitopes of H5N1 HA, we have produced a neutralizing monoclonal Ab (mAb) termed mAb12–1G6 against the pre-pandemic vaccine, NIBRG-14, for H5N1 clade 1 influenza viruses. Our epitope mapping revealed that mAb12–1G6 recognized the loop of A/Viet Nam/1194/2004 (VN1194, the parental strain of NIBRG-14) HA located near the receptor-binding domain in site 5. Our data include that mAb12–1G6 cross-neutralized the clade 2.2.1 of H5N1 virus, suggesting that mAb12–1G6 recognized the corresponding epitope of HA conserved in clade 1 and clade 2.2.1 viruses.

2. Materials and methods

2.1. Virus strains and cell culture

The following strains of influenza virus were used in this study: A/Viet Nam/1194/2004 (VN1194) (H5N1), its attenuated vaccine strain NIBRG-14, A/Indonesia/05/2005 (Ind05/PR8-RG2) (H5N1), A/turkey/Turkey/1/2005 (NIBRG-23) (H5N1), A/Anhui/01/2005 (Anhui01/PR8-RG5) (H5N1), A/duck/Pennsylvania/10128/84 (dP) (H5N2), and A/NewCaledonia/20/99 (NC) (H1N1). Madin–Darby canine kidney (MDCK) and 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

2.2. Virus neutralization test

A hundred 50% tissue culture infectious dose (TCID50) of influenza A H5N1 viruses was mixed with serial dilutions of mAb12–1G6 in Eagle's minimum essential medium containing 0.42% bovine serum albumin at 37 °C for 30 min. The virus-mAb mixture was inoculated to MDCK cells in a 96-well microtiter plate and the cells were incubated at 37 °C for 3–5 days. Neutralizing titers were defined as the minimum concentrations of mAb required to neutralize 100 TCID50 of H5 viruses in MDCK cells.

2.3. Selection of escape mutant

NIBRG-14 virus (10^6 $TCID_{50}$/1.2 ml) was incubated with 1 mg of mAb12–1G6 for 30 min at 37 °C. Serial dilutions of the virus-mAb mixture were inoculated to MDCK cells in a 96-well microtiter plate and the cells were incubated for 4–7 days at 37 °C. The virus culture (10^{4-5} $TCID_{50}$) was subjected to plaque purification in the presence of 2.5 μg of mAb12–1G6.

2.4. Western blot and slot blot analyses

Cells were lysed with sample buffer and subjected to SDS–PAGE followed by Western blotting. After blocking, the membrane was incubated with primary Abs and subsequently with secondary Abs conjugated with horseradish peroxidase. For slot blotting, cells were sonicated and blotted onto the membrane. The membrane was incubated with mAb12–1G6 (test Ab) and mAb25–40 (control Ab). mAb25–40 recognized a conserved region, far apart from the receptor binding pocket, in VN1194 and dP. Signal intensities by test Ab were normalized to those by control Ab. In some experiments, expression levels of HA were initially semi-quantified with control Ab using serial dilutions of cell lysates. After normalization, equivalent levels of HA proteins were blotted onto the membrane and probed with test Ab.

2.5. Expression of recombinant HA proteins

The cDNAs of the H5 HA gene were synthesized from the total virion RNAs of the NIBRG-14 and dP strains by RT-PCR. DNA construction of chimeric HAs between VN1194 and dP was carried out by PCR. Site-directed mutagenesis was carried out by using a Gene Tailor Site-Directed Mutagenesis System (Invitrogen) with the relevant primer sets. Amino acid numbering was based on the structure of H5 HA [12]. The resultant HA cDNAs were cloned into eukaryotic and *Escherichia coli* expression vectors pCDNA3 (Invitrogen) and pET14B (Novagen), respectively. Protein expression in *E. coli* was carried out by addition of isopropyl-β-D-thiogalactoside. 293T cells were transfected with the pcDNA3 vectors using Lipofectamine 2000 or LTX (Invitrogen).

2.6. Immunofluorescence assay

mAb12–1G6 was pre-labeled by using the Zenon Alexa Fluor 488 mouse IgG1 labeling kit (Invitrogen). mAb25–40 was similarly labeled with Alexa Fluor 568 as a control mAb. At 24 h post-transfection with HA expression vectors, MDCK cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Following blocking, cells were incubated with the Alexa Fluor 488-labeled mAb12–1G6. After refixation, cells were incubated with the Alexa Fluor 568-labeled mAb25–40 and were observed by a microscope (BZ8000; Keyence, Osaka, Japan).

3. Results

3.1. Mapping of the epitope recognized by mAb12–1G6

We immunized mice with NIBRG-14, pre-pandemic vaccine for H5N1 clade 1 influenza virus and isolated mAb12–1G6. MAb12–1G6 reacted with HA of its parental virus, VN1194 in ELISA (Fig. S1D) and completely neutralized 100 $TCID_{50}$ of the VN1194 virus at 5 ng/50 μl. Western blotting confirmed that mAb12–1G6 reacted with VN1194 HA1 but not HA2 (data not shown).

To identify the region of VN1194 HA that is bound with mAb12–1G6, we expressed recombinant HAs with N-terminal deletions (termed del1 and del2) in *E. coli* (Fig. 1A). Western blotting revealed that mAb12–1G6 reacted with del1 (containing the globular head domain of HA1, amino acid positions 53–271) but not del2 (with a deletion of the globular head domain) (Fig. 1B), suggesting that mAb12–1G6 recognized the globular head domain of HA1. However, the *E. coli* expression system lacks post-translational modifications (e.g., glycosylation and disulfide bond formation), leading to possibly incorrect protein folding.

To assess the epitope in a more relevant expression system, we expressed full-length HA in mammalian 293T and MDCK cells. We constructed chimeric HAs between VN1194 (H5N1) and dP (H5N2), the latter of which did not react with mAb12–1G6 (Fig. 1C). mAb12–1G6 reacted with chimeras A and C containing the globular head domain derived from VN1194 HA but not with chimeras B and D containing that of dP HA (Fig. 1C). mAb12–1G6 still failed to bind chimeric HA containing a smaller region of dP HA (amino acid positions 116–256) (chimera E) but recovered when the region was limited to amino acid positions 183–227 (chimera G) (Fig. 1C). These findings were confirmed when the signal intensity obtained with mAb12–1G6 was normalized by that obtained with mAb25–40. These data suggest that mAb12–1G6 recognized the amino acid positions 116–183, a region partly composed of the receptor-binding pocket in HA1.

To determine the epitope for mAb12–1G6, we carried out alanine scanning mutagenesis at the surface residues within amino acid positions 128–228 (see Fig. 3D). Western blotting with 293T

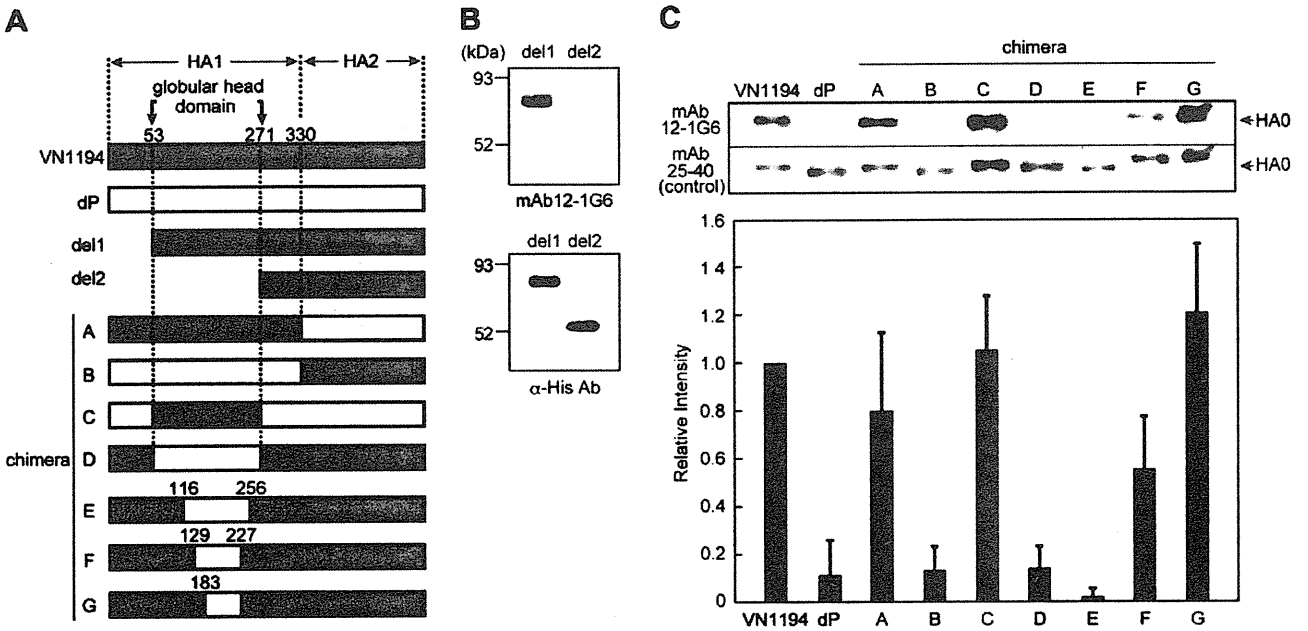

Fig. 1. Reactivities of mAb12–1G6 with truncated and chimeric VN1194 HAs. (A) Schematic representation of truncated/chimeric HAs of influenza VN1194 (H5N1) viruses. Amino acid numbering is based on the H5 HA. The globular head domain of HA1 corresponds to a 53–271 region. The position 330 indicates the HA1/HA2 cleavage site. VN1194 HA is colored in gray and dP (H5N2) HA is in white. (B) Reactivities of mAb12–1G6 with truncated VN1194 HAs. The HAs (del1 and del2) with 6 histidine tags were expressed in *E. coli* and subjected to Western blotting using mAb12–1G6 and anti-histidine-tag Ab. (C) Reactivities of mAb12–1G6 with chimeric HAs between VN1194 and dP. 293T cells expressing chimeric HAs were sonicated and subjected to Western blotting using mAbs12–1G6 and 25–40. Intensities of signals were measured using ImageJ software and relative intensities (mAb12–1G6 to mAb25–40) were calculated. Data were shown as means with standard deviations from three independent experiments.

cell lysates showed that mAb12–1G6 did not react with the VN1194 HA mutants only when they contained single Y141A, Q142A, G143A, K144A, and K193A substitutions (Fig. 2A). Since Western blotting is inappropriate for the analysis of conformational epitopes, cell lysates without protein denaturation were subjected to slot blotting. In this assay, mAb12–1G6 similarly failed to react with the Q142A, G143A, or K144A mutants (Fig. 2B). The use of mAb25–40 (as a control) showed that comparable levels of HAs were applied in both assays. Consistent with the data by these assays, when the HA mutants were expressed in MDCK cells and were subjected to indirect immunofluorescence analysis, mAb12–1G6 did not detect the Q142A, G143A, or K144A mutants (Fig. 2C). Thus, our surface scanning mutagenesis defined the amino acids 142, 143, and 144 of VN1194 HA as determinants for the mAb12–1G6 binding. In the H5 HA1 structure analyzed by crystallography [5,12], the region we mapped was located near the receptor-binding pocket (Fig. 3C), suggesting that mAb12–1G6 may conformationally inhibit HA binding to sialic acid receptor.

To determine accurately the epitope for mAb12–1G6, 3 escape mutants of the VN1194 virus were selected in the presence of mAb12–1G6. Sequencing of the entire HA1 region revealed that all escape mutants carried only S145P substitution.

3.2. Neutralizing activity of mAb12–1G6 against clade 2 viruses and reactivity of mAb12–1G6 with clade 1 H5 HA (VN1194) derivatives containing the corresponding epitope of clade 2.1.3.2 H5 HA (Ind05)

To test whether the mAb12–1G6 generated against the clade 1 VN1194 virus also neutralized various clade 2 H5N1 viruses, VN1194 (clade 1), Ind05/PR8-RG2 (clade 2.1.3.2), NIBRG-23 (clade 2.2.1), and Anhui01/PR8-RG5 (clade 2.3.4) were used. mAb12–1G6 did not neutralize clade 2.1.3.2 or 2.3.4 viruses even at 10 μg/50 μl but did cross-neutralize clade 2.2.1 virus at 5 ng/50 μl, a concentration which was comparable with the concentration to neutralize clade 1 VN1194 virus.

To investigate whether the neutralization failure of mAb12–1G6 against the clade 2.1.3.2 Ind05 virus was due to amino acid variations at the corresponding epitope, the amino acids at positions 142, 144, and 145 of VN1194 HA were replaced with the corresponding residues of the clade 2.1.3.2 Ind05 HA (Q142L, K144S, and S145P, respectively) (Fig. 3A). Slot blotting analysis revealed that mAb12–1G6 did not recognize the H5 HA with S145P substitution. Single Q142L and K144S substantially reduced but did not abolish the reactivity with mAb12–1G6. A combination of these substitutions abolished the reactivity with mAb12–1G6 (Fig. 3B). Collectively, our data indicate that the S145P substitution is crucial for the mAb12–1G6 reactivity, whereas K144R substitution (e.g., clade 2.2.1 NIBRG-23) is well tolerated. A careful observation of the amino acid sequences of the corresponding epitope among the clade 2 viruses indicated that glutamine at position 142 and serine at position 145 were conserved in clade 1 and 2.2.1 viruses and at least, the latter was mutated to proline in the clade 2.1.3.2 and 2.3.4 viruses (Fig. 3A).

3.3. Reactivity of scFv12–1G6 with H5 and H1 HAs

To further confirm the epitope recognized by mAb12–1G6, we expressed and purified single-chain variable fragment (scFv) of mAb12–1G6 in which the genes of the variable heavy and light chains were joined with a polypeptide linker. When the reactivity of scFv12–1G6 was tested by sandwich-ELISA using H5 HAs expressed in 293T cells, scFv12–1G6 showed higher binding affinity to clade 1 VN1194 and clade 2.2.1 NIBRG-23 HAs than dP HA and almost lost the affinity to the VN1194 HA derivative containing Q142L, K144S, and S145P substitutions (Fig. S1A). These binding profiles were similar to those of our original mAb12–1G6. Slot blotting showed that mAb12–1G6 cross-reacted with both VN1194 and NC (H1) viruses (Fig. S1B), possibly through the binding avidity

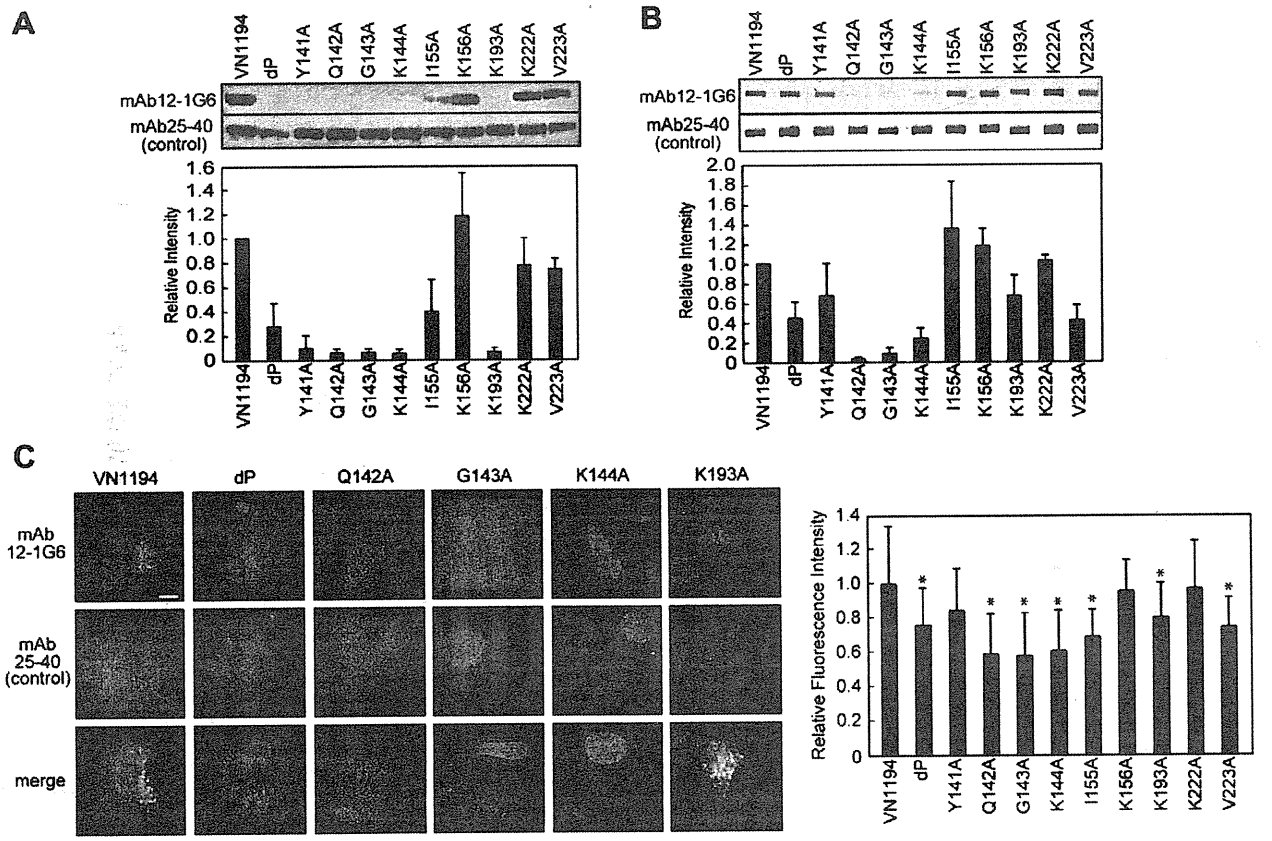

Fig. 2. Reactivities of mAb12–1G6 with VN1194 HA mutants containing amino acid substitutions. 293T cells expressing HA constructs containing amino acid substitutions (indicated) were sonicated and subjected to Western blotting (A) and slot blotting (B) using mAb12–1G6 and mAb25–40 (as a control). In each assay, signal intensities were measured using ImageJ software and relative intensities (mAb12–1G6–mAb25–40) were calculated. (C) MDCK cells expressing HA constructs with amino acid substitutions (indicated) were subjected to indirect immunofluorescence assay using mAb12–1G6 (green) and mAb25–40 (as a control) (red). Nuclei were stained with DAPI (blue). Fluorescence intensities were measured using ImageJ software with the "RGB Measure" plug-in and relative fluorescence intensities (mAb12–1G6–mAb25–40) were calculated. All micrographs were shown at the same magnification. Bar, 10 μm. All graph data were indicated as means with standard deviations from three independent experiments. *, $p < 0.01$.

of mAb12–1G6. To test this possibility, the VN1194 and NC viruses were slot-blotted to the membrane and probe with scFv12–1G6, indicating that scFv did not bind to the NC virus but only detected the VN1194 virus (Fig. S1C). When the binding affinities of mAb12–1G6 and scFv12–1G6 were compared by sandwich-ELISA, the results indicated that scFv12–1G6 showed an approximately 1000-fold lower binding affinity, although both mAb12–1G6 and scFv12–1G6 bound to VN1194 HA and NIBRG-23 HA in a dose-dependent manner (Fig. S1D and E). Much lower affinity of scFv than affinity of mAb has been reported elsewhere [11]. ScFv12–1G6 failed to bind to VN1194 HA derivative containing Q142L, K144S, and S145P substitutions, confirming that mAb12–1G6 and scFv12–1G6 specifically recognized the epitope composed of an exposed loop near the receptor-binding site [12].

4. Discussion

In this study, we obtained mAb12–1G6, which neutralized the clade 1 (VN1194) and clade 2.2.1 (NIBRG-23) but not clade 2.1.3.2 (Ind05) or clade 2.3.4 (Anhui01) H5N1 influenza viruses. Epitope mapping on VN1194 HA revealed that mAb12–1G6 recognized the exposed loop (amino acid positions 140–145 of HA1) corresponding to antigenic site A in H3 HA (Fig. 3C).

Phylogenetic analysis of H5 HA genes from 2004 and 2005 has shown two distinct lineages, termed clade 1 and 2 [3]. Comparison of their amino acid sequences and analysis of crystal structures have identified 13 antigenic variations, many of which are mainly clustered around the receptor binding domain [12]. Indeed, the epitope in clade 1 VN1194 HA recognized by mAb12–1G6 exhibited substitutions Q142L, K144S, and S145P in clade 2.1.3.2 Ind05 HA (Fig. 3A). A comparison of the amino acid sequence in the globular head domain between the VN1194 and Ind05 HAs revealed 12 amino acid differences (Fig. 3D). In the three-dimensional structure of the globular domain, the 6 amino acids (at positions 133, 142, 143, 144, 145, and 193) out of the 12 amino acids were located near the epitope and/or the receptor-binding pocket (Fig. 3C, left). Site-directed substitutions L133S (data not shown) and K193A (Fig. 2B and C) showed no reduction in the reactivity by mAb12–1G6, suggesting that the amino acid differences in the proximity of the epitope were not involved in the reactivity by mAb12–1G6.

A careful sequence comparison around the epitope (Fig. 3A) showed that clade 2.1.3.2 Ind05 and clade 2.3.4 Anhui01, neither of which was neutralized by mAb12–1G6, had common amino acid propensities (polarity or hydrophobicity) in the epitope. For example, lysine (hydrophilic, basic) at position 144 in clade 1 VN1194 HA was substituted for serine in clade 2.1.3.2 Ind05 and for threonine in clade 2.3.4 Anhui01, both of which were hydrophilic and polar. However, when the glutamine at position 142 and the lysine at 144 were singly substituted by leucine and serine within the clade 1 HA, respectively, the reactivity with mAb12–1G6 was reduced but not abolished (Fig. 3B), suggesting that these substitutions, unless combined, may be tolerated. The K144R substitution showed no reduction in mAb12–1G6 binding (Fig. 3B) and in fact, mAb12–1G6 neutralized the clade 2.2.1 (NIBRG-23) strain, which contains a K144R substitution in the epitope.

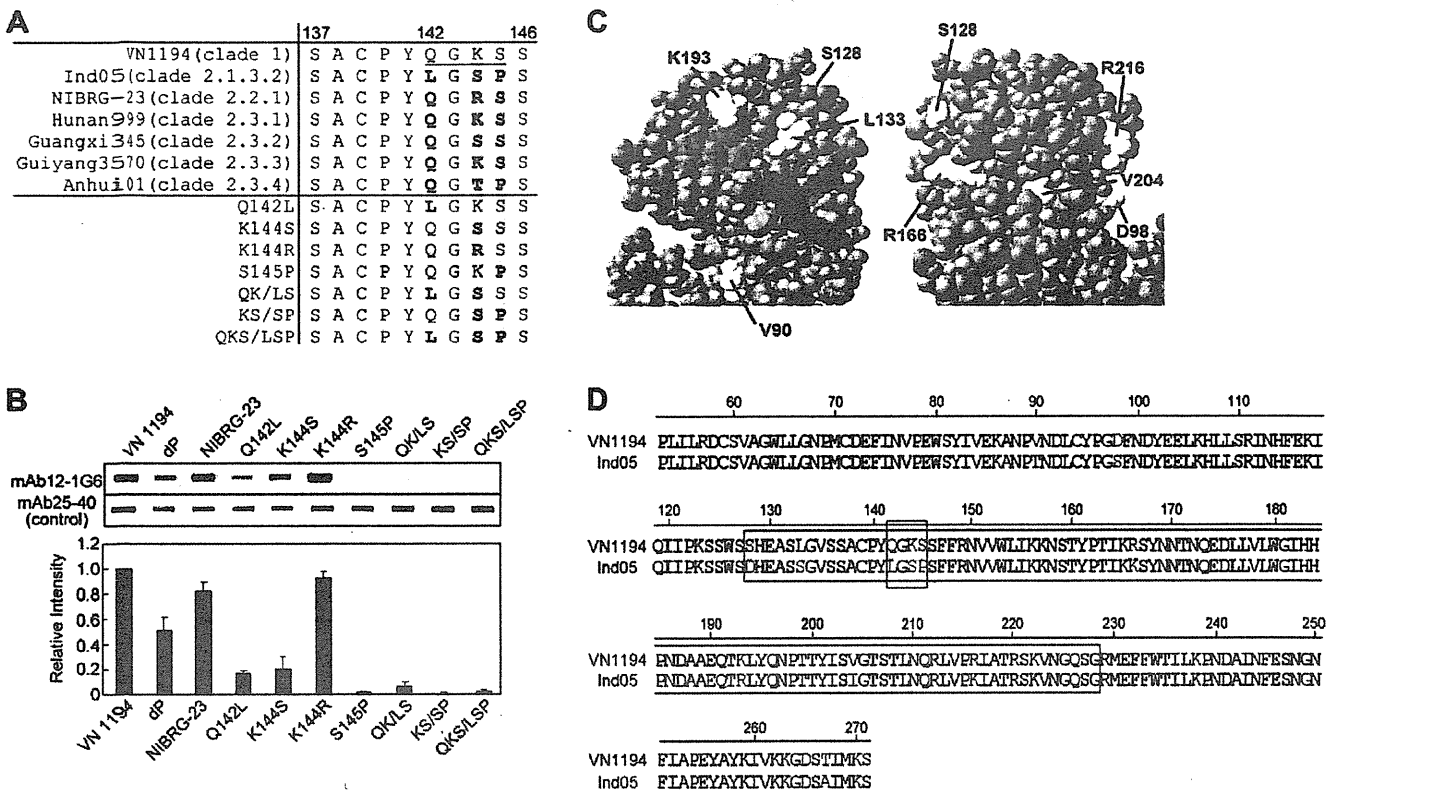

Fig. 3. Defining of the amino acids recognized by mAb12–1G6. (A) Amino acid sequences in and around the epitope recognized by mAb12–1G6 in VN1194 (clade 1), Ind05 (clade 2.1.3.2), NIBRG-23 (clade 2.2.1), A/chicken/Hunan/999/2005 (Hunan999) (clade 2.3.1), A/Goose/Guangxi/345/2005 (Guangxi345) (clade 2.3.2), A/chicken/Guiyang/3570/2005 (Guiyang3570) (clade 2.3.3), and Anhui01 (clade 2.3.4) HAs, and VN1194 HA derivatives containing the corresponding amino acid substitutions. Amino acid diversity is observed at positions 142 (yellow), 144 (blue), and 145 (orange). The epitope recognized by mAb12–1G6 was underlined and mutated amino acids were indicated by bold. (B) The reactivity of mAb12–1G6 with VN1194 HA derivatives containing amino acid substitutions to clade 2 (at positions 142, 144, and 145). 293T cells expressing the HAs containing the single, double, and triple amino acid substitutions were subjected to slot blotting as described in the legend for Fig. 2B. Signal intensities were measured using ImageJ software and relative intensities (means with standard deviations) were calculated form three independent experiments. (C) The epitope recognized by mAb12–1G6 and the location of amino acid differences between VN1194 and Ind05 HAs on the globular head domain. The image of the head domain in H5 HA was generated from the Protein Data Bank (PDB accession number 2IBX) and with Swiss-Pdb Viewer. The epitope recognized by mAb12–1G6 (red), the mutated amino acids between VN1194 and Ind05 HAs (yellow), and the receptor-binding pocket (blue) were highlighted. (D) The amino acid sequences of the globular head domain of the VN1194 and Ind05 HAs. The residues boxed in yellow indicate different amino acids between VN1194 and Ind05. The epitope recognized by mAb12–1G6 was boxed by red line. The region boxed by black line includes the surface amino acids examined by alanine scanning mutagenesis in this study.

More importantly, the serine at position 145 in clade 1 was replaced by proline in clade 2.1.3.2 and clade 2.3.4. The S145P substitution, when introduced into VN1194 HA, completely abolished the reactivity by mAb12–1G6 (Fig. 3B). This finding was further highlighted when the escape mutants of VN1194 were selected in the presence of mAb12–1G6. Consistent with our data, some neutralizing mAbs described previously failed to react with escape mutants from clade 1 A/Viet Nam/1203/04 (H5N1) virus, only when the viruses contained amino acid substitutions at position 145 [7]. Altogether, the data suggest that the proline at position 145 is significantly involved in the loop structure in antigenic site A. Our escape mutants only contained the S145P substitution, suggesting that mAb12–1G6 may specifically recognize the loop structure. Alternatively, it is possible that the substitution at position 145 has initially been selected in the presence of mAb12–1G6. A comparison of the amino acids at position 145 in H5N1 HA sequences showed proline in clade 2.1.3.2 (Ind05) and clade 2.3.4 (Anhui01) but serine in clades 2.3.1, 2.3.2, and 2.3.3. Since the serine at position 145 is conserved in clade 2.3.1–2.3.3 viruses, it is possible, although is not proven, that mAb12–1G6 may have cross-clade neutralizing activity against these clade viruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.108.

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