J Mol Neurosci (2008) 35:339–343
DOI 10.1007/s12031-008-9052-z

The H5N1 Influenza Variant Fujian-Like Hemagglutinin Selected Following Vaccination Exhibits a Compromised Furin Cleavage

Neurological Consequences of Highly Pathogenic Fujian H5N1 Strains

Antonella Pasquato · Nabil G. Seidah

Received: 14 November 2007 / Accepted: 7 February 2008 / Published online: 28 February 2008

Abstract The outbreak of H5N1 avian influenza strains infectious to human has dire neurological and pathological consequences. This led to the massive vaccination of host poultry, resulting in a Fujian-like variant (vFJ) resistant to immunization with two mutations at the furin-processing site of hemagglutinin: loss of the P2 Lys and P9 substitution of Gln to Leu within the cleavage site. We synthesized 14mer peptides mimicking the processing site of Fujian-like strains. We found that the peptide with the vFJ sequence is less cleaved as compared to the parent FJ-derived peptide by furin at either neutral or acidic pH values. We hypothesize that the double hemagglutinin mutations in vFJ may result in viruses with less processed hemagglutinin, thereby providing a mechanism for evading immune neutralization.

Keywords Hemagglutinin HA · Furin · Heparin · Influenza virus · Fujian · Neurological diseases

Introduction

Viral diseases are a major threat to the social and economic fabric of society, with a quarter of all deaths in the world each year attributed to infectious diseases, including HIV, hepatitis C, and influenza virus, the latter disease affecting ~20% of children and ~5% of adults each year. In the USA, during an average epidemic season, influenza results in >300,000 deaths and >100,000 hospitalizations each year (Lewis 2006). A more serious outbreak of pandemic influenza could kill millions and grind international travel and trade to a halt, triggering a major global depression. Factors such as augmented mobility, climate change, and urbanization have increased the viral diseases pose. Influenza A is classified into serologically defined antigenic subtypes of the hemagglutinin (HA) and neuraminidase (NA) major surface glycoproteins. Sixteen HA and 9 NA influenza A subtypes were identified in birds, with all possible combinations. All influenza A subtypes circulate in wild birds providing a natural reservoir, but only the HA subtypes H1, H2, and H3 and the NA subtypes N1 and N2 have circulated widely in humans. Pandemic influenza results when an avian influenza strain infects humans. Three major influenza pandemics occurred during the last century causing widespread death: 1918 H1N1 (Spanish flu), 1957 H2N2 (Asian flu) and 1968 H3N2 (Hong Kong flu). Recently, an H5 virus caused illness in a limited number of people in Hong Kong (Claas et al. 1998). H5N1 is one of the most pathological strains found to infect humans and, since 2003, with an alarming 100-fold increase in the number of deaths attributed to this virus (http://www.who.int/whr/2007/en/index.html).

The main functional viral surface glycoprotein HA precursor (HA0) requires prior cleavage into its subunits HA1 and HA2 by host proteases for viral infectivity. While HA1 contains the sialic acid receptor-binding sequence, the hydrophobic N terminus of HA2 is the fusogenic domain that allows cellular entry of the virus. The widely expressed basic amino acid-specific furin-like proprotein convertases (Seidah and Chretien 1999) are the main secretory proteases implicated in the processing of HA in highly pathogenic avian influenza A virus at a multibasic processing site critical for efficient cleavage (Stieneke-Grober et al.

A. Pasquato · N. G. Seidah (✉)
Laboratory of Biochemical Neuroendocrinology,
Clinical Research Institute of Montreal,
Montreal, Quebec H2W 1R7, Canada
e-mail: seidahn@ircm.qc.ca

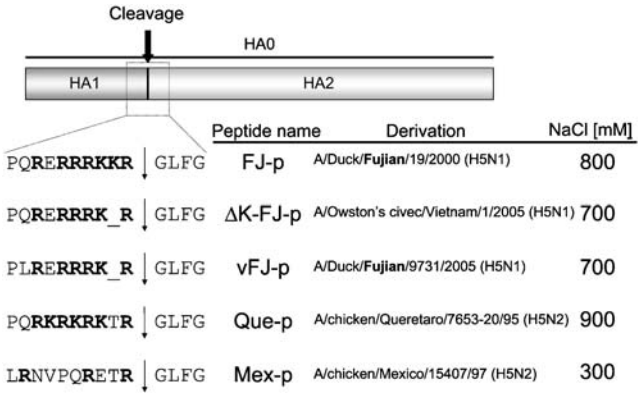

Figure 1 Amino acid sequences of the HA-derived peptides used in this study and the annotation of their corresponding strains, with emphasis on the predicted furin-cleavage site. The concentrations of NaCl needed to elute these peptides from a heparin column are shown

1992). Indeed, the emergence of the Hong Kong H5N1 strain in 1997 with an additional tetrapeptide RERR inserted before the cleavage site (Fig. 1) resulted in an approximately fivefold increase in the efficacy of furin cleavage (Basak et al. 2001). Viruses with a single basic residue separating the HA1 and HA2 domains are much less virulent, likely because the HA0 is cleaved only in certain tissues by trypsin-like enzymes (Garten and Klenk 1999). The primary sequence of the HA0 processing site of various H5N1 strains are shown in Fig. 1 together with some H5N2 strains. All sequences, including that of the most common Asiatic Fujian-like strain [A/duck/Fujian/19/2005(H5N1), herein called FJ], reveal the presence of the canonical furin-recognition motif RX(K/R)R↓. We note that since 2004, more virulent H5N1 strains appeared that lost the basic amino acid Lys at the P2 position [A/duck/Yunnan/5813/2005(H5N1), herein called ΔK-FJ]. Starting from September 2005, the Chinese government undertook a massive vaccination of poultry against the deadly influenza H5N1 strain. Amazingly, this resulted in a selection of a highly infectious and damaging new Fujian-like H5N1 strain [A/duck/Fujian/9731/2005(H5N1), herein called vFJ; Smith et al. 2006], which, in addition to losing the P2 Lys, also contains a substitution of Gln to Leu at position P9 of the HA0 furin-processing site (Fig. 1).

Neurological manifestations associated with influenza have been reported more than 100 years ago. Neurologic complications due to an epidemic influenza were reported in Japan (Togashi et al. 2004), and more recently, numerous cases of severe central nervous system symptoms that are influenza-related were diagnosed (Maricich et al. 2004). In particular, patients showed encephalopathy and seizures that were associated with the influenza virus, mainly the A/Fujian/411/2002 (H3N2)-like strain, excluding the possibility of "postinfluenza symptoms", as this virus was isolated from human cerebrospinal fluid (Maricich et al. 2004). In addition, it has been shown that avian influenza viruses belonging to H5N1 strains can infect and replicate both in mice and ferret brains, inducing severe neurologic sign and death upon intranasal infection (Rowe et al. 2003). In these experiments, various strains of the virus were isolated in multiple systemic organs, including the brain. The mechanism through which the virus is able to infect the nervous system is presently unknown, even though olfactory nerves and ethimoid plate were suggested to be possible routes of

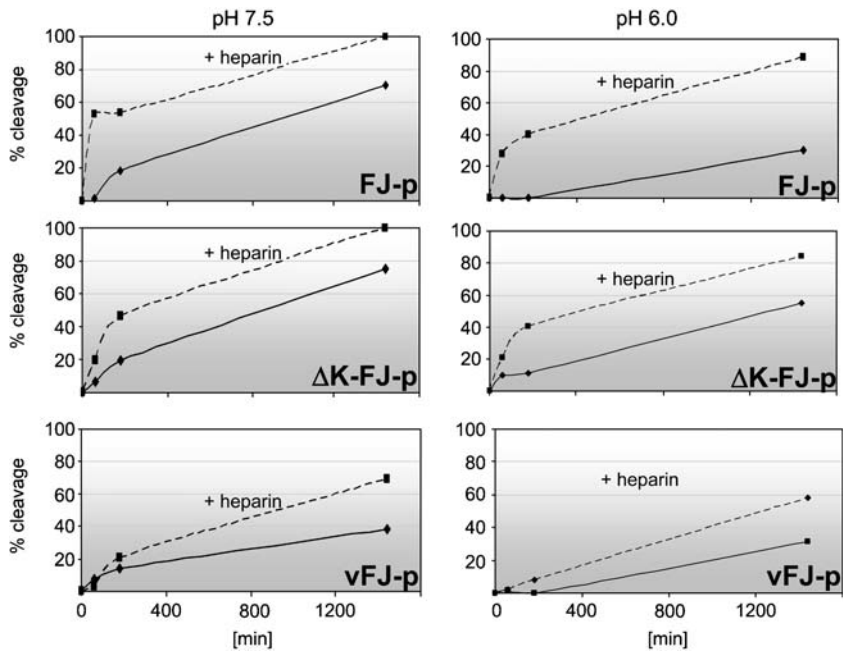

Figure 2 Time course of the percent digestion by furin of the FJ-, ΔK-FJ-, and vFJ-derived 14mer peptides in the absence and presence (+) of 20 μM heparin (*dashed line*)

entry into the central nervous system since the viruses were herein detected (Govorkova et al. 2005; Park et al. 2002).

Since the HA of Fujian H5N1 strains is processed by furin-like convertases and the latter enzymes are well expressed in the brain (Seidah et al. 1994), in this study we investigated the processing of peptides mimicking the HA0 cleavage site found in different H5N1 strains, including the vFJ-like one. The data show that, while the ΔK-FJ sequence is best cleaved at all pHs, the cleavage of the vFJ-like peptide at neutral, but not acidic, pH is approximately twofold less efficient than that of the parent FJ sequence. This suggests that vaccination may have resulted in a vFJ-strain selected for poor processing, likely providing an enhanced escape route from immune response.

Table 1 Percent cleavage of the various peptides after overnight incubation with furin at pH 6 and 7.5, in the presence (+) or absence (-) of heparin

Heparin	–		+	
pH	6	7.5	6	7.5
Peptides	% Cleavage			
FJ-p	30	70	90	100
ΔK-FJ-p	55	76	86	100
vFJ-p	30	38	58	69
Mex-p	0	5	10	100
Que-p	0	0	4	10

Methods

Recombinant hFurin The medium of cells (BSC40) infected with a vaccinia virus recombinant of a soluble form of hfurin (VV:hFurin-BTMD; Decroly et al. 1996) was collected 18 h post-infection and concentrated (Centriprep YM-30). Activity was measured with the fluorogenic substrate Pyr-RTKR-MCA with a Spectra MAX GEMINI EM microplate spectrofluorometer, Molecular Devices (360 nm excitation, 460 nm emission).

Heparin-binding assays Peptide (200 μM) in 500 μl buffer 10 mM phosphate were loaded on a HiTrap Heparin HP column (GE Healthcare) and eluted with a 0.1–2 M NaCl gradient. Each fraction (500 μl) was analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) as above.

Enzymatic assays Assays were performed on 200 μM of each custom-synthesized peptide (GenScript, NJ, USA) in 2 mM $CaCl_2$, 25 mM Tris–HCl buffer pH 7.0, 1 mM β-mercaptoethanol, 1 μl furin at 37°C. When specified, 20 μM heparin (Sigma, low molecular weight) was added. Heparin alone shows no enzymatic activity (*not shown*). Samples (20 μl) taken at time zero (immediately after substrate addition), 2 h, and overnight, were analyzed by RP-HPLC on a Varian C_{18} column (5 μm, 100 Å, 4.5× 250 mm). The enzymatic reaction was interrupted treating with 5 μl aqueous 0.1% trifluoroacetic acid. The digestion products were identified by matrix-assisted laser desorption/ionization mass spectrometry. Cleavage percent was calculated from RP-HPLC peak areas.

Results and Discussion

It has become apparent that many infectious viruses do enter the brain and cause neurological dysfuntions. The molecular basis of the neurotropism of certain influenza virus strains can be influenced by the host and the virus strain (Starick and Werner 2003). After penetrating the blood–brain barrier, influenza infection is propagated to glial cells and neurons (Silvano et al. 1997). Since influenza H5N1 strains do enter the mammalian brain resulting in

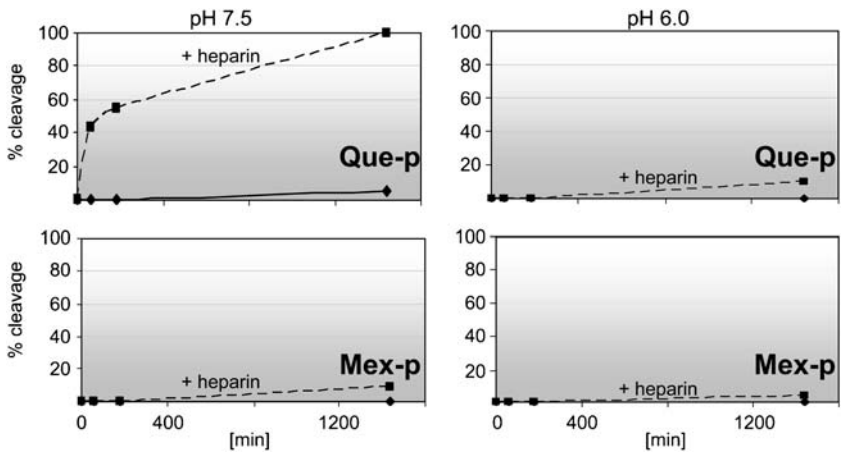

Figure 3 Time course of the percent digestion by furin of the Que-p and Mex-p 14mer peptides in the absence and presence (+) of 20 μM heparin (*dashed line*)

severe neurological disorders (Rowe et al. 2003), we decided to study in more detail the furin-like processing of HA0 of various strains, especially the Fujian one that has appeared following vaccination.

In order to compare the processing efficacy of HA0 of the various H5N1 strains, we opted to synthesize three 14mer peptides mimicking the corresponding furin-like cleavage site of FJ, ΔK-FJ and vFJ strains (Fig. 1). For comparison, we also synthesized two 14mer peptides containing the processing site of the highly pathogenic Queretaro and low pathogenic Mexico H5N2 strains (Garcia et al. 1996). As all these peptides contain multibasic residues, and since our published data showed that heparin can enhance the efficacy of furin-cleavage of peptides mimicking the HIV-1 gp160 processing site (Pasquato et al. 2007), we tested the ability of these basic peptides to bind immobilized heparin on a column. Indeed, the data show that the high pathogenic strains are eluted from the heparin column at 700–900 mM NaCl concentrations, as compared to 300 mM for the low pathogenic strain peptide Mex-p (Fig. 1, right). A negative control 16mer acidic peptide (QEDEDGDYEELVLALR) did not bind heparin (*not shown*).

We next investigated the ability of furin to cleave these peptides in the presence or absence of 20 μM heparin at pH 7.5 and 6 (Figs. 2 and 3 and Table 1). Cleavage of the FJ-like peptides was monitored by RP-HPLC as a function of time (Fig. 2) and overnight (Table 1). Mass spectrometric analyses of the products (*not shown*) confirmed their identity and demonstrated that cleavage occurred exclusively at the expected physiological sites (Fig. 1). The data showed that at pH 7.5 both ΔK-FJ-p (76%) and FJ-p (70%) were approximately twofold better processed than vFJ-p (38%; Table 1). At pH 6, all peptides are cleaved with lower kinetics as compared to pH 7.5 (Fig. 2 and Table 1). In addition, at pH 6 both FJ-p and vFJ-p are cleaved with similar kinetics, whereas the ΔK-FJ-p is still the best furin substrate.

Since most cells expose negatively charged heparin-sulfate proteoglycans, we tested the effect of heparin on the furin processing of the selected peptides (Fig. 1). Interestingly, while both Que-p and Mex-p peptides are not processed by furin at either pH 7.5 or 6, addition of 20 μM heparin dramatically enhanced the processing of the basic Que-p, albeit at neutral pH, while Mex-p was still poorly cleaved under the conditions tested (Fig. 3, Table 1). This emphasizes the effect of heparin in optimizing the furin processing of substrates containing multibasic residues at strategic P-positions within the cleavage site. Different from Que-p and Mex-p, addition of heparin to the incubation medium of the FJ-like peptides resulted in an approximately two- to threefold enhancement of processing at both pH values (Fig. 2, Table 1). Nevertheless, even under optimized processing conditions by heparin, the vFJ sequence is still the least cleaved one (~60% versus ~100%; Table 1).

As processing of HA0 by furin can occur both at the cell-surface (neutral pH) and in intracellular acidic endosomes (Thomas 2002), our data suggest that vaccination against the parent FJ-strain resulted in a vFJ strain with a double mutation (loss of Lys at P2 and replacement of Gln at P9 by Leu), exhibiting a less efficiently processed HA0 at neutral but not acidic pH values. The selection for the vFJ strain may confer an advantage to the virus by possibly exposing more unprocessed HA0 at its envelope. The latter would adopt a different conformation of HA than its processed form and hence may be the major antigen that is immuno-neutralized by vaccination, sparing the highly infectious virus with processed HA. Finally, it is notable that the single mutation resulting in ΔK-FJ conferred a higher furin-cleavability to the HA0 at both pH values tested. This may rationalize the widespread multiple strains that carry this mutation, as evidenced by database BLAST analyses (not shown). In contrast, we could not identify any influenza strain with a single Gln-to-Leu mutation at the P9 position of the furin-cleavage site of HA0. In accordance, a peptide with this single Gln-to-Leu mutation results in at least a fivefold lower furin-processing (not shown). It is thus a matter of speculation that a single Gln-to-Leu mutation may be too deleterious to the infectivity of the virus, and the double mutation observed in the vFJ strain may alleviate this process, rendering the virus less infectious but with an enhanced ability to evade immuno-neutralization of its infectious particles. While all the data presented in this work were obtained in vitro, further investigations using live viruses would surely enhance our understanding of the selection pressures that led to the mutations resulting from the FJ-strain vaccination. Finally, the neurological consequences of the emergence of new strains will need careful analysis.

Acknowledgements We would like to thank Brigitte Mary for her secretarial help. This work was supported by a CIHR grant (# MGP-44363), a Canada Chair (# 201652) and by a generous gift from the Strauss foundation.

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