Molecular Biology, Vol. 35, No. 3, 2001, pp. 423–425. Translated from Molekulyarnaya Biologiya, Vol. 35, No. 3, 2001, pp. 500–503.

MISCELLANEOUS

UDC 578.2

Functional Match between Influenza Virus Hemagglutinin and Neuraminidase Is Restored after Gene Reassortment

I. A. Rudneva[1], A. S. Gambaryan[2], A. A. Shilov[1], B. V. Sinitsyn[1], E. A. Kropotkina[1], N. A. Il'yushina[1], and N. V. Kaverin[1]

[1] *Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow, 123098 Russia; E-mail: kaverin@online.ru*

[2] *Chumakov Institute of Poliomyelitis and Virus Encephalites, Russian Academy of Medical Sciences, Moscow Region, 142782 Russia*

Received November 2, 2000

Abstract—Influenza virus A (FluA) reassortants with low-functional neuraminidase (NA) of subtype N1 and hemagglutinin (HA) of subtypes H2, H3, H4, and H13 display virion aggregation and accumulate to a lower titer because sialyl residues are not completely removed from virion components. Nonaggregating variants of FluA (H13N1) were shown to result from a mutation that reduces the HA affinity for sialyl substrates. Amino acid substitution K156E, which increases a negative charge at the edge of the receptor-binding pocket of HA large subunit (HA1), was revealed in two independent variants. This substitution was the only difference between HA1 of the original reassortant and one of its variants and, therefore, accounted for restoration of the functional match between HA and NA.

Key words: influenza virus, gene, sequencing, mutation, hemagglutinin, neuraminidase

INTRODUCTION

Two coat glycoproteins, hemagglutinin (HA) and neuraminidase (NA), of the influenza virus A (FluA) are functionally related: HA interacts with terminal sialyl residues of cell oligosaccharides and thereby provides for virus binding to the cell surface, and NA removes sialyl residues from viral and cell components during virus reproduction. When NA is inactivated by a temperature-sensitive mutation [1], a deletion [2], or a chemical inhibitor [3, 4], HA interacts with nonremoved sialyl residues, and virus particles aggregate. Aggregation and sorption of virus particles on the surface of infected cells impair dissemination and reproduction of the virus. This has also been observed with reassortants combining low-functional human FluA NA (N1) and avian Flu HA (H2, H3, H4, and H13) [5, 6]. The reassortants tend to aggregate and accumulate to a low titer in embryonated chicken eggs and in cell cultures because of aggregate adhesion to the cell surface [7]. Serial passaging results in selection of variants that do not aggregate and accumulate to a normal level. Like original reassortants, the nonaggregating variants carry nonremoved sialyl residues on the virion surface, and their HA has a markedly reduced affinity for high-molecular-weight sialyl substrates [8]. Sequencing of the gene region for HA large subunit (HA1), which contains a receptor-binding site, has revealed amino acid substitutions that increase a local negative charge at the edge of the receptor-binding pocket. Such substitutions are associated with a lower receptor-binding activity of HA and restore accumulation of avian Flu of subtypes H2, H3, and H4 [8, 9]. Here we analyze avian Flu of subtype H13, which is of special interest owing to its evolutionary and biological characteristics.

EXPERIMENTAL

Viruses. Influenza virus A/Pilot whale/Maine/328/84 (H13N2) was obtained from the virus collection of the Institute of Virology. Reassortant RWB1 was human influenza virus A/USSR/90/77 (H1N1) carrying the HA gene of influenza virus A/Pilot whale/Maine/328/84 (H13N2) [5]. Viruses were propagated in 10-day embryonated chicken eggs. Virus-containing allantoic fluid was stored at 4°C.

Virus purification. Allantoic fluid was clarified by low-speed centrifugation. Virus was purified by centrifugation through 20% sucrose (w/w) at 23,000 rpm for 90 min at 4°C in an SW27-1 rotor. The pellet was resuspended in 0.01 M Tris-HCl (pH 7.4) supplemented with 0.15 M NaCl (to assay the affinity for sialyl-containing substrates) or with 0.1 M NaCl, 1 mM EDTA (to extract RNA).

Affinity for sialyl substrates. Fetuin binding was estimated in the direct solid-phase assay with immobilized virus and fetuin conjugated to horseradish peroxidase [10]. Affinity for sialic acid, 3-sialyllactose,

0026-8933/01/3503-0423$25.00

Table 1. Affinity of RWB1 variants for sialyl substrates

Virus	K_a, μM sialic acid			
	fetuin	3'-sialyllactose attached to polyacrylic carrier	sialic acid	3'-sialyllactose
RWB1	0.492 ± 0.116	0.183 ± 0.022	2229.3 ± 133.7	308.3 ± 16.3
RWB1-XII	1.174 ± 0.166	0.961 ± 0.051	4766.7 ± 185.8	151.0 ± 17.9
RWB1-Xa	1.254 ± 0.227	0.815 ± 0.051	4270.3 ± 175.8	169.8 ± 10.5

Note: In each case, K_a (mean ± SE) was obtained in four independent experiments [9].

and 3-sialylglycopolymer (a conjugate of 1-N-glycyl-sialyllactose with poly-4-nitrophenylacrylate) was estimated from competitive inhibition of fetuin conjugate binding [12].

Sequencing. The HA gene was amplified via reverse transcription–polymerase chain reaction. The products were cloned in pGEM-T as recommended by Promega. Recombinant plasmids were propagated in *Escherichia coli* JM109 and sequenced according to Sanger. Primers directed to the HA gene of Flu A/Pilot whale/328/84 (GenBank accession no. M26091) were obtained from Litekh.

RESULTS

Selection of reassortant RWB1 variants. Like other reassortants carrying the NA (N1) gene of Flu A/USSR/90/77, reassortant RWB1 showed virion aggregation and accumulated to a low titer in embryonated chicken eggs [5]. Serial passaging allowed selection of variants that lost the ability to aggregate and restored normal accumulation [6]. In two independent experiments, we passaged RWB1 10 and 12 times in embryonated eggs infected at 3000 50% embryo infective doses (EID_{50}). Being 16–32 hemagglutinating units (HU) with original RWB1, accumulation increased to 128–256 HU and became comparable with that of parental Flu A/Pilot whale/Maine/328/84 after three or four passages. The resulting two variants were designated RWB1-XII and RWB1-Xa.

Affinity for sialyl substrates. Original RWB1 and its two variants were tested for affinity to sialyl substrates (Table 1). Compared with RWB1, both RWB1-XII and RWB1-Xa had similarly low affinities for peroxidase-conjugated fetuin. The decrease in affinity was even greater with a high-molecular-weight synthetic substrate (sialyllactose attached to a carrier). With low-molecular-weight substrates, the affinity of the two variants was only slightly lower or even higher than in RWB1.

Table 2. Amino acid substitutions in HA1 of RWB1 variants

Virus	Amino acid substitution
RWB1-XII	K156E
RWB1-Xa	Y50C; E83G; K156E

Note: Amino acid positions are given according to the HA (H3) sequence.

Amino acid substitutions in HA1. We sequenced the RWB1, RWB1-XII, and RWB1-Xa gene regions that code for HA1 forming the receptor-binding pocket. A comparison with the corresponding sequence of Flu A/Pilot whale/Maine/328/84 (GenBank M26091) revealed amino acid substitutions L182I and A325V in HA1 of RWB1. The substitutions were also found in the original strain A/Pilot whale/Maine/328/84 (Institute of Virology) which was used to obtain RWB1. Hence the substitutions characterize our subline of this isolate, rather than being reassortment artifacts. Compared with RWB1, both variants contained substitution K156E, which probably increases the negative charge at the edge of the receptor-binding pocket [13]. In addition, RWB1-Xa had substitutions Y50C and E83G (Table 2).

DISCUSSION

Surface glycoproteins HA and NA greatly vary in antigenic properties among FluA isolates obtained from humans, mammals, and birds. In total, HA of 15 subtypes combine with NA of 9 subtypes [14]. The HA–NA combinations vary in frequency, some being never found. The selectivity of subtype combination is still poorly understood, while the problem is related to the origin of pandemic FluA variants.

We have previously shown that low-functional NA of strain A/USSR/90/77 (H1N1) fails to completely remove sialyl residues from virion components [5, 8]. Reassortants that combine this NA and avian Flu HA, which has a high affinity for sialyl residues, display virion aggregation [5] and accumulate to a lower titer in embryonated chicken eggs [5] and in cell cultures [7]. In their nonaggregating variants obtained via serial passaging, mutations in the HA gene compensate for a low NA function [6, 8, 9]. With subtypes H2, H3, and H4, the compensation is a decrease in affinity for high-molecular-weight sialyl substrates as a result

of amino acid substitutions that increase a negative charge at the edge of the receptor-binding pocket of HA. Thus we have found substitutions K156E and K165Q in two independent H2 variants, N145D and N248D in two independent H3 variants, and N160D in an H4 variant [8, 9]. Here we for the first time obtained such data for subtype H13.

Interestingly, FluA (H13) occupies a specific ecological niche. Strains with other HA subtypes have all been isolated from waterfowl (ducks, geese), a major natural FluA reservoir [14]. In contrast, FluA (H13) isolates originate from gulls, shorebirds, and, in some cases (including our strain A/Pilot whale/Maine/328/84), from dolphins. We previously studied only duck isolates of FluA (H2, H3, and H4), and it was unclear whether the molecular mechanism of HA adaptation to low-functional NA is shared by viruses differing in the host range. Here this mechanism was observed for H13, as the character and position of amino acid substitution in HA (H13) proved the same as in one of the H2 variants. Thus, the functional match between HA and NA is restored after reassortment via a common mechanism, which is neither restricted to any evolutionary branch of HA (as our previous data demonstrate) nor specific for FluA with a certain narrow host range.

ACKNOWLEDGMENT

This work was supported by the Russian Foundation for Basic Research (project no. 98-04-48013).

REFERENCES

1. Palese, P., Tobita, K., Ueda, M., and Compans, R.W., *Virology*, 1974, vol. 61, pp. 397–410.
2. Liu, C. and Air, G.M., *Virology*, 1993, vol. 194, pp. 403–407.
3. Palese, P. and Compans, R.W., *J. Gen. Virol.*, 1976, vol. 33, pp. 159–163.
4. Gubareva, L.V., Bethell, R., Hart, G.I., *et al.*, *J. Virol.*, 1996, vol. 70, pp.1818–1827.
5. Rudneva, I.A., Kovaleva, V.P., Varich, N.L., *et al.*, *Arch. Virol.*, 1993, vol. 133, pp. 437–450.
6. Rudneva, I.A., Sklyanskaya, E.I., Barulina, O.S., *et al.*, *Arch. Virol.*, 1996, vol. 141, pp. 1091–1099.
7. Kropotkina, E.A., Rudneva, I.A., Rusanova, O.S., *et al.*, *Vopr. Virusol.*, 1998, vol. 43, pp. 17–20.
8. Kaverin, N.V., Gambaryan, A.S., Bovin, N.V., *et al.*, *Virology*, 1998, vol. 244, pp. 315–321.
9. Kaverin, N.V., Matrosovich, M.M., Gambaryan, A.S., *et al.*, *Virus Res.*, 2000, vol. 66, pp. 123–129.
10. Gambaryan, A.S. and Matrosovich, M.M., *J. Virol. Methods*, 1992, vol. 39, pp. 111–123.
11. Gambaryan, A.S., Tuzikov, A.B., Piskarev, V.E., *et al.*, *Virology*, 1997, vol. 232, pp. 345–350.
12. Matrosovich, M.M., Gambaryan, A.S., Tuzikov, A.B., *et al.*, *Virology*, 1993, vol. 196, pp. 111–121.
13. Wilson, I.A., Skehel, J.J., and Wiley, D.C., *Nature*, 1981, vol. 289, pp. 366–373.
14. Webster, R.G., Bean, W.J., Gorman, O.T., *et al.*, *Microbiol. Rev.*, 1992, vol. 56, pp. 152–179.