ISSN 0026-8933, Molecular Biology, 2015, Vol. 49, No. 2, pp. 303–311. © Pleiades Publishing, Inc., 2015.
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CELL MOLECULAR BIOLOGY

UDC 578.74

Dedicated to the Enlightened Memory of Professor Nikolai Veniaminovich Kaverin, Member of the Russian Academy of Sciences

Effect of Amino Acid Substitutions in the Small Subunit of the Avian H5N2 Influenza Virus Hemagglutinin on Selection of the Mutants, Resistant to Neutralizing Monoclonal Antibodies

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Received October 9, 2014; in final form, October 28, 2014

Abstract—Mutations in the hemagglutinin (HA) protein molecule of influenza A viruses associated with the virus's resistance to physical and chemical factors can play important roles in the selection of influenza variants during circulation in nature. In this study, we examined escape mutants of the A/mallard/Pennsylvania/10218/84 (H5N2) influenza virus, which were selected with a monoclonal antibody specific to an epitope in the large HA subunit (HA1). We obtained escape mutant m4F11(4), which carries a single amino acid substitution $S145P_1$ in the HA1 subunit and two other mutants, m4G10(10) and m4G10(6), which accumulated additional mutations in the small subunit (HA2), i.e., $L124F_2$ and $L124F_2$+$N79D_2$, respectively. We demonstrate that amino acid substitutions in HA2 of m4G10(10) and m4G10(6) viruses can compensate for the negative effect of the $S145P_1$ mutation, which manifested in a significant increase in the capacity for viral replication at the early stages of infection in chicken embryos, as well as in growth in the HA thermostability compared with the m4F11(4) escape mutant. We propose that these variations in phenotypic properties, which provide advantages during viral replication, play a role in the positive selection factor and are retained in the viral population.

DOI: 10.1134/S002689331502003X

Keywords: influenza A virus, hemagglutinin H5, escape mutants, monoclonal antibodies, phenotypic properties

Antigenic drift in surface glycoproteins, i.e., hemagglutinin (HA) and neuraminidase, are some of the most important evolutionary processes that occur in human influenza A virus. A similar phenomenon has been described for avian influenza viruses of the H5N1 subtype, which are the cause of epizootics in birds and can result in sporadic cases of severe illness in humans [1, 2]. At present, antigenic drift is usually modeled by selecting escape mutants that are resistant to the neutralizing action of a monoclonal antibody (mAb). In this process, in addition to amino acid substitutions in the large subunit (HA1), responsible for the resistance to the action of neutralizing mAb, escape mutants may acquire amino acid substitutions in other HA regions that do not react with the antibody. These secondary mutations are often considered random, and their appearance in escape mutants has been attributed to the high frequency of mutations in influenza viruses.

We characterized a large set of escape mutants in previous studies, during antigenic mapping of the HA subtype H5 [3, 4]. Many mutants obtained by a single selection carried two or three amino acid substitutions, some of which were located in the small subunit (HA2), which does not react with the mAb used in this selection [4]. In cases, when the same substitutions in HA2 were detected in mutants obtained in independent selection experiments with a specific mAb, the probability of these events was considered to be unlikely. In this study, we examined the effects of amino acid substitutions in HA2 on the phenotypic characteristics of escape mutants of influenza virus subtype H5.

MATERIALS AND METHODS

Viruses

Escape mutants m4F11(4) and m4G10(10) were selected previously [4, 6] based on a mouse-adapted virus A/mallard/Pennsylvania/10218/84 (H5N2) (Mlrd/

† Deceased.

PA-MA) in the Laboratory of Subviral Structures at the Ivanovsky Institute of Virology, Russian Ministry of Health [5]. Viruses were accumulated in 10-day-old chicken embryos, which were infected with virus-containing material into allantoic cavity at multiplicity of infection at 1000 EID_{50} per embryo. Infected embryonated chicken eggs were incubated for 48 h at 37°C, then cooled overnight at 4°C. Virus-containing allantoic fluid was collected and the viral particle content was measured by titration in hemagglutination assay (HA assay) [7]. Viral titers were expressing in hemagglutination units (HAU). Preparations were stored at –80°C.

Monoclonal Antibodies

Antibodies 3G9, 4G10, 4F11, 5F12, 6E2, 5G9, and 7E11 against A/duck/Novosibirsk/56/05 (H5N1) influenza virus were used as ascites fluids obtained in mice [8, 9].

Selection of Escape Mutants

Escape mutants of influenza virus were obtained using methodology developed by Webster and Laver [10] with some modifications. Allantoic fluids were diluted to a concentration of infectious virus at 2×10^7 EID_{50}/mL, mixed with mAb and incubated for 1 hour at room temperature. The resulting suspensions were inoculated into 10-day-old chicken embryos, and incubated at 37°C for 48 h. Viral variants resistant to this mAb were selected using HI [11] and passaged five to six times by cloning chicken embryos using methodology of limiting dilutions to obtain homogeneous virus populations.

Determination of Infectivity of Influenza Viruses in Eggs

To determine the 50% embryo infection dose (EID_{50}), 10-day-old chicken embryos were inoculated into allantoic cavities with a series of tenfold dilutions. Five embryos were used for each dilution. Infected chicken embryos were incubated for 48 h at 37°C and cooled overnight. Then, allantoic fluid was collected from each embryo. Presence of virus was evaluated using the HA assay method with a 0.75% solution of chicken erythrocytes. The average EID_{50} value for each viral variant was calculated using the methodology of Reed and Muench [12].

Purification and Concentration of Influenza Viruses

Virus-containing allantoic fluids were centrifuged at 3000 rpm for 15 min at 4°C. Then, viral fluids were layered on 4 mL of 20% sucrose in 0.01 M Tris-HCl buffer (pH 7.2) containing 0.15 M NaCl. Virus particles were precipitated by ultracentrifugation (Beckman, United States) in a SW27 rotor at 22000 rpm for 90 min at 4°C. Viral particle precipitate was resuspended in 1/64 of the original volume the same buffer in a Dounce homogenizer and clarified by centrifuging at 3500 rpm for 8 min at 4°C. The viral content in the resulting preparations was determined by titration in HA assay. Preparations were stored at –80°C.

Serological Assays

Solid-phase enzyme-linked immunosorbent assay (ELISA) was performed by the methodology variant of Philpott et al. [13] with some modifications. Purified virus diluted to 100 HAU in PBS (pH 7.4) was added to each well of a 96-well plate and incubated at 4°C for 18 h. Nonbound virus was removed by washing the plate with PBS-0.05% Tween-20 (PBST). Free surface portions of the plate were blocked with a 2% solution of bovine serum albumin (BSA) in PBS for 1 h at 37°C. Then, blocking solution was removed and mAb diluted in 1% BSA in PBS were added to the wells and incubated for 2 h at 37°C. Then, plates were thoroughly washed with PBST and processed for binding with goat anti-mouse antibodies conjugated with horseradish peroxidase (Sigma Chemical, United States). Then, wells were rinsed, filled with 100 μL of tetramethylbenzidine solution in substrate buffer (MDL, Russia). Reaction was stopped with 2 M H_2SO_4. Absorbance of the product was measured in a spectrophotometer Microplate Reader (BioRad, United States) at a wavelength of 450 nm. The efficacy of mAb binding to virus was calculated, using the formula by Philpott et al. [13]. Hemagglutination inhibition assay (HI) was carried out by conventional methodology [11] using a 0.75% suspension of chicken erythrocytes.

Kinetics of Virus Accumulation in Chicken Embryos

The determination of virus replicative kinetics in chicken embryos was performed as described previously [6] in the presence and absence of mAb 4G10 obtained towards virus A/duck/Novosibirsk/56/05 (H5N1). Virus-containing allantoic fluid (1000 EID_{50}) was used to infect 10-day-old chicken embryos, followed by incubation at 37°C for 18, 24, 36, or 48 h. Five embryos were used for incubation at each time interval. At the end of incubation, embryos were cooled, allantoic fluid collected, and the virus content in each sample was determined using the HA assay method.

To determine the kinetics of virus accumulation in the presence of mAb, infected chicken embryos were incubated for 1 h at 37°C. Then, allantoic cavities were filled with 0.2 mL of mAb 4G10 (diluted 1 : 100 in lactalbumin hydrolyzate medium), and subsequent procedures were carried out as described above.

Reproductive Activity of Viruses at Different Temperatures

Ten-day-old chicken embryos were inoculated with virus at a dose of 1000 EID_{50} per embryo (and incubated in a thermostat (Binder, Germany) at 33, 37, or 39°C for 48 h. Four embryos were used at each temperature point. After the incubation period, allantoic fluid was collected from each embryo under sterile conditions, and the viral content in it was measured with HA assay. Next, combined samples were prepared from viral preparations obtained at a particular temperature by combining equal volumes of individual allantoic fluids. Combined samples were titrated to determine infectivity at 37°C for 48 h (see section Determination of Infectivity of Influenza Viruses in Eggs). The reproductive capacity of each virus at a test temperature (RCT marker) was calculated as the ratio of infectious titers ($lgEID_{50}$) at the test temperature and at 37°C.

Analysis of Viral Thermostability

Virus-containing allantoic fluids were clarified by low speed centrifugation, diluted in PBS to 128 HAU, and dispensed by 120 μL in 0.5-mL, thin-walled PCR tubes (SSI, United States). Tubes were placed in a Mastercycler Gradient 5331 thermal cycler (Eppendorf, Germany). The temperature range was set at 51.5–63.0°C. Tubes were heated for 40 min, then transferred to ice. Control samples containing 120 μL of virus were incubated for 40 min at 0°C. Virus content in each sample was determined by HA assay using 0.75% suspension of chicken erythrocytes.

Determination the pH Optimum of Fusion of Hemagglutinin (Hemolysis Test)

Virus-containing allantoic fluids were clarified by low-speed centrifugation and diluted with PBS to 128 HAU. Then, 50 μL of 2% chicken erythrocyte solution in PBS were added to 250 μL of clarified viral preparation. Mixtures were incubated with shaking at +4°C for 1 h. Then, erythrocytes with virus adsorbed on their surface were centrifuged at 2500 rpm for 1 min at +4°C. Supernatant was discarded and precipitate was resuspended in 250 μL of 0.1 M MES buffer (pH 5.0–7.0) and incubated with shaking for 1 h at 37°C. Erythrocyte pellet without the addition of the virus was resuspended in 250 μL of 0.5% Tween-20 in PBS served as the positive control, and erythrocyte pellet without the virus that was resuspended in 250 μL of MES buffer (pH 7.0) served as the negative control. After incubation, samples were centrifuged for 1 min at 2500 rpm. Then, 170 μL of supernatant were collected and transferred to a 96-well plate for measuring the optical density at 450 nm using the iMark Microplate Reader (Bio-Rad, United States). Results of measurements were plotted in a graph and used to determine the pH of fusion [14].

Polymerase Chain Reaction and Sequencing

Viral RNA was isolated from virus-containing allantoic fluid using a set of reagents in RNeasy Mini Kit (Qiagen, United States). Reverse transcription and PCR were performed with primers for Influenza A virus HA gene [4]. Amplification products were purified using QIAquick PCR Purification Kit (Qiagen, United States). DNA sequencing was carried out using a DNA ABI Prism 3130 automated DNA sequencer (Applied Boisystems, United States), and a set of reagents in BigDye Terminator v3.1 kit (Applied Boisystems, United States). Nucleotide sequences were analyzed using DNASTAR Sequence Analysis Software Package (DNASTAR, United States).

RESULTS

Selection of Escape Mutants and Amino Acid Substitutions in Hemagglutinin Molecules

We carried out a selection of escape mutants based on wild-type virus Mlrd/PA-MA (H5N2). Selection and characterization of escape mutants, m4F11(4) and m4G10(10), with amino acid substitutions, $S145P_1$, and $S145P_1$ and $L124F_2$, respectively, is described in our previous reports [4, 6]. Escape mutant m4G10(6) was selected, using a neutralizing antibody mAb 4G10, directed to an epitope located on the HA1 subunit of H5 HA. We determined HA gene sequence of this mutant, and identified nucleotide changes, responsible for amino acid substitutions in the large (HA1) and a small (HA2) subunits. One of these mutations, $S145P_1$, was localized to the antigenic site A of the HA1 subunit of HA of H3 subtype [15, 16]. Two other mutations were identified in HA2: $N79D_2$ and $L124F_2$.

Interaction of Escape Mutants with Monoclonal Antibodies

Interactions of Mlrd/PA-MA virus and its escape mutants, m4F11(4), m4G10(10) and m4G10(6), with neutralizing mAb, obtained against virus A/Duck/Novosibirsk/56/05 (H5N1), were examined, using HI and ELISA assays (Table 1). We have previously characterized mAb 7E11 and 4F11 [4, 9], which reacted with antigenic site A, whereas mAb 3G9, 5F12, 6E2 and 5G9, interacted with antigenic site B, while 4G10 was found to bind to the site A and, partially, to the B site in HA molecule.

As seen from results shown in Table 1, mAb 4F11, 4G10, 7E11 directed towards the antigenic site A of HA1 did not significantly inhibit the binding of escape mutants carrying amino acid substitution $S145P_1$ at site A to erythrocytes. HI titers for these mutants showed a 16-fold or higher reduction compared to the parental virus.

The HI method is based on noncompetitive inhibition. Antibody binds to an epitope on HA, while sialy-

Table 1. Results for cross-immunological assays (HI and ELISA[1]) for Mlrd/PA-MA (H5N2) virus and its escape mutants selected with mAb to virus A/duck/Novosibirsk/56/05 (H5N1)

Viruses	Amino acid substitutions	mAbs to antigenic sites of HA													
		A				A + B		B							
		7E11		4F11		4G10		3G9		5F12		6E2		5G9	
		HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA
Mlrd/PA-MA[2]		12800	+	25600	+	6400	+	1600	+	6400	+	6400	+	12800	+
m4F11(4)[2]	$S145P_1$	<200	–	<200	–	200	+	3200	+	3200	+	6400	+	12800	+
m4G10(10)	$S145P_1$ $L124F_2$	<200	–	200	+	200	+	800	+	12800	+	12800	+	12800	+
m4G10(6)	$S145P_1$ $N79D_2$ $L124F_2$	<200	–	200	+	200	+	1600	+	12800	+	12800	+	6400	+

[1] ELISA results are presented as + (binding > 75%) and – (binding < 25%). Percentages of bound viral particles were calculated using the formula by Philpott et al. [13]. Binding efficiency of wild-type virus with each mAb was considered as 100%.

[2] HI data for indicated viruses are cited from the study by Rudneva et al. [4].

lated carbohydrate chains on the surface of red blood cells interact with the HA receptor-binding site. Therefore, HI results are affected not only by the interaction of the HA variant with an antibody, but also by the proximity of the antibody epitope to the HA receptor-binding site, as well as by the HA affinity to receptors on the surface of red blood cells and the valency of these interactions. Valency is defined as the multiplicity of HA molecules on a virion and the multiplicity of receptors on the erythrocyte surface compared to a monovalent interaction of mAb with and HA molecule. That is why a negative HI result in a virus/mAb/erythrocyte system does not necessarily indicate the absence of interactions between HA and mAb. For this reason, we employed a more sensitive approach, i.e., the ELISA method, to access direct binding. As can be seen from the data presented in Table 1, all escape mutants interacted with mAb 3G9, 5F12, 6E2, and 5G9, which recognize site B on HA, as well as the antibody 4G10, which recognizes sites A and B. In addition, m4G10(10) and m4G10(6) mutants also interacted with 4F11 antibody to site A on the HA molecule.

Thermal Stability of Escape Mutants

The examination of the thermal stability of HA of viral variants uncovered differences in the temperature dependence of virus inactivation between Mlrd/PA-MA and its escape mutants (Fig. 1). The parent virus Mlrd/PA-MA ability to agglutinate erythrocytes was abolished after heating at 56.7°C [6], whereas, for escape mutants, the temperature for HA inactivation was higher. For instance, m4F11(4) mutant with $S145P_1$ substitution was inactivated at 61.4°C, while m4G10(10), which contains an additional amino acid replacement, $L124F_2$, was inactivated at 59.5°C [6]. Escape mutant m4G10(6), which contains two additional substitutions, $N79D_2$ and $L124F_2$, was the most thermostable. Its inactivation temperature was identified as 63.0°C. We propose that the $N79D_2$ substitution or both substitutions in HA2, i.e., $N79D_2$ and $L124F_2$, lead to the stabilization of the HA structure, which affects the resistance of the virus to heat inactivation.

Kinetics of Accumulation of Viral Escape Mutants in Chicken Embryos

The growth kinetics of the original virus and its escape mutants in chicken embryos were evaluated by HA assay at time points of 18, 24, 36, and 48 h [6]. Only escape mutant m4F11(4) with a single substitution at $S145P_1$ was not detected 18 h after infection and, at the 24-h mark, its HA titter was lower than for other viruses (Fig. 2a). Virus variants m4G10(10) and m4G10(6) with amino acid substitutions in HA2 manifested a level of replication similar to that of parental strain at all time points. However, the content of m4G10(10) mutant with a single replacement in HA2 surpassed all other variants 48 h after infection. These result indicates that amino acid substitutions in HA2 can compensate for the negative effect of the $S145P_1$ amino acid substitution, which results in the efficient replication of escape mutants in chicken embryos at the early stages of infection.

In order to find out the role of amino acid substitutions in the HA2 subunit of HA in the process of selection of escape mutants, we carried out a series of experiments to investigate the kinetics of virus accumulation in the presence of mAb 4G10, which recognizes antigenic site A in the HA molecule. We did not

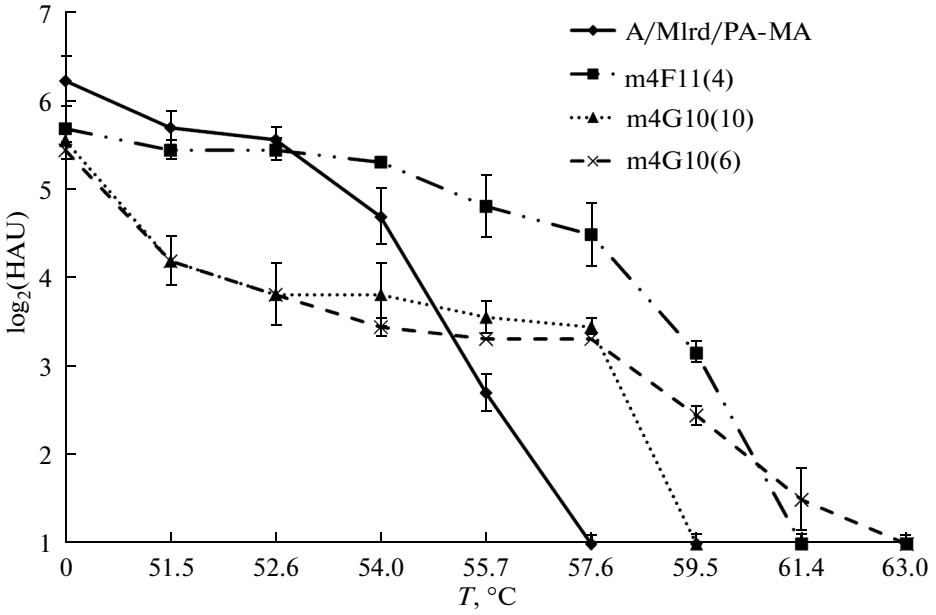

Fig. 1. Thermal stability of Mlrd/PA-MA (H5N2) virus and its escape mutants. This and subsequent graphs present results as average values with standard deviations calculated based on three independent experiments.

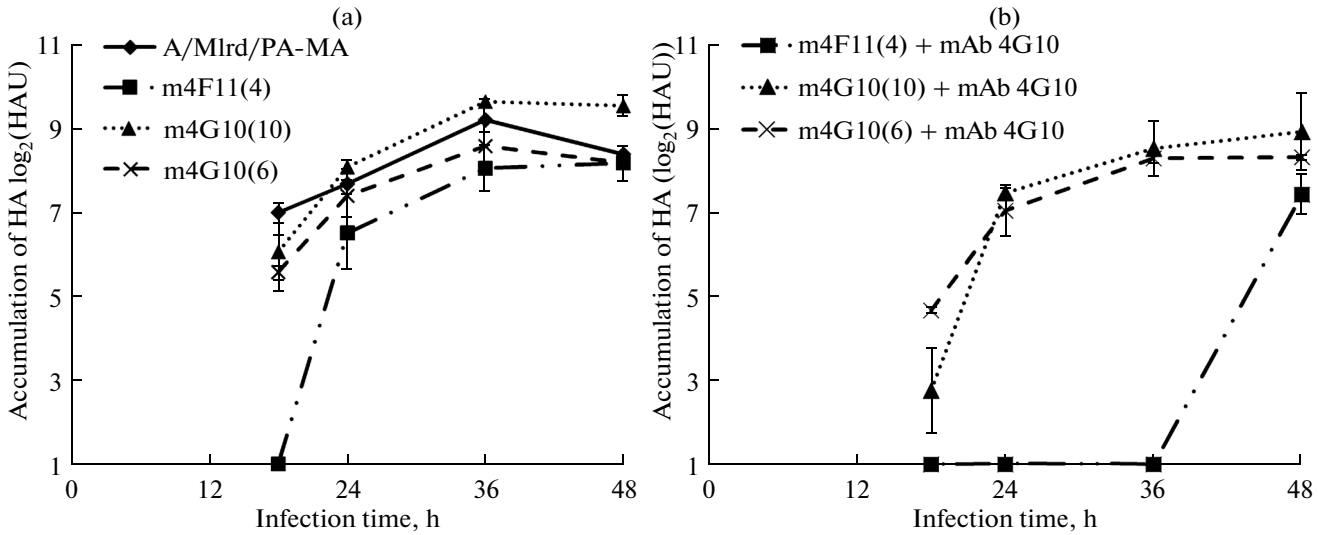

Fig. 2. Changes in hemagglutinin accumulation in Mlrd/PA-MA virus and its escape mutants in infected chicken embryos during multiple cycles of infection in the (a) absence and (b) presence of mAb 4G10.

test the original Mlrd/PA-MA virus in these experiments, since the mAb 4G10 neutralizes it completely. The results of these experiments are presented in Fig. 2b. No replication of m4F11(4) virus with a single $S145P_1$ substitution can be seen in the presence of 4G10 mAb at time points at 18, 24, and 36 h. However, m4G10(10) and m4G10(6) mutants with additional substitutions in HA2 have been detected by HA assay at 128–256 HAU. Thus, we conclude that amino acid substitutions $N79D_2$ and $S145P_1$ can compensate for the negative effect of $S145P_1$ mutation, which affects the ability of the virus to replicate actively during the selection process under the influence of mAb 4G10.

Reproductive Activity of Mlrd/PA-MA Virus and Its Escape Mutants at Different Temperatures (RCT Marker)

The investigation of RCT markers showed that the parental Mlrd/PA-MA virus manifested the highest activity ($\log EID_{50}$) during reproduction in chicken embryos at a low temperature of 33°C (Table 2). In contrast, infectious titers of escape mutant m4F11(4)

Table 2. Reproductive ability of Mlrd/PA-MA virus and its escape mutants at different temperatures*

Viruses	Amino acid substitutions	$\log EID_{50}$		
		33°C	37°C	39°C
Mlrd/PA-MA	–	8.7 ± 0.4	8.0 ± 0.3	7.7 ± 0.2
m4F11(4)	$S145P_1$	7.6 ± 0.3	7.3 ± 0.3	7.9 ± 0.2
m4G10(10)	$S145P_1$, $L124F_2$	7.8 ± 0.3	7.7 ± 0.2	8.3 ± 0.3
m4G10(6)	$S145P_1$, $N79D_2$, $L124F_2$	8.5 ± 0.2	7.7 ± 0.2	7.7 ± 0.2

* Results are presented as mean values ±SD calculated based on three independent experiments.

($S145P_1$) decreased by almost an order (0.7–1.1 $\log EID_{50}$) at 33 and 37°C compared with Mlrd/PA-MA virus. Same tendency was observed for m4G10(10) ($S145P_1$ and $L124F_2$). However, at 39°C, the activity of the latter increased. In m4G10(6) mutant ($S145P_1$, $N79D_2$, and $L124F_2$), the reproduction levels at all temperatures did not differ from the parental Mlrd/PA-MA virus. Thus, we detected differences in the effects of one or two amino acid substitutions in HA2 on the ability of the respective escape mutants to replicate in chicken embryos at a given temperature. In all cases, these changes compensated for the negative impact of the $S145P_1$ mutation on the replicative ability of the virus.

DISCUSSION

We selected a large set of escape mutants in previous studies in the course of antigenic mapping of the H5 subunit of HA [3, 4]. Several of these mutants, which were generated in a single round of selection, had two or three amino acid substitutions in the HA molecule, including within the small subunit. We reported earlier [6] that amino acid substitution at position 145, localized in a lateral loop of HA1 subunit, which corresponds to antigenic site A in the HA molecule of the H3 subtype, has an effect on viral phenotypic parameters, such as the thermal stability and the kinetics of viral accumulation in chicken embryos. Amino acid substitutions $N79D_2$ and $L124F_2$ in the HA2 subunit are located fairly far from each other. Figure 3 shows that position 79 is localized at the top of a long α helix stalk in HA. In turn, position 124 is located near the fusion of the HA α-helical domain with cell membrane. In HA trimer, these amino acids are localized to the contact surfaces between monomer subunits, which participate in formation of hydrogen bonds and hydrophobic interactions, and may affect the process of fusion of the viral and cellular membranes.

The roles of amino acid substitutions in HA2 subunit during selection of escape mutants using mAb is poorly understood. Furthermore, phenotypic characteristics of viruses with altered HA2 subunit are not well studied. At this point, it is known that amino acid substitutions in the HA2 of influenza H5 virus may lead to changes in multiple parameters, such as pH of conformational transition, thermal stability, transmissivity, and infectivity [14, 18–20]. However, the association of antigenic and phenotypic properties of mutant viruses with amino acid substitutions in the HA2 was not reflected in previous studies. Our investigation represents the first attempt to describe the relationship between antigenic and phenotypic properties of escape mutants with amino acid substitutions in the large and small subunits of the H5 molecule of HA, and the role of amino acid substitutions in HA2 in the process of selection of escape mutants.

A comparison of the results of cross-immunological reactions (ELISA and HI) revealed certain differences, the causes of which were summarized briefly in the Results section. An earlier study by Ilyushina et al. [21] discussed how resistance to mAb may be acquired by escape mutants. The resistance of escape mutants are presumed to be caused mainly by changes in the primary sequence of the protein within the antibody recognition epitope as a result of mutations and the consequent conformational changes in the epitope area or the appearance of an additional glycosylation site. In the examined escape mutants, amino acid substitutions in HA did not lead to the formation of glycosylation sites. Thus, the increased resistance to mAb in these mutants is presumably caused by changes in the structure and/or the conformation of HA1 epitopes. The appearance of a proline residue in a protein generally results in a kink in the polypeptide chain and, hence, a local disruption in the secondary and tertiary structure. Let us discuss the results of our ELISA assays, which uncovered interactions between escape mutants and 4F11mAb, which recognizes antigenic site A at the location of the $S145P_1$ substitution, in more detail. In this case, the observed absence of mAb binding to m4F11(4) mutant is quite logical. However, the detected interactions with m4G10(10) and m4G10(6) are surprising. This is an interesting result, which indirectly indicates the possibility of an alternate HA1 conformation in mutants with substitutions in HA2 compared with the spatial structure of m4F11(4) with a single $S145P_1$ substitution.

An analysis of the thermal stability of escape mutants with amino acid substitutions $N79D_2$ and $L124F_2$ revealed their ability to agglutinate erythro-

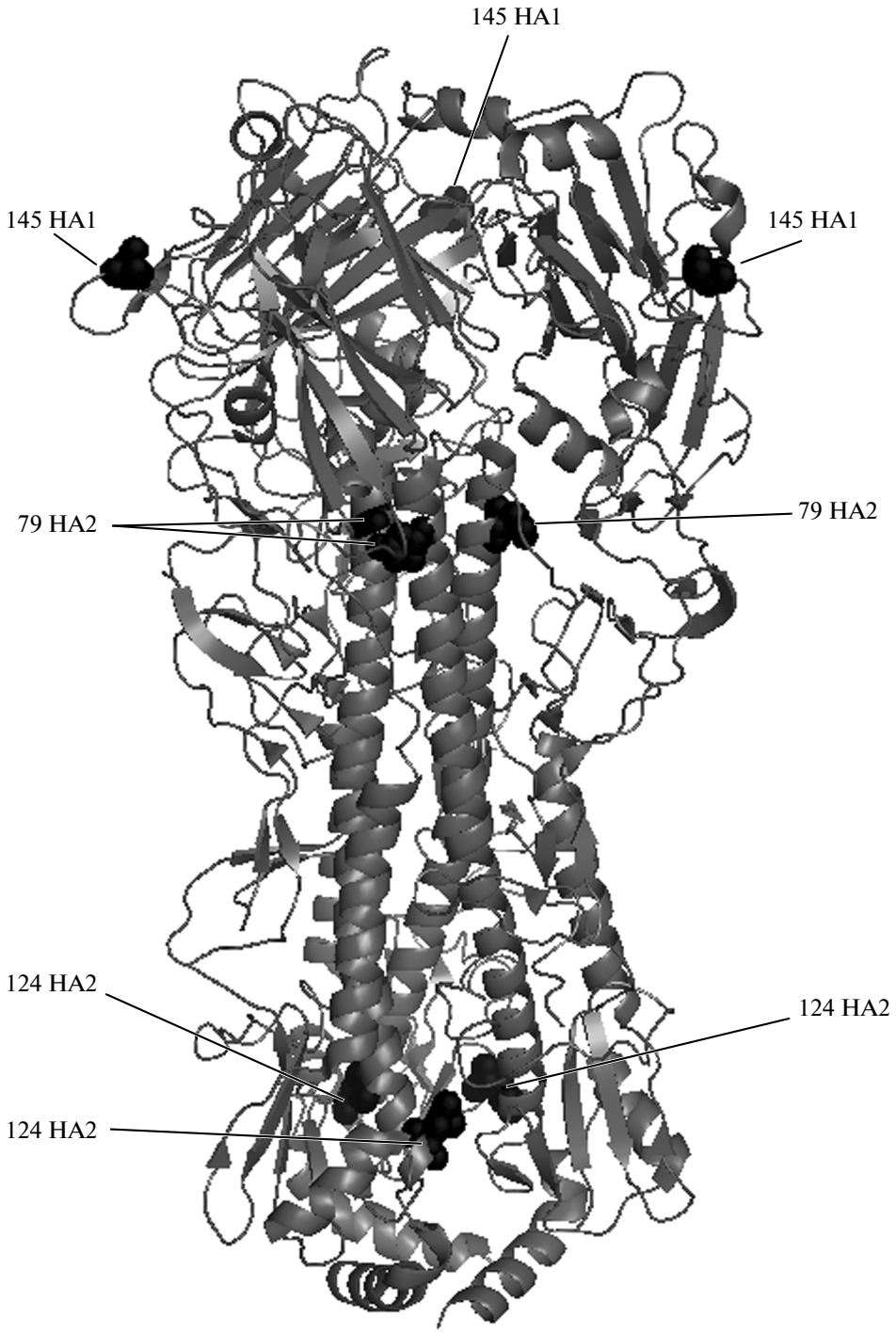

Fig. 3. Localization of amino acid substitutions in Mlrd/PA-MA virus escape mutants in a three-dimensional structure model of H5 HA (PDB 1FK0) [17]. Image processing was performed with PyMol 0.99c software.

cytes at elevated temperatures (Fig. 1). Apparently, substitutions in HA2 molecule provide for a more rigid structure, and ensure its stability at elevated temperatures. The amino acid residue at position 124 is located near the base of the HA stalk. The appearance of phenylalanine residue at this position can potentially stabilize the contacts between the neighboring HA2 subunits of the monomers [22], since phenylalanine (unlike leucine) can participate in stacking interactions. Furthermore, asparagine at position 79, which is devoid of a negative charge (inherent to aspartic acid residues at physiological pH), can make the tip of the stalk region less accessible to water molecules, which can in turn lead to an increase in the overall stability of

the structure of the HA trimer. The study by Xu et al. [23] showed that, just before the virus fuses with the cell membrane, the D79 side chain interacts with the K68 residue of the α helix, which is located on another HA2 monomer. Interestingly, analyses of highly pathogenic strains of H5 subtype with a number of bioinformatics algorithms uncovered a so-called internal disorder in the upper segment of the long chain of the HA2 α subunit (near position 79) and, at the adjacent hinge that joins the long and short α chains [24]. In that study, Goh et al. showed that the mobility of the HA2 molecule in the region of amino acid residues 68–79 correlates with the increased virulence of highly pathogenic strains of influenza virus, H5N1 and 1918 H1N1. Thus, changes in this area of the HA2 subunit most likely affect the viability of the virus and its replicative capacity.

We found that the appearance of amino acid substitutions in HA2 subunits affected replicative abilities of escape mutants m4G(10) and m4G(6) at the initial stages of infection in chicken embryos. In these cases, the levels of viral accumulation 18 h after infection were higher than that of the m4F11(4) mutant with a single substitution in HA1 (Fig. 2a). In the presence of 4G10 mAb, the only replication of m4G(10) and m4G10(6) mutants was detected in chicken embryos at 18, 24, and 36 h. In contrast, m4F11(4) virus was not detected. It is known that the increase in the replicative activity of the influenza virus may be associated with the change of the optimal pH of fusion [25]. Therefore, we tested this possibility in the studied escape mutants, m4F11(4), m4G10(6), and m4G10(10), and found that optimum pH of fusion did not differ from that of the wild-type virus (data not shown). Thus, the observed changes in replicative phenotypes of escape mutants are not associated with changes in the pH values of viral fusion and are due to other factors.

Our analysis of the effects of temperature on replicative activity of virus mutants in chicken embryos revealed that the reproduction of the m4G10(6) virus, which carries two additional substitutions $N79D_2$ and $L124F_2$, did not differ from the parental Mlrd/PA-MA virions at any temperature and was maximal at 33°C. In contrast, the m4G10(6) mutant with an additional substitution at $L124F_2$ demonstrated a different nature of the temperature dependence of replication and reached a maximum at 39°C. Thus, amino acid substitutions in the small subunit of HA can affect the optimum temperature of the replication of escape mutants, which can provide them with advantages in the process of antigenic drift.

Based on our results, we propose that amino acid substitutions in the HA2 subunit, which appear during the selection of escape mutants using mAb directed to HA1 subunit, are not accidental, but rather play the role of compensatory mutations by providing advantages to these viral variants during replication in chicken embryos and by promoting escape effects by neutralizing the action of mAb during the selection process. What are the molecular mechanisms underlying these phenomena? We can speculate that these mechanisms are based on conformational changes in HA molecule and/or intermolecular interactions between HA2 subunits of the adjacent monomers, which ultimately affects the efficiency of virus replication. Detailed studies in the near future should define the molecular mechanisms of acquired resistance to mAb by escape mutants under the influence of these substitutions.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research project no. 12-04-00257.

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Translated by I. Grishina