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Extraction of catalytically active neuraminidase of H5N1 influenza virus using thrombin proteolytic cleavage

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The stalk of influenza neuraminidase (NA) has been a target of cleavage by various proteases, resulting in the release of catalytically active globular heads from virus particles. However, despite successful cases in a number of influenza subtypes, this strategy could not be applied to all influenza viruses due to high variation of the NA stalk. In the present study, reverse genetics was employed to construct non-pathogenic recombinant influenza A viruses, termed rgH1N1$_{LVPR}$ and rgH1N1$_{LVPR-GS}$, that harbor the NA of H5N1 virus engineered to contain a specific thrombin cleavage site at the stalk region. By using thrombin to cleave NA at its stalk, a productive extraction of NA globular heads could be obtained from purified rgH1N1$_{LVPR}$. Furthermore, it was found that the NA of rgH1N1$_{LVPR-GS}$ could be cleaved by endogenous thrombin present in embryonated chicken eggs, resulting in the release of NA globular heads into allantoic fluids. These data highlight the use of thrombin cleavage as an effective strategy for extraction of active NA heads directly from live viral particles not only of H5N1 but, theoretically, of any subtype of influenza A viruses.

1. Introduction

Known for its sialidase activity, the influenza virus neuraminidase (NA), a tetrameric type II transmembrane glycoprotein, facilitates release of newly formed virions and prevents them from self-aggregating after budding from infected cells (Air and Laver, 1989; Colman, 1994). All 9 subtypes of NA share a common structure comprising a globular head domain, a stalk region, and a transmembrane domain. The globular head contains the active site consisting of a pocket of 15 charged amino acids that are conserved in all type A and B influenza viruses (Colman et al., 1983). NA has been an attractive target for anti-influenza drug screening as its inhibitors, e.g. oseltamivir and zanamivir, have been proven effective for treatment of influenza (Crusat and de Jong, 2007). However, with recurrent outbreaks of highly pathogenic avian influenza H5N1 virus and the emergence of oseltamivir-resistant strains (Lackenby et al., 2008), new therapeutic agents that inhibit NA activity are needed.

One of the strategies currently being employed to obtain catalytically active NA for subsequent uses in the screening of anti-NA active compounds involves extraction of native NA globular heads directly from live virus particles through cleavage at the NA stalk by proteases such as pronase (Franca de Barros et al., 2003; McKimm-Breschkin et al., 1991; Wu et al., 1995) or bromelain (Aitken and Hannoun, 1980; Cabezas et al., 1982). Since both proteases cleave their substrates non-specifically (Rowan et al., 1988; Trop and Birk, 1970) and the stalk region constantly changes as the virus evolves, the use of proteases to release active NA globular heads is very likely limited to those of a few subtypes. In particular, the stalk region of the NA of H5N1 virus has a unique deletion of 20-amino acids (Keawcharoen et al., 2005; Shinya et al., 2005) that might disrupt cleavage by currently used proteases, rendering the release of NA from virus particles not adequately effective. Even though the stalk of the NA of H5N1 has been reported to be cleaved by bromelian (Collins et al., 2008; Russell et al., 2006), it is not clear how much NA globular head was obtained from the treatment or whether the method was applicable with all subtypes of H5N1. Moreover, due to its non-specific cleavage nature, bromelain has also been used to extract hemagglutinin (HA), a more abundant surface glycoprotein, from influenza virus particles (Siniakov et al., 1979), which, as a result, often complicates the downstream purification of NA. Therefore, using proteases that cleave specifically at the NA stalk should serve as an ideal strategy for obtaining homogenous populations of released NA molecules. With its highly specific cleavage nature, thrombin was selected as the protease of choice.

In the present study, reverse genetics was utilized to construct non-pathogenic recombinant influenza A viruses, termed rgH1N1$_{LVPR}$ and rgH1N1$_{LVPR-GS}$, that harbor the NA of H5N1 virus

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engineered to contain a specific thrombin cleavage site at the stalk region. Through cleavage of purified rgH1N1$_{LVPR}$ by thrombin, a productive extraction of NA globular heads could be achieved. Furthermore, the NA of rgH1N1$_{LVPR-GS}$ was shown to be cleaved by endogenous thrombin present in embryonated chicken eggs, resulting in the release of NA globular heads into the allantoic fluid. These findings thus highlight the usefulness of thrombin cleavage site as an effective strategy for releasing active NA globular heads directly from influenza virus particles.

2. Materials and methods

2.1. Cell culture and virus propagation

Human embryonic kidney 293T and Madin-Darby canine kidney (MDCK) cells were maintained in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (PAA, Cölbe, Germany). Both cell lines were obtained from the American Type Culture Collection (ATCC). The propagation of viruses was carried out in MDCK cells in the presence of 2.0 μg/ml of TPCK-treated trypsin (Sigma–Aldrich, St Louis, MO, USA) or in 10-day-old embryonated chicken eggs.

2.2. Plasmid construction

The pHW2000 plasmid expressing full-length NA of A/Vietnam/DT-036/2005 (H5N1) (GenBank accession no. DQ094291, kindly provided by Dr. Erich Hoffmann, St. Jude Children's Research Hospital) was used as a template. The insertion of LVPR or LVPRGS into the stalk region of the NA was performed by PCR-mediated site-directed mutagenesis. For insertion of LVPR motif, the primer sequences used for PCR were as follows: LVPR-F (5′AGCAATGCTAATTTTCTTGTACCGCGTACTGAGAAAGCTGTG3′) and LVPR-R(5′CACAGCTTTCTCAGTACGCGGTACAAGAAAATTAGCATTGCT3′). The PCR reaction was carried out using Phusion high-fidelity DNA polymerase (Finnzyme, Finland) by 30 cycles with the following conditions: 98 °C for 7 s, 63 °C for 20 s, and 72 °C for 2 min 20 s. The PCR products were subsequently digested with *DpnI* (Fermentas, Vilnius, Lithuania) prior to transformation into XL1-Blue competent cells (Stratagene, La Jolla, CA, USA). For the insertion of LVPRGS, the pHW2000-NA containing LVPR insertion described above was used as a template with a large molar excess of complementary mutagenic primers as follows: LVPR-GS-F (5′CTTGTACCGCGTGGTTCGAAAGCTGTCGCTTCAG3′) and LVPR-GS-R (5′CTGAAGCCACAGCTTTCGAACCACGCGGTACAAG3′). The PCR reaction was performed using *pfu* DNA polymerase (Fermentas) with the following PCR cycling regimen: 95 °C for 30 s following by 16 cycles of 95 °C for 30 s; 56 °C for 30 s; 68 °C for 5 min. The obtained plasmids were subsequently sequenced to ensure that the LVPR or LVPRGS was properly inserted and that unwanted mutations were not introduced by PCR.

2.3. Reverse genetics for recombinant viruses

The 8-plasmid transfection system for generating recombinant influenza A viruses was performed following previously described protocols (Hoffmann et al., 2002, 2000). Briefly, for the generation of rgH1N1$_{LVPR}$, rgH1N1$_{LVPR-GS}$ and rgH1N1$_{wt}$, the co-culture of 293T-MDCK cells were transfected with 7 plasmids expressing all genes, except NA, derived from A/PR/8/34 (kindly provided by Dr. Erich Hoffmann), and the NA plasmids described in the previous section. The transfection was done with a DNA–lipid complex containing 1 μg of each plasmid, 18 μl of TransIT-LT1 (Mirus Corporation, Madison, WI, USA) in a final volume of 1 ml of Opti-MEM medium. Cells were incubated with the transfection mixture for 6–8 h before being washed and replaced with fresh medium. The co-cultured cells were incubated for an additional 24 h, and 2.0 μg/ml of TPCK-treated trypsin was added. After 72 h, the supernatant was harvested from the cell culture, and inoculated into 10-day-old embryonated chicken eggs, and the eggs were incubated at 37 °C for 48 h. Allantoic fluids were harvested and subjected to hemagglutination assay to determine the presence of viruses.

2.4. Plaque assay

The plaque assay was performed as described previously (Yen et al., 2006). Briefly, six-well tissue culture plates were seeded with MDCK cell monalayers. Once cells reached 100% confluence, they were washed twice with PBS and incubated with various dilutions of virus stocks for 1 h at 37 °C. After adsorption, each well was overlaid with 2× MEM medium containing 0.3% BSA, 0.9% Bacto Agar, and 1 μg/ml of TPCK-treated trypsin and incubated at 37 °C in a CO_2 incubator. After 48 h, the overlay was removed and the monolayer was stained with 0.1% crystal violet in 10% formaldehyde.

2.5. Thrombin cleavage and Western blot analysis

Viruses were grown in 10-day-old embryonated chicken eggs at 37 °C for 2 days. Allantoic fluids were harvested, and the viruses were purified through a sucrose-gradient ultracentrifugation at 150,000 × *g* for 2 h at 4 °C. Purified viruses were subsequently resuspended in 1× thrombin cleavage buffer (150 mM NaCl, 2.5 mM $CaCl_2$, 20 mM Tris–HCl, pH 8.4) and treated overnight at 25 °C with human thrombin (Sigma–Aldrich) at 0.04, and 0.2 units/μl, respectively. To access the released NA, treated virus samples were boiled after incubation and resuspended with 5× SDS loading buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Bioscience, Little Chalfont, Bucks, UK). After blocking in 5% skim milk (Difco Laboratories, Detroit, MI, USA), the presence of NA or HA was detected using rabbit anti-N1 (ProSci Incorporated, Poway, CA, USA) or rabbit anti-H1 (eEnzyme, Montgomery Village, MD, USA), followed by HRP-conjugated goat anti-rabbit IgG (Santacruz Biotechnology, Santacruz, CA, USA). The visualization of the band was performed by using Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. To detect the presence of virus particles, NP was detected using rabbit anti-NP (eEnzyme), followed by HRP-conjugated goat anti-rabbit IgG antibodies.

2.6. Virus elution assay

The elution of recombinant virus bound on human red blood cells was carried out to determine the receptor-destroying enzyme (RDE) activity as described previously (Gulati et al., 2005). Briefly, 50 μl of dilutions of allantoic fluids or supernatants of infected MDCK cells containing each recombinant virus, with the HA titers of 1:64, was incubated with 50 μl of 0.75% human red blood cells in U-shape microtiter plates at 4 °C for 1 h. Subsequently, the plates were incubated at 26 °C for 2 h or 12 h. Complete reduction of HA titers within 2 and 12 h were recorded as fast and slow elution, respectively. To test the ability of the virus to rebind to red blood cells, the wells were mixed, stored at 4 °C for 90 min, and agglutination was monitored.

2.7. Separation of intact virus particles from allantoic fluids

Allantoic fluids containing virus were subjected to ultracentrifugation at 200,000 × *g* for 90 min to sediment virus particles. To remove residual virus particles, supernatants were incubated with anionic polymer-coated magnetic beads as described by Sakudo