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ORIGINAL ARTICLE

Analysis of a point mutation in H5N1 avian influenza virus hemagglutinin in relation to virus entry into live mammalian cells

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Abstract Binding to and infection of human cells is essential for avian influenza virus transmission. Since virus binding is not always predictive for efficient infection of the cells, here we wished to investigate how hemagglutinin (HA) mutations of avian influenza virus H5N1 influence virus post-binding events in a single cycle of replication. One mutation observed in H5 HA of avian and natural human isolates from mainland China, Hong Kong, Vietnam and Thailand was identified and analyzed. The effects of the mutation on receptor binding, fusion and virus entry into cultured cells were investigated using hemadsorption, polykaryon formation and pseudotyped virus that express luciferase in the cytoplasm of transduced cell. Our results revealed that replacing aspartic acid at residue 94 with asparagine enhanced virus fusion activity and increased the binding of HA to sialic acid α2,6 galactose, while it decreased pseudotyped virus entry into cells expressing the avian type receptor, sialic acid α2,3 galactose. Our result may have implications for the understanding of the role of HA mutations in virus entry into live cells that exclusively display one type of receptor.

Abbreviations

AIV	Avian influenza virus
HA	Hemagglutinin
Wt	Wild type
SA	Sialic acid
RBS	Receptor binding site

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Introduction

Continuous outbreaks of highly pathogenic H5N1 influenza A virus in poultry and humans across numerous countries on different continents are unprecedented. Current evidence suggests that all fatal human cases result from direct transmission of virus from birds to humans [6], and so far these viruses are unable to be transmitted efficiently from human to human [32]. Clearly, the potential for the emergence of a human-adapted H5N1 virus remains a worldwide public-health risk.

It has been suggested that binding to and infection of human airway epithelial cells [23, 26] is essential for avian influenza virus transmission to humans. The HA mediates attachment of the virus to sialic-acid-containing receptors on the host cell surface, as well as fusion of the virus envelope with the cellular membrane [15, 25].

Generally, human isolates preferentially bind to terminal sialic acids with an α2,6 linkage (SAα2,6Gal), while avian isolates prefer an α2,3 linkage (SAα2,3Gal) [2, 13, 14]. Many studies have reported that the switch in receptor specificity from SAα2,3Gal (avian) to SAα2,6Gal (human) is a key factor for influenza A virus to cross the species barrier [18, 29], and mutating just one or two amino acids in HA is sufficient to switch AIV receptor specificity from avian to human [8, 21, 30]. The E190D, Q226L, or G228S mutation in H1,H2, or H3 have been demonstrated to switch their preferences from avian-type to human-type

receptor. Yet, none of these mutations causes a similar receptor preference switch for the H5 subtype, even though they reduce the binding of H5 to the avian type SAα2,3Gal [27, 28]. Recent studies have identified mutations at amino acids 182 and 192, that independently convert the receptor preference of H5 HA from avian to human type [33]. Despite the fact that human trachea primary epithelial cells express both sialyl(alpha2-3)Gal receptor for avian influenza viruses and sialyl(alpha2-6)Gal receptor for human influenza viruses [17], HA mutations play an important role in virus transmission from avian to human. Also it was anticipated that other mutations in H5 HA, particularly those occurring in the circulating virus isolated from human, may enable the virus to be transmitted across species.

The receptor-binding preference of influenza virus has been studied in cell-based assays [8], competition assays with soluble receptor analogues [21] and glycan microarray assays [27, 28]. Although these studies provided valuable information on virus receptor binding specificity, they may not be predictive for subsequent infection of the target cells. Thus, a greater understanding is clearly needed of the post-binding function that may contribute to differences in viral infection and tropism. Recently, it was reported that, by carrying the green fluorescent protein (GFP) as a heterologous membrane-anchored protein, the interaction between native HA molecule and live cells and subsequent infection of cells could be determined quantitatively [24]. However, this method cannot investigate the entry of infectious virus in a single cycle of replication, which may be helpful for interpreting infection data. In addition, this method cannot rule out the influence of other genes of the virus. In an earlier study, pseudotyped retroviral vectors were used to analyze the receptor-binding pocket of H7 avian influenza virus. However, the binding and transduction of pseudotyped virus was not compared with the expression of receptors on the cells that were used [19].

In the present study, in order to quantitatively investigate how an H5 HA mutation may affect the entry of virus into cells in a single cycle of replication, we constructed pseudotyped HIV-HA-luc viruses that carry a mutated HA and luciferase reporter gene. The entry of these HA mutants into live mammalian cells was quantitated by the amount of luciferase expressed and compared with the expression of the sialic acid receptors on these live cells. Moreover, our assay separates the HA and NA from the rest of viral genes, allowing a focused study of the HA factor in viral infection. The simplicity and the lower biosafety requirement of this method provide a convenient way to monitor the influence of each identified HA mutation on virus entry into target cells. We also analyzed the binding and polykaryon formation properties of the HA mutants. Our results revealed that the D94 N mutation increased the recognition of H5 HA by SAα2,6Gal human type-receptor and decreased entry of pseudotyped virus HIV-HA-luc into cells with SAα2,3Gal avian-type receptor.

Materials and methods

Cells lines

293T and COS cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL). HeLa cells were maintained in minimal essential medium (Gibco). In all cases, the culture medium was supplemented with 10% fetal bovine serum (FBS) (Hyclone, UT), 100 μg/ml streptomycin and 100 unit/ml penicillin.

Sequence comparison of HA and construction of HA mutants

In order to identify mutations of HA that are located near the receptor-binding domain and observed in both avian and human natural isolates, the sequence encoding the entire HA gene from A/chicken/Fujian/1042/2005 was used as wild type (Wt) and compared with isolates identified in both poultry and humans in mainland China, Hong Kong, Thailand, and Vietnam during outbreaks between 1996 and 2005. Viral RNA of A/chicken/Fujian/1042/2005 (H5N1) was extracted from allantoic fluids using the RNeasy RNA extraction kit (Qiagen, Chatsworth, CA) according to manufacturer's protocol. The H1 HA was amplified from the cDNA of A/WSN/33(H1N1) and inserted between the XhoIand Not I sites of the pCI-neo vector (Promega) to generate pCI- HA (Hu). The H5 HA and neuraminidase (NA) genes were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from viral RNA according to a previously described method [11]. Amplified full-length viral HA, NA and ion channel protein M2 cDNA were inserted between the Xho I and Not I sites of the pCI-neo vector to generate pCI-HA (Wt), pCI-NA and pCI-M2. Mutants were generated, using pCI-HA as a template, by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) and verified by DNA sequencing.

Hemadsorption assay for HA receptor-binding activity

For detecting the receptor-binding specificity of these HA mutants, hemadsorption was performed with erythrocytes from different species that vary in the types of SA they display. HeLa cells ($\sim 3\times 10^5$ cells in a 35-mm-diameter plate) were transfected with 2 μg purified pCI-HA and

2 μg pCI-NA plasmids plus 0.25 μg pCI-M2 (which was co-expressed with HA to support HA transport). After a 40-h incubation at 37°C, the medium was removed. Cells were washed twice with PBS, treated with *Vibrio cholerae* sialidase (5.5 mU/ml, Roche) for 1 h at 37°C, washed twice again, and incubated respectively with chilled 0.5% horse or guinea pig erythrocyte suspensions in PBS for 1 h at 4°C. The cells were washed five times with PBS, adsorbed erythrocytes were disrupted with distilled water, and the concentration of hemoglobin in the lysate was evaluated by measuring the absorbance at 540 nm.

To determine whether mutations made in the HA affected the level of expression of the proteins on the cell surface, surface expression of HA was determined in parallel with the hemadsorption assay on HeLa cells. Briefly, HeLa cells ($\sim 3\times10^5$ cells in a 35-mm-diameter plate) were transfected with 2 μg of pCI-HA or its mutant plasmids. Forty hours after transfection, cells were washed with PBS and incubated for 30 min at 4°C with anti-H5 chicken serum at 1:1,000 dilutions. The cells were then washed once with PBS and incubated for 30 min at 4°C with fluorescein-labeled goat anti-chicken immunoglobulin antibodies (Sigma, St Louis, MI) at 1:200 dilutions. After two washes, cells were analyzed on a FACSSCAN (FACScan, Becton Dickinson, Heidelberg, Germany).

Polykaryon formation assay

A polykaryon formation assay was performed to investigate whether HA mutations had any influence on the efficiency of HA-directed fusion. We used HeLa cells for the polykaryon formation assay because they possess both the SA < alpha > 2,3 Gal and SA < alpha > 2,6 Gal receptors, which are important for both avian and human influenza virus infection. On the other hand, it was easy for us to observe and count polykaryon formation using HeLa cells. The capacity of HeLa cells transfected with HA expression plasmid to fuse together to form polykaryons was assayed as described previously [7]. HeLa cells ($\sim 3\times10^5$ cells per well; six-well plate) were transfected with 2 μg of pCI-HA plasmid or its mutant plasmids. Two days after transfection, cells were washed and incubated for 2 min at 37°C with fusion buffer (10 mM HEPES, 10 mM MES in PBS, pH 5.0) and then incubated for another 6 h at 37°C with complete medium to allow the formation of polykaryons. The cells were then fixed and stained with crystal violet, and polykaryons were counted in five randomly chosen fields. To determine whether mutations made in the HA affected the level of expression of the proteins on the cell surface, surface expression of HA was determined in parallel with the polykaryon formation assay on HeLa cells.

Production and quantification of HIV-like particle release

These pseudotyped viruses were constructed by incorporating HA mutants and NA into the HIV-luciferase vector, pNL43LucE$^-$R$^-$ (HIV-luc) [3], which encodes all of the HIV gene products except Env and Nef (a generous gift from Dr. Hongkui Deng). Pseudovirus HA-HIV-luc was produced by transient transfection of 1 μg each of pCI-HA and pCI-NA envelope expression plasmid plus 1 μg pNL43-luc-E$^-$R$^-$ plasmid on 293T cells using the calcium phosphate precipitation method [19]. Forty-eight hours after transfection, the culture supernatants were harvested and filtered through a 0.45-μm filter (Millipore, Watford, UK), and aliquots were stored at −80°C. One milliliter of filtered viral supernatant was further purified by centrifugation through 400 μl of 20% sucrose at 14,000 rpm for 30 min at 4°C.

The resulting pellet was resuspended in 2 × loading buffer, and the sample was split into two and subjected to SDS-PAGE. For the detection of HA proteins, one blot was probed with a chicken anti-HA polyclonal antibody (purchased from the Veterinary Institute of Harbing, China) at a dilution of 1:500, and horseradish peroxidase-labeled goat anti-chicken IgG (Sigma) was used as secondary antibody at a dilution of 1:1,000. For detection of the HIV p24 protein, the second blot was incubated with mouse anti-p24 monlclonal antibody (purchased from Wandayin Biotech, BJ) at 1:1,000 dilution followed by HRP-conjugated goat anti-mouse IgG as the secondary antibody (1:10,000) (Wandayin Biotech). Blots were developed with the enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Next, the amount of HA on HIV-like particles released into the culture supernatants was quantified by HA antigen capture ELISA. In brief, 96-well polyvinyl choride microplates (Costar) were coated with 1:200 diluted chicken anti-H5 polyclonal antibody (purchased from the Veterinary Institute of Harbing, China) at 4°C overnight (100 μl/well), and washed with washing buffer (0.1% Tween20 in PBS). The HIV-like particles, diluted with phosphate-buffered saline (PBS), were adsorbed to the wells of an antibody-coated plate (100 μl/well) at 37°C for 1 h. After unbound particles were removed with washing buffer, anti-HA monoclonal antibody (kindly provided by Wenbo Liu) at 1:3,000 dilution were added to the plate (100 μl/well) and incubated at 37°C for 1 h. After washing with washing buffer, a horseradish-peroxidase-labeled goat anti-mouse IgG (Sigma) was used as secondary antibody at a dilution of 1:20,000 (100 μl/well), followed by 1 h incubation at 37°C for 1 h. After washing, the amount of labeled pseudovirus was quantified by evaluating the

perodoxidase activity present in the wells by using the standard o-phenylenediamine substrate. The amount of HA in the cell culture supernatant was calculated according to the HA antigen standard (purchased from the Veterinary Institute of Harbing, China).

Transduction by pseudotyped HA-HIV virus on 293T and COS cells

To investigate the effects of these HA mutants on virus entry into cells, pseudotyped virus carrying these mutants was used to transduce two different mammalian cell lines (293T or COS cells). The entry of these HA mutants was assessed by measuring the luciferase activity of the pseudotyped virus HIV-HA on live cells. Sub-confluent 293T or COS cells (in 24-well plates) at a density of 2×10^4 per well were transduced with an equal amount of pseudotyped virus (0.5 HA units). Inoculum of pseudoparticles in serum-free DMEM was added to the well and cells were incubated for 6 h at 37°C before addition of regular serum-containing medium. Two days after transduction, the cells were lysed, and the luciferase activity in 20 µl lysate was assayed in a TD-20/20 luminometer (Promega) using commercially available reagents (Promega).

Lectin staining

For the detection of the relative amounts of SAα2,3Gal and SAα2,6Gal moieties on the surface on three different cells lines, cells were analyzed using linkage-specific lectins (Glycan Differentiation Kit, Roche). Briefly, cultured 293T, COS or HeLa cells were resuspended and washed twice in PBS and once with buffer 1 (50 mM Tris–HCl, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ A). Then, the cells were incubated at 4°C for 1 h with 0.1 mg/ml digoxigenin (DIG)-labeled *Sambucus nigra* agglutinin (SNA), which specifically binds to the SAα2,6Gal/GalNAc moiety, or with 0.5 mg/ml DIG-labeled *Maackia amurensis* agglutinin (MAA), which specifically binds to the SAα2,3Gal/GalNAc moiety. After washing with PBS, the cells were incubated with fluorescein-conjugated anti-DIG antibodies (Roche) at 1:500 dilution, then washed and analyzed for fluorescene intensity.

Results

Effects of the D94 N mutation on the receptor binding specificity

After comparing the amino acid sequences of wild-type (Wt) HA with some of those circulating in mainland China, Hong Kong, Vietnam, and Thailand between 1996 and 2005 (Fig. 1a), we identified six amino acid substitutions outside the receptor-binding domain of HA, which are K35R, D45 N, D94 N, K35R/D45 N, K35R/D45 N/D94 N, A267T. Preliminary analysis of these six mutations revealed that, with the exception of D94 N mutant, none of these mutants displays an apparent influence on receptor binding and post-binding events (data not shown). To determine the contribution of D94 N to viral receptor-binding, fusion and entry into cells, we performed further analysis and analyzed the D94 N mutation by comparing the D94 N single mutant with K35R/D45 N double (dMT)

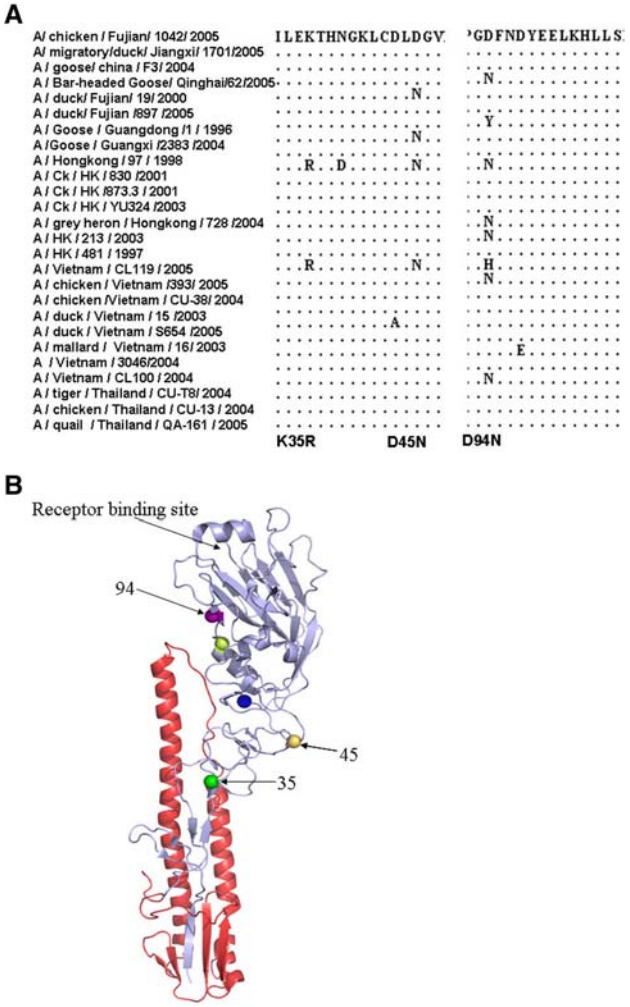

Fig. 1 **a** Amino acid sequence comparison of HA1 of AIV A/chicken/Fujian/1042/05 to the other HAs of H5N1 avian influenza viruses from mainland China, Hong Kong, Vietnam, Thailand human and avian isolates. The sequence data were obtained from the GenBank database. Differences with respect to the top sequence are shown. The H3 numbering system [9] is used, and mutated residues in HA are highlighted. The figure was generated with BioEdit 7.0 software. **b** Location of amino acid mutations in the three-dimensional structure of H5 HA. The structure of H5 HA was produced with reference to the three-dimensional structure of A/Vietnam/1203/04 H5 HA[28]. The positions of amino acid substitutions are indicated in *color*

Table 1 Sequence changes of mutants and cell-surface expression of Wt and mutant HAs analyzed by FACS

HA	HA1 amino acid residue at position			HA expressing	
	35	45	94	(Percentage of total cells)	MFI
Mock				0.1	661
Wt	**K**	**D**	**D**	62.8	1417
D94 N	–	–	**N**	55.8	1469
dMT(K35RD45 N)	**R**	**N**	–	63.7	1406
tMT(K35RD45ND94 N)	**R**	**N**	**N**	65.1	1380

HeLa cells were transfected with pCI-HA plasmids containing the wild-type or mutant HA genes. The surface expression levels (expressed as mean fluorescence intensity, MFI) and percentage of HA-expressing cells were determined by FACS analysis. Mock represents pCI-neo empty vector. The results presented here are the average of three experiments, *Wt* represents wild-type HA gene

and K35R/D45 N/D94 N triple (tMT) mutants, which occurred naturally in human and avian isolates (Fig. 1b). These HAs were expressed in HeLa cells, and FACS analysis revealed that there was no significant difference in either the percentage of cells expressing these HAs or their mean fluorescence intensities (Table 1), suggesting that these mutations did not significantly alter expression of the HA protein. The similar cell-surface expression levels of different HAs allowed us to compare their receptor-binding abilities.

The binding preferences of HA to different linkages of sialic acid were detected according to a previously reported method, the binding of cell surface HA to erythrocytes of different animal species. Horse erythrocytes display mainly SAα2,3Gal, while guinea pig erythrocytes predominantly display SAα2,6Gal [10, 22]. A hemadsorption assay was used to measure red blood cell binding to exogenously expressed H5 HA [10]. We compared the receptor-binding preferences of expressed human H1 subtype HA (Hu), H5 HA (Wt), and its mutants by their abilities to adsorb horse or guinea pig erythrocytes. As expected, cells expressing H1 human virus HA (Hu) preferably bound guinea pig erythrocytes ($p < 0.01$, t test, CI 95%), whereas the AIV HA (Wt) adsorbed horse erythrocytes much more efficiently ($p < 0.01$, t test, CI 95%). Notably, introducing the D94 N mutation in Wt HA dramatically changed its receptor preference. The amount of guinea pig erythrocytes adsorbed was almost as high as that of H1 human virus HA (Hu), while the binding of D94 N HA to horse erythrocytes was reduced (Fig. 2). These results indicated that the substitution of aspartic acid 94 with asparagine enhanced the receptor preference of Wt HA to human type SAα2,6-Gal receptor and decreased its receptor binding to avian type SAα2,3Gal receptor. This conclusion was further supported by analyzing the double and triple mutants. The double mutant K35R/D45 N (dMT) had a binding preference similar to that of wild-type AIV HA (Wt), adsorbing more horse erythrocytes (Fig. 2). Yet, the triple mutant, K35R/D45 N/D94 N (tMT), bound a significantly higher

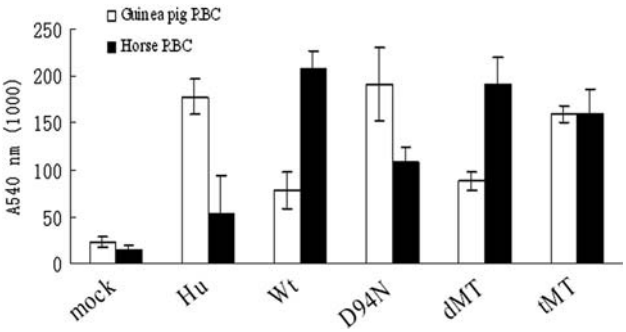

Fig. 2 Hemadsorption activity of HA. HeLa cells were transfected with 2 μg each of plasmids expressing H1 (A/WSN/33/H1N1, Hu) HA, H5 (A/chicken/Fujian/1042/05, Wt) HA, mutants H5 HA proteins plus plasmid pCI-M2 (0.25 μg). Hemadsorption activity was measured with horse (*black bars*) or guinea pig erythrocytes (*open bars*). All transfections were performed in triplicate, and the results presented here represent the average of three independent experiments. *Mock* represents pCI-neo empty vector, *Hu*: pCI-HA (A/WSN/H1N1), *Wt*: pCI-Wt:HA(A/chicken/Fujian/1042/05), *tMT*: K35R/D45 N/D94 N, *dMT*: K35R/D45 N

amount of guinea pig erythrocytes ($p < 0.02$, t test, CI 95%). The different phenotypes of the dMT and D94 N mutants may due to interaction between the double mutation K35R/D45 N and the single mutation D94 N. Therefore, the double mutation has some influence on the function of the single D94 N mutant. These results revealed that the D94 N mutation could enhance binding of HA to human-type SAα2,6Gal receptor.

Polykaryon formation of HeLa cells

In addition to receptor preference, we also determined the effects of these mutations on HA-mediated membrane fusion activity. Using the polykaryon formation assay, we found that the number of polykaryons formed by D94 N mutant in HeLa cells was clearly higher than that of Wt (Fig. 3) ($p < 0.05$, t test, CI 95%). Furthermore, the amount of polykaryon formed by the triple mutant K35R/D45 N/D94 N (tMT) was also higher than that of double

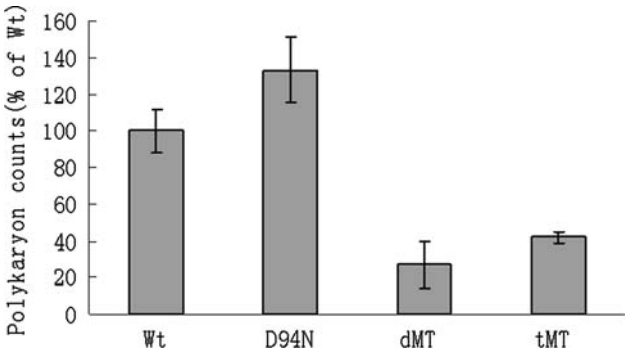

Fig. 3 Polykaryon formation of Wt and mutant HAs in HeLa cells at pH 5.0. HeLa cells were transfected with pCI-HA plasmids containing wild-type or mutant HA genes. Forty-eight hours post-transfection, the cells were treated with low-pH buffer, then fixed and stained

mutant K35R/D45 N (dMT) ($p < 0.05$, t test, CI 95%). These observations suggest that the D94 N mutation was able to enhance HA-mediated membrane fusion in mammalian cells.

Incorpration of mutant HA protein in pseudotyped virus

Next, we determined the relative ability of the HA protein to be incorporated into the pseudotyped virus. Analysis of the vector particles generated by western blotting revealed that Wt and HA mutants were incorporated into HIV-like particles (Fig. 4a). In addition, the amount of HA on HIV-like particles released into the culture supernatants was quantified. The luciferase activity (indication of pseudotyped virus titers) depends on the amount of virus that was allowed to transduce the cells. Thus, 0.5 HA units of pseudotyped virus was used in subsequent experiments.

Differential effects of HA mutation on virus post-attachment events correlate with receptor expression

The post-attachment events of these HAs mutants were assessed by titering these HA pseudotyped viruses on 293T and COS cells, which are mammalian cell lines derived from human embryo kidney and monkey kidney, respectively. On 293T cells (Fig. 4b), the D94 N mutant displayed higher viral titers than wild-type HA ($p < 0.01$, t test, CI 95%). Similarly, the titers of the triple mutant K35R/D45 N/D94 N (tMT) were also higher than that of the double mutant K35R/D45 N (dMT) ($p < 0.01$, t test, CI 95%) (Fig. 4b). These results indicated that the D94 N mutation could increase the binding capacity of HA. However, in COS cells, the D94 N mutant produced much lower titers than wild-type HA ($p < 0.05$, t test, CI 95%) (Fig. 4b). We infer that these two cell types express different levels of 2,6- and 2,3-linked sialic acid receptors.

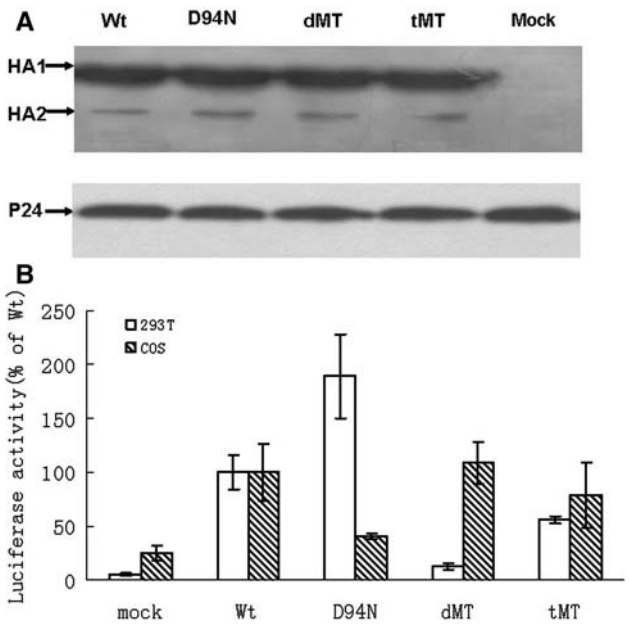

Fig. 4 **a** HA incorporation into HIV-luc pseudovirus. HIV-like particles pseudotyped with mutant HAs were generated and analyzed by Western blotting. The HA was identified by chicken anti-HA antibody; the p24 protein of HIV was detected by a mouse anti-p24 monlclonal antibody. *Mock* represents HIV-like pseudotyped particles with pCI-neo empty vector. *Wt* represents HIV-like pseudotyped particles with HA of A/chicken/Fujian/H5N1. **b** Transduction of HIV-HA-luc particles pseudotyped with HA or its mutants in 293T and COS cells. Pseudoviruses were generated by transfecting 293T cells with envelope expression plasmid pCI-HA, pCI-NA plus HIV reporter plasmid pNL-luc-$E^{-}R^{-}$. 293T and COS cells were transduced with an equal amount (0.5 HA units) of HA-HIV-luc pseudovirus. After 2 days, the cells were lysed, and luciferase activity was assayed in a luminometer (Promega). The results presented here were the average of three independent experiments ± SD. *Mock* represents HIV-like pseudotyped particles with pCI-neo empty vector. *Wt* represents HIV-like pseudotyped particles with HA of A/chicken/Fujian/H5N1

We then characterize the nature and relative abundance of influenza-virus-binding receptor (Sia2-3Gal and Sia2-6Gal linkages) on the surface of 293T and COS cells by fluorescence-activated cell sorter (FACS) analysis. Using linkage-specific SNA and MAA lectins, which bind specifically to 2,6- and 2,3-linked sialic acids, respectively, the presence of sialic-acid-containing receptors on the surface of COS and 293T cells was investigated. COS cells contained mainly the SAα2,3Gal linkage, and only 6.8% cells displayed SAα2,6Gal (Table 2). Therefore, COS cells probably comprise two populations of receptor-bearing cells, one (6.8% of cells) containing both types of Sia-Gal determinants and the other containing primarily SAα2,3-Gal. In the same experiment, 293T cells expressed both types of Sia-Gal determinants, and 82.6% of 293T cells expressed sialic acids in the α2-6 linkage. Moreover, the mean fluorescence intensity of FACS analysis verified a similar difference in the absolute amount of sialic acid

Table 2 The distribution of SAα2,3Gal and SAα2,6Gal on the surface of COS and 293T cells

Cell	Mock		SAα 2,6 Gal		SAα 2,3 Gal	
	Percentage of total cells	MFI	Percentage of total cells	MFI	Percentage of total cells	MFI
COS	0.2 ± 0.02	559 ± 34	6.8 ± 0.8	897 ± 68	85.2 ± 66	2017 ± 159
293T	0.7 ± 0.04	817 ± 70	82.6 ± 5.5	1581 ± 165	94.7 ± 56	1989 ± 201

The percentage of lectin-stained cells was calculated according to the relative fluorescence intensity in the FACScanfluoro spectrometer. The results presented here represent the average of three experiments. *MFI* represent mean fluorescence intensity. Mock represent unstained control cells

receptors on COS and 293T cells (Table 2). These findings could well explain why 293T cells displayed higher transduction efficiency than COS cells. Moreover, these findings indicate that the different distribution of viral receptor on the cell surface may be one of important factors affecting virus transduction, and COS cells could be used to evaluate the ability of HA to recognize the SAα2,3Gal receptor.

On the other hand, we infer that HA mutation may be another important factor affecting virus transduction. The higher transduction efficiency of the D94 N mutant in 293T cells is in accordance with its higher affinity with receptor and higher membrane fusion activity, whereas the lower transduction efficiency of the D94 N mutant in COS cells is likely due to their preferential expression of the avian type SA alpha2,3Gal receptor. We could therefore deduce that the D94 N mutation decreases the ability of HA to recognize the avian-type SAα2,3Gal receptor.

Discussion

Monitoring HA mutations among avian and humans isolates is essential for evaluating possible adaptive changes that could favor interspecies transmission of avian influenza virus. Most of the structural and functional information concerns HA-receptor interactions and binding properties. Moreover, it has been suggested that strong receptor binding does not always lead to high viral infectivity [19] and is not always sufficient for virus entry into cells [1]. Thus, a greater understanding of the post-binding functions that occur with pathogenic avian strains of influenza virus is apparently needed.

An earlier study has described the effects of substitutions of residues in the receptor-binding pocket (Y98, S/T136, W153, H183, E/D190, L/I194, H3 numbering) and of a residue at position 228 in the HA protein from the H7-subtype avian influenza virus using pseudotyped virus. Most of the mutations were found to affect receptor binding activity to some extent [19]. In other studies, virus receptor binding preference of various HAs was investigated, and it has been demonstrated that the Q226L mutation increased the binding of H2 and H3 to the SAα2,6Gal receptor, while for H1 strains, the E190D and G225E mutations increased the affinity of the virus for the SAα2,6Gal receptor [21]. Using a glycan microassay, mutations at amino acids 182 and 192 have been found to independently convert the receptor preference of H5 HA from avian to human type [33]. Overall, these studies focused mainly on the receptor-binding ability and preference of HA, and there is a growing need to study the post-binding and subsequent infection of target cells.

Using human airway epithelial cells, amino acid 226 in the HA of H9N2 influenza viruses was demonstrated to determine cell tropism and replication [31]. More recently, recombinant viruses that carried GFP and various HAs (H1, H3 and H5) were constructed to study the binding and infection of influenza virus to a variety of target cells, which would help to elucidated the cell tropism and infection of H5N1 virus [24]. It is probably important to know the effects of H5 HA mutations on virus binding and infection of various cells.

In the current study, the D94 N mutation was observed in two Hong Kong avian cases (2003 and 2004), one Hong Kong human case (1998), two Vietnam human cases (2004 and 2005), and the recently reported virus isolated from wild waterfowl in mid-2005 from Qinghai Lake in China (Fig. 1a). In addition, this site is located near the Y98 residue, which has been suggested to impair the receptor-binding activity [20]. We therefore undertook an investigation of the influence of this mutation on receptor recognition, fusion and virus entry into cells. Our results suggest that the emergence of the D94 N mutation may represent one of the evolutionary features of influenza virus, which offers an advantage for avian virus to bind to the cells with a human-type receptor and decreased its entry into cells with an avian-type receptor.

Studies by other researchers have also indicated that certain binding-site mutations influence the post-binding function of the HA protein [19]. To interpret the attachment and post-attachment events, we also analyzed the fusion activity of these mutants. Our findings from polykaryon formation of HeLa cells indicate that the D94 N mutation could influence the fusion function.

According to previous structural studies, residue 94 is not directly involved in sialic acid binding [4]. Yet, it caused apparent changes in receptor binding and virus entry into cells in our study. From a structural perspective, residue 94 is located close to the 220 loop of the receptor-binding domain and is near amino acid 98, which has been implicated in binding the sialic acid receptor [20] (Fig. 1b). Thus, the D94 N mutation may indirectly influence the HA-receptor interaction by altering the protein conformation. Moreover, changing aspartic acid to asparagine at residue 94 would result in a decreased negative charge in the vicinity of the receptor-binding pocket, which has been reported to remarkably affect the receptor-binding affinity through electrostatic repulsion [16]. Both effects may contribute to the increased affinity of HA for the SA receptor observed in our study. Therefore, although the D94 N mutation is located outside of the receptor-binding site, it could contribute to structural changes that result in alteration in receptor recognition and cell tropism. Supporting this notion, other scientists have reported that mutations farther from HA receptor-binding site could change the receptor-binding specificity and fusion function of influenza viruses in subtle, but biologically important ways [5, 12].

It should be noted that the D94 N mutation increased the entry of pseudotyped virus into 293T cells and enhanced the fusion activity of HAs (Table 3), which is in agreement with the expression of both SAα2,6Gal and SAα2,3Gal receptors on 293T cells. However, the luciferase expression of these mutants in COS cells was not consistent with their fusion function. This suggests that, in the pseudotyped virus post-attachment step, these HA mutants are poorly compatible with COS cells. The COS cells expressed mainly the SAα2,3Gal receptors and the transduction efficiency with these cells was apparently lower than with 293T cells when luciferase activity was measured. Combined with its enhanced fusion activity, a possible reason for the difference in transduction efficiency between 293T and COS cells could be the different distribution of sialic-acid-containing receptors on these two cell types.

Table 3 Comparison of results obtained from different tests

	Hu	Av Wt	Av D94 N	Av dMT	Av tMT
Ads[a] horse rbc[b]	+	+++	++	+++	+++
g.p.[c] rbc	+++	+	+++	+	++
Fusion	ND	+++	++++	+	++
Luc act[d] 293T 2,3++ 2,6++	ND	+++	++++	+	++
COS2,3++ 2,6+	ND	++	+	++	+

++++, 100–150%; +++, 75–100%; ++, 25–75%; +, 0–25%; *ND* not done

[a] Hemadsorption assay was performed with horse or guinea pig erythrocytes

[b] RBC represents red blood cells

[c] Represents guinea pig erythrocytes

[d] Luciferase activity was measured on 293T and COS cells

In other studies, QT-6 cells were found to have a receptor distribution similar to that of COS cells. In addition, infection rates of the QT-6 cells by a recombinant virus carrying H3 molecules of a human influenza virus were much lower than those with H1 and H5 viruses [24]. These results suggested that cell lines that express the SA in 2-3 or 2-6 linkage exclusively may better allow binding and infection properties of viral HA mutants to be studied. However, their infection was not a strict single cycle of replication and could not exclude the influence of other genes. In the present study, the combination of pseudotyped virus that carry HA and NA molecules and that express luciferase reporter upon transduction of COS cells that exclusively express the SAα2,3Gal receptor allows us to analyze virus entry into cells in a single cycle of replication. Furthermore, the availability of cell lines that express the SAα2,6Gal receptor only would be helpful for investigating HA mutations that would enable virus entry into cells with a human-type receptor, which in turn may help to understand the possible contribution of the HA mutation to the adaptation of the H5 avian strain to a human host.

In summary, using hemadsorption, polykaryon formation and pseudovirus transduction, we demonstrated that the D94 N mutation in HA enhanced HA binding to a human-type receptor but decreased pseudotyped virus entry into cells with an avian-type receptor. Moreover, our results demonstrate that the method we used in this study, using pseudotyped virus to analyze the entry of HA mutants of the highly pathogenic (HP) avian influenza virus H5N1 into live cells, is more convenient, safer and more sensitive than those of other assays with live virus. All together, our findings might provide a basis for further comprehensive studies of HA mutations on pandemic potential and interspecies transmission.

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