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Characterization of a trypsin-dependent avian influenza H5N1-pseudotyped HIV vector system for high throughput screening of inhibitory molecules

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ABSTRACT

In this study, we have generated and characterized an avian influenza H5N1 hemagglutinin (HA), neuraminidase (NA) and M2 ion channel pseudotyped HIV-based vector system (HaNaM-pseudotyped HIV vector). The cleavage site of the HA protein was modified to necessitate trypsin-dependent maturation of the glycoprotein. HA, NA and M2 were efficiently incorporated in HIV vector particles which could transduce different cell lines in a trypsin-dependent manner. Results also showed that the presence of avian influenza M2 and NA proteins maximized both vector production and transduction and that transduction was highly sensitive to the specific NA inhibitor oseltamivir (Tamiflu). H5N1 HaNaM-pseudotyped HIV vector system was also adapted for cell-based high throughput screening of drug candidates against influenza virus infection, and its high sensitivity to the specific oseltamivir validates its potential utility in the identification of new influenza inhibitors. Overall, the trypsin-dependent H5N1-pseudotyped HIV vector can mimic avian influenza virus infection processes with sufficient precision to allow for the identification of new antivirals and to study avian influenza virus biology in a lower biosafety level laboratory environment.

1. Introduction

The avian influenza H5N1virus is a subtype of the influenza A virus that can cause illness in humans and many other animal species. The earliest infections of humans by H5N1 coincided with an epizootic of H5N1 influenza in Hong Kong's poultry population, infecting 18 people with a fatality rate of 33%. Since then, the H5N1 virus has spread to over 60 countries or regions and infected several million wild and domestic birds. Between 2003 and June 2007, there were a total of 317 confirmed human cases of H5N1 viral infection, 191 of which were fatal, according to the World Health Organization (2007). At present, even though H5N1 viruses are not readily transmissible between humans, it is quite possible that they can acquire such transmissibility via mutations and/or gene reassortment from circulating human influenza A viruses. Due to the high virulence of this virus, its endemic presence and its high mutational rate, the H5N1 virus is the world's largest current pandemic threat and preparations are being made for a potential outbreak.

Although vaccination is the primary means for controlling influenza pandemics, antivirals provide an additional line of defense, particularly important for controlling a rapidly spreading pandemic (Ferguson et al., 2005; Longini et al., 2005). Currently, two classes of influenza antivirals are available; inhibitors for viral M2 ion channel proteins (amantadine and rimantadine) and neuraminidase (NA) inhibitors (zanamavir and oseltamivir). However, their extensive use coupled with the adaptability of the influenza genome has promoted the emergence of drug resistant strains (Kiso et al., 2004). Thus, there is a strong need for developing novel drugs against influenza viruses. Several *in vitro* screening assays have been developed to identify compounds with specific antiviral properties, such as a solid-phase binding assay with the sodium salts of sialylglycopolymers to analyze HA binding to human-receptor (Yamada et al., 2006), assays for M2 ion channel function or NA activity. However, these analyses are based on fastidious non-viral *in vitro* assays and require several additional manipulations of live H5N1 virus for validation.

Recently, the influenza HA-pseudotyped lentiviral/MLV vectors have been successfully used to study gene transfer into lung

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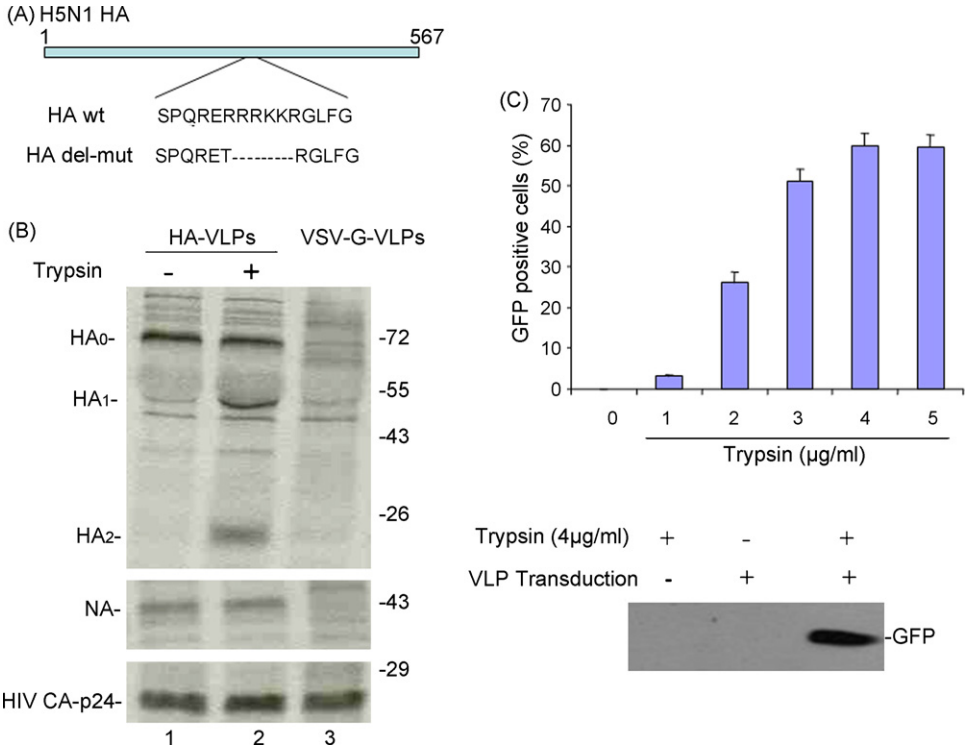

Fig. 1. Biochemical and functional analysis of H5N1-VLP harboring HA cleavage site modification. (A) Upper panel: modification of H5 HA cleavage. (B) 3 μg of H5N1 HA, NA and M2 protein expressors or VSV-G protein expressor were co-transfected with HIV vector containing GFP gene and HIV Gag-Pol expressing plasmid, as indicated in Section 2. At 48 h transfection, cells were labeled with [^{35}S]-methionine for 12 h. Purified H5N1-VLPs were treated (lane 2) or non-treated (lane1) with 4 μg of trypsin/ml for 1 h. Then, labeled VLPs were lysed with RIPA lysis buffer, immunoprecipitated by human anti-influenza serum (upper panel), rabbit anti-NA (middle panel) and mouse anti-P24 antibodies (lower panel) and analyzed by SDS-PAGE followed by autoradiography of gel. HA0: non-cleaved HA protein; HA1: HA surface subunit; HA2: HA transmembrane subunit; NA: neuraminidase; CA: HIV capsid p24 protein. (C) Dose-dependent trypsin activation of H5N1 HaNaM-pseudotyped VLP transduction. Equal amounts of purified HaNaM-pseudotyped VLPs were used to infect 293T cells in the absence or presence of various concentrations of trypsin, as indicated. The transduction efficiency (upper panel) was determined by measurement of GFP-positive cells with fluorescence-activated cell sorter (FACS) analysis at 48–72 h after infection. Intracellular GFP protein expression was detected by immunoprecipitation with anti-GFP antibody, followed by SDS-PAGE and anti-GFP western blot (lower panel). The results are representative for two independent experiments.

epithelial cells or for vaccination against the H5N1 virus (McKay et al., 2006; Szécsi et al., 2006). The present study describes a HIV-based vector pseudotyped with the HA, NA and M2 viral membrane proteins of a H5N1 avian influenza virus. Characterization of vector production and infectivity indicate that this vector can be used for different applications including high throughput drug screening.

2. Materials and methods

2.1. Plasmid constructs

The avian influenza H5N1 HA, NA and M2 cDNAs were generated by gene synthesis of codon optimized sequences for translation in mammalian cells. Sequences corresponded to HA (NCBI accession No. AB239125.1), NA (NCBI accession No. AB239126.1) and M2 of the Influenza A/Hanoi/30408/2005 virus obtained from the Influenza Sequence Database (Le et al., 2005). The protease cleavage site of H5N1 HA was modified to delete five basic amino acids (RRRKK) and add a threonine residue proximal to the cleavage site of the protein (Fig. 1A), as previously described (Li et al., 1999). Each synthesized HA, NA or M2 cDNA was cloned into a modified pCAGGS vector. The HIV-based vectors encoding for GFP gene (pHxEGFPWP) or encoding for lacZ gene (pHx*Lac*ZWP) and the helper packaging construct pCMVΔR8.2 encoding for the HIV helper function, were described previously (Kobinger et al., 2001). The vesicular stomatitis virus G (VSV-G) glycoprotein expressor was also previously described (Yao et al., 1998).

2.2. Cell culture and transfection

Human embryonic kidney 293T cells, the canine kidney MDCK cells and A549 lung carcinoma cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin. The CD4+ C8166 cells were maintained in RPMI-1640 medium containing 10% FCS and antibiotics. DNA transfection in 293T cells was performed with a calcium phosphate precipitation method.

2.3. Antibodies and chemicals

Several antibodies were used in immunoprecipitation procedures. The rabbit polyclonal antibodies against avian influenza NA protein were purchased from CedarLane Lab (Hornby, Ontario, Canada). Human anti-influenza serum was obtained from a confirmed influenza immunized individual in Winnipeg, Canada. The anti-HIVp24 monoclonal antibody used in this study was previously described (Ao et al., 2007). The rabbit anti-GFP antibody was obtained from Molecular Probes Inc. The NA inhibitor oseltamivir (Tamiflu) was obtained from Hoffmann-La Roche Limited. Trypsin (TPCK-treated) was purchased from Sigma Inc. The enhanced β-galactosidase assay kit (CPRG) was purchased from Genlantis Inc. (San Diego, CA). HIV-1 p24 ELISA Kit was obtained from the AIDS Vaccine Program of the Frederick Cancer Research and Development Center.

2.4. Generation of lentiviral vector and transduction

H5N1 HaNaM-pseudotyped HIV-based vector was produced by triple transfection of 293T cells using calcium phosphate precipitation. Briefly, 293T cells were transfected with the appropriate envelope expression plasmids, the HIV packaging plasmid pCMVΔR8.2 and the HIV-based vector pHxEGFPWP or pHxLacZWP (Kobinger et al., 2001). After 48 h of transfection, viral vectors were concentrated from the supernatant by ultracentrifugation with a Beckman Ti70 rotor at 32,000 rpm for 2 h, and virus titers were quantified by using a RT activity assay or HIV-1 p24 Antigen Capture Assay Kit. For the HA peptides screening experiments, the H5N1 HaNaM-pseudotyped vector was pre-activated by trypsin treatment (4 μg/ml) for 1 h at 37 °C before ultracentrifugation.

Transduction: 293T, MDCK or A549 cells were plated at 2×10^4 cells/well in 24-well plates or 4×10^3 cells/well in 96-well plate for 24 h prior to transduction. Equal amounts of H5N1 or VSV-G-pseudotyped virus-like particles (VLPs) (adjusted by p24 level) or serial dilutions (2-fold) of VLPs were incubated with target cells in DMEM medium containing 0.5% BSA and 4 μg/ml trypsin. After overnight incubation, DMEM (containing 10% FCS) was added to transduced cells, and between 48 and 72 h following transduction, the percentage of transduced (GFP-positive) cells was determined by using fluorescence-activated cell sorter (FACS; Becton Dickenson FACS Calibur) analysis. The relative transduction efficiency of the LacZ-containing vector was evaluated by measuring β-galactosidase (β-Gal) in cell lysates with the β-Gal assay kit (CPRG Spectra max plus; Molecular Devices Corporation) or by detecting β-Gal positive cells using the assay as described previously (Kimpton and Emerman, 1992) and counted with an Elispot Reader (AID Autoimmun Diagnostika GmbH).

2.5. Pharmacologic inhibition of H5N1-pseudotyped HIV-based vector production and infection

293T cells were plated and transfected with HIV vector encoding plasmids together with HA, NA and M2 expressors as described above. After 12 h, the medium was changed with fresh medium containing the NA inhibitor oseltamivir (100 μg/ml) and incubated for additional 36 h prior to harvesting VLPs. To evaluate the effect of oseltamivir on the early stage of VLP transduction, 293T cells were transduced with VLPs in the absence or presence of oseltamivir for 2 h at 37 °C. Cells were then washed with DMEM to remove the remaining oseltamivir and cultured in fresh DMEM for 48 h at 37 °C. Transduced EGFP-positive cells were analyzed by FACS.

2.6. Immunoprecipitation and fluorescent microscopy analysis

To analyze the incorporation of HA and NA in VLPs, HaNaM-VLP producing 293T cells were starved in methionine-free DMEM for 30 min at 48 h post-transfection and then metabolically labeled with 200 μCi of [^{35}S]-methionine for 16 h. Labeled VLPs were pelleted through a 20% sucrose cushion, lysed by RIPA lysis buffer (8 mM Na_2HPO_4, 2 mM NaH_2PO_4 pH 7.4, 140 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.05% SDS, 1× Roche complete protease inhibitor) and immunoprecipitated using human anti-influenza serum and the supernatant was sequentially immunoprecipitated with rabbit anti-NA protein followed by mouse anti-p24 antibodies. Immunoprecipitates were resolved on 10% SDS-PAGE and visualized by autoradiography. To visualize the GFP-expressing cells in the culture, 293T and A549 cells were grown on glass coverslips (12 mm^2) in 24-well plates. Transduced cells were fixed with PBS-4% paraformaldehyde for 5 min. Cells were then analyzed by confocal microscopy with an Olympus IX-70.

3. Results

3.1. H5N1 HA protein harboring a modified cleavage site is efficiently incorporated into HIV-based vector particles and leads to trypsin-dependent transduction

To establish a H5N1 avian influenza envelope protein pseudotyped HIV vector system, three H5N1 membrane protein expressors were generated by gene synthesis of HA, NA and M2 cDNAs according to H5N1 influenza A virus (A/Hanoi/30408/2005) sequences (Le et al., 2005). The HA open reading frame was mutagenized to convert the furin-dependent cleavage site to a trypsin-dependent activation site characteristic of low pathogenicity influenza envelope glycoprotein (Fig. 1A) (Li et al., 1999). Each synthesized cDNA was cloned into a pCAGGS vector under the control of a CMV enhancer/chicken β-actin promoter as described in Section 2. The pseudotyped HIV-based vector was generated by transfection of H5N1 HA, NA and M2 expressors together with a HIV-based vector encoding for the enhanced green fluorescence protein (EGFP) (pHx-EGFPWP, see Section 2) and a HIV-1 Gag-Pol-Tat and Rev packaging plasmid in 293T cells. In parallel, a VSV-G-pseudotyped HIV-based vector was produced as a control. To examine the association of H5N1 HA and NA proteins in VLPs, transfected 293T cultures were radiolabed with S^{35}-methionine, untreated or treated with trypsin, and immunoprecipitated with human anti-influenza serum, rabbit anti-NA or mouse anti-P24 antibodies as indicated in Fig. 1B. HA1 and HA2, the tryptic-cleavage products of the HA precursor protein HA0, were detected in trypsin-treated VLP samples (Fig. 1B, lane 2) whereas only the single high molecular weight HA0 was detected in untreated VLP samples (Fig. 1B, lane 1), demonstrating appropriate trypsin sensitivity of HA. A band of molecular weight corresponding to NA was also detected from HaNaM-pseudotyped VLPs only (Fig. 1B, middle panel, compare lanes 1 and 2 with lane 3). HaNaM- and VSV-G-pseudotyped VLPs shared only one band corresponding to the HIV capsid (Gagp24) protein in VLP preparations (Fig. 1B, lower panel).

To test whether the HaNaM-pseudotyped vector was able to transduce 293T cells in a trypsin-dependent manner, equal amounts of HaNaM-pseudotyped vector (as adjusted by HIV-1 $p24^{gag}$ antigen level) were incubated with 293T cells in the absence or presence of various concentrations of trypsin. Transduction efficiency was evaluated by measuring the percentage of EGFP-positive cells by fluorescence-activated cell sorter (FACS) analysis. Results showed that the HaNaM-pseudotyped vector was able to efficiently transduce 293T cells in the presence of exogenous trypsin. The maximal transduction efficiency (approximately 60% GFP-positive cells) was achieved when 4 μg/ml of trypsin was present during transduction, while no GFP-positive cells were detected in the absence of trypsin (Fig. 1C, upper panel). Consistently, high level of GFP expression was detected only in 293T cells tranduced by HaNaM-pseudotyped VLPs in the presence of trypsin (Fig. 1C, lower panel). These results clearly indicate that vector transduction requires trypsin activation.

3.2. Both H5N1 NA and M2 are required for maximal pseudotyped HIV VLP production and transduction

Previous studies have shown that the presence of influenza membrane M2 and NA proteins significantly increases the transduction efficiency of influenza HA-pseudotyped equine infectious anemia virus (EIAV)-based vector (McKay et al., 2006) or MLV vector (Szécsi et al., 2006). To further investigate the effect of H5N1 NA and M2 proteins on HA-pseudotyped HIV vector production and transduction, HIV-based vector was co-transfected with the HA expressor alone or in combination with HA, NA and/or M2 expressors in 293T cells. Production of HA-pseudotyped HIV vec-

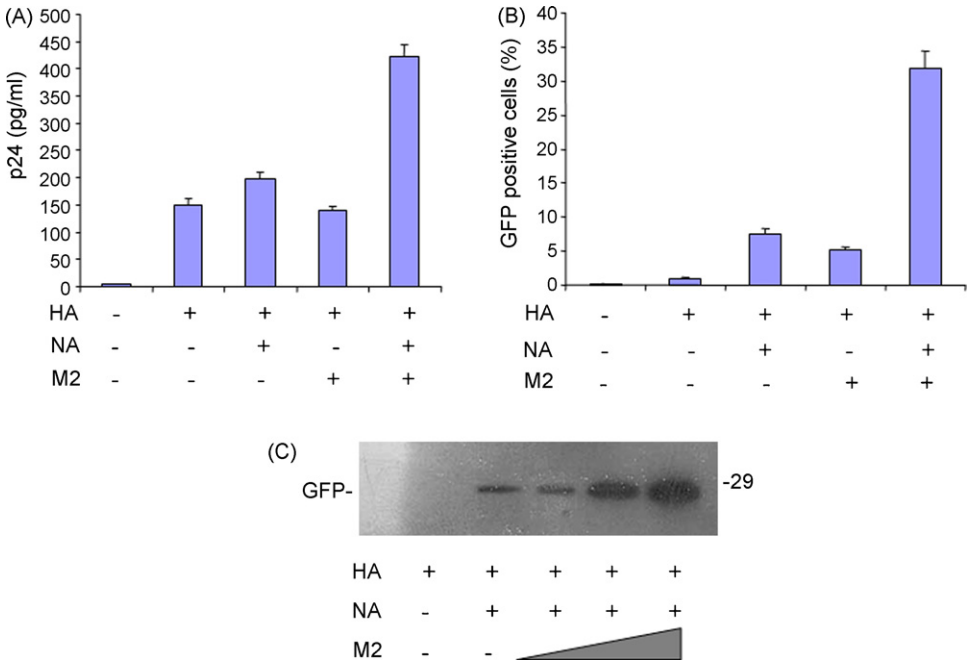

Fig. 2. Both NA and M2 proteins are required for a maximal HaNaM-pseudotyped VLP transduction. (A) The HIV vectors were co-transfected in 293T cells with H5N1 HA (3 μg) expressor alone, or co-transfected with different combinations of HA (3 μg), NA (3 μg) and M2 (3 μg) expressors, as indicated. At 48 h post-transfection, VLPs were collected from supernatants by ultracentrifugation and quantified by HIV-1 p24 detection ELISA. (B) Equal amounts of VLPs from panel A were used to transduce 293T cells, and at 48–72 h post-transduction, the percentage of GFP-positive cells was measured by FACS analysis. (C) The HA-, HA/NA- or HA/NA/M2-pseudotyped VLPs were produced in 293T cells that were co-transfected with HIV vectors, H5N1 HA(3 μg), NA (3 μg) and increasing amount of M2 expressing plasmids (0.5, 1, 3 μg). Then, equal amounts of VLPs were used to infect 293T cells and the expression of GFP protein in transduced cells was detected by immunoprecipitation with an anti-GFP antibody, followed SDS-PAGE and anti-GFP western blot. The results are representative for two independent experiments.

tor was evaluated by measurement of HIV capsid p24 levels 48 h post-transfection. Results show that the individual expression of NA modestly increased HIV vector production while M2 did not have a significant effect (Fig. 2A). Interestingly, co-expression of NA and M2 resulted in a marked increase of vector production (Fig. 2A). To further test the effect of NA and/or M2 on pseudotyped vector transduction efficiency independent of their effects on VLP production, equal amounts of the produced pseudotyped vector (normalized by HIV-1 p24 levels) produced from the culture described in Fig. 2A were used to transduce 293T cells. After 48 h, the transduction efficiency was analyzed by measurement of EGFP-positive cells by FACS. Results showed that the presence of NA or M2 alone enhanced transduction efficiency by approximately 3- and 5-fold, respectively (Fig. 2B). Interestingly, the presence of both NA and M2 resulted in a 30-fold increase in transduction efficiency (Fig. 2B). The detection of vector-mediated EGFP expression by western blot analysis in transduced cells also showed a robust increase in transgene expression when all three influenza membrane proteins were present in the vector preparation (Fig. 2C).

3.3. Transduction of HaNaM-pseudotyped HIV VLPs in different cell types

To test the transduction ability of H5N1 HaNaM-pseudotyped HIV vector in different cell types, equal amounts of HaNaM-pseudotyped VLPs (normalized by p24 levels) were incubated with different cell lines, including human embryonic kidney 293T cells, canine kidney MDCK cells, A549 human lung carcinoma cells, and CD4+ C8166 T-lymphoid cells. In parallel, the similar amount of VSV-G-pseudotyped vector was used as control. Two to three days after transduction, each cell culture was monitored for the presence of GFP-positive cells by FACS analysis. The VSV-G-pseudotyped vector transduced between 30% and 50% of all target cell lines tested (Fig. 3A). In contrast, HaNaM-pseudotyped vector transduced 293T and MDCK cells with efficiencies reaching up to 55% approximately (Fig. 3A and B). However, in CD4+ C8166 T-lymphoid cells, transduction ability of HaNaM-pseudotyped VLPs was very low (approximately 3% GFP-positive cells), whereas the transduction mediated by VSV-G-pseudotyped VLPs reached approximately 50% (Fig. 3A). These results clearly indicate the different tropisms of H5N1 and VSV-G envelope proteins.

3.4. The NA inhibitor oseltamivir (Tamiflu) interferes with HaNaM-pseudotyped vector production and transduction

The susceptibility of HaNaM-pseudotyped vector to a specific anti-influenza (NA) compound, oseltamivir (Tamiflu), was evaluated. First, HaNaM-pseudotyped vector was produced in 293T cells in the presence or absence of oseltamivir. The presence of oseltamivir (100 nM) in transfected cells decreased HaNaM-pseudotyped vector production by approximately 3-fold (Fig. 4A). In contrast, oseltamivir treatment did not significantly affect VSV-G-pseudotyped vector production. To evaluate the transduction ability of released pseudotyped VLPs, equal amounts of VLPs produced from oseltamivir-treated or non-treated 293T cells were used to transduce 293T cells. After 48 h, the transduced cells (EGFP-positive cells) were monitored by FACS analysis. Strikingly, while the non-treated HaNaM-pseudotyped VLP's induced 35% transduction in 293T cells, VLPs produced from oseltamivir treated cells lost their transduction ability (Fig. 4B). However, the presence of oseltamivir only moderately affected the transduction efficiency of VSV-G-pseudotyped vector. These results clearly indicate that the treatment of oseltamivir not only specifically inhibits HaNaM-pseudotyped VLP's production, it also drastically disrupts the transduction ability of the released VLPs.

Some recent studies have reported that the presence of NA is also required for the initiation of influenza virus infection (Matrosovich et al., 2004; Ohuchi et al., 2006). To test whether oseltamivir

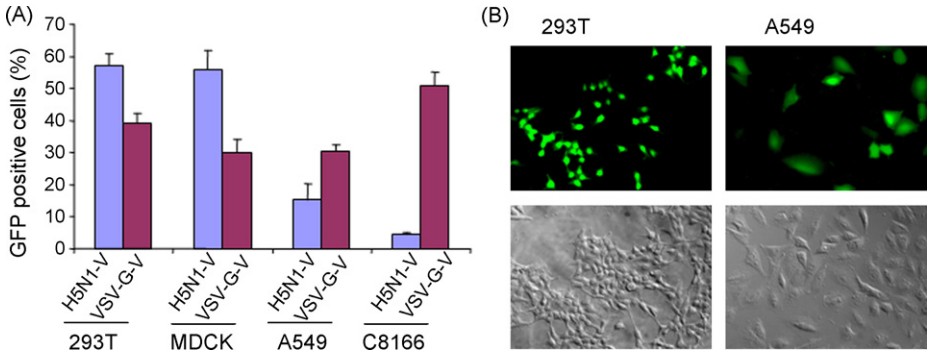

Fig. 3. The transduction efficiency of HaNaM-pseudotyped HIV VLPs in different cell types. (A) Equal amounts of HaNaM- or VSV-G-pseudotyped HIV VLPs (as adjusted by the level of HIV p24 antigen) were used to transduce human embryonic kidney 293T cells, canine kidney MDCK cells, A549 lung carcinoma cell and CD4+ C8166 T cells. The percentage of the GFP-positive cells in each cell population was measured by FACS analysis 48–72 h after transduction. (B) Fluorescence microscopic images of GFP-positive 293T (a) and A549 cells (b) 48 h post-transduction with HaNaM-pseudotyped VLPs. The results presented here were obtained from two independent experiments.

could also affect HaNaM-pseudotyped vector transduction, equal amounts of HaNaM- or VSV-G-pseudotyped vector were incubated with 293T cells in the presence or absence of oseltamivir (100 nM) for 2 h. Vector and oseltamivir were then removed and transduced cells were cultured in fresh DMEM without oseltamivir for 48 h and then, the EGFP expression was monitored by FACS analysis. The presence of oseltamivir was shown to inhibit HaNaM-pseudotyped vector transduction by approximately 37%, while it did not affect VSVG-pseudotyped vector transduction (Fig. 4C). Overall, these results indicate that HaNaM-pseudotyped vector production and transduction are susceptible to oseltamivir.

3.5. *Adaptation of HaNaM-pseudotyped vector transduction into a microplate assay*

Given that HaNaM-pseudotyped HIV vector transduction provides a safe and sensitive way for testing antivirals against H5N1 membrane proteins, we set up a quantitative 96-well plate transduction system for a potential high throughput screening assay. A HaNaM-pseudotyped HIV vector containing a LacZ reporter gene was produced in 293T cells and used in this assay. To test the sensitivity of 96-well transduction system, different amounts of HaNaM-HIV-LacZ vector were incubated with 293T cells in 96-well plates for 48 h and transduction efficiency was monitored by measurement of vector-produced β-Gal activity with an enhanced β-Gal assay kit (Fig. 5A, upper panel) or by staining of β-Gal positive cells by using a staining method as previously described (Kimpton and Emerman, 1992) (Fig. 5A, lower panel). The results in Fig. 5A showed a liner correlation between the input of HaNaM-pseudotyped vector and the level of β-Gal activity (Fig. 5A, upper panel). Measurement of the number of β-Gal positive cells with an Elispot Reader also showed a close correlation between the amount of input vector and the number of β-Gal positive cells (Fig. 5A, lower panel). Similar results were obtained when MDCK cells were used instead of 293T cells in 96-well plates (data not shown).

The inhibitory effect of oseltamivir on HaNaM-pseudotyped vector transduction in this microplate system was also tested. Equal amounts of vector produced from oseltamivir-treated or non-treated 293T cells were serially diluted and used to transduce 293T cells in 96-well plate. After 48 h, the transduction levels were monitored by using the enhanced β-Gal assay kit, as described above. The results showed that while HaNaM-pseudotyped VLPs produced in the absence of oseltamivir efficiently transduced 293T cells and showed a dose-dependent transduction, the VLPs produced in the presence of oseltamivir drastically lost their transduction ability (Fig. 5B). Taken together, this micro-well transduction assay is highly sensitive to the action of oseltamivir, and it can be developed as a high throughput system for rapid screening of compounds and/or polypeptides against the H5N1 avian influenza virus entry step.

4. Discussion

Because of the highly pathogenic features of H5N1 influenza virus, a high biosafety containment facility is required for H5N1 influenza infection studies, including antiviral screening experiments. In addition, precautions have to be taken when using cDNA encoding a wild-type H5N1 HA gene in the laboratory due to the potential risk of recombination. In this study, we have developed a H5N1 HaNaM-pseudotyped HIV vector system, in which the proteolytic cleavage site of HA was replaced with a trypsin sensitive site, resulting in a trypsin-dependent "low-pathogenic" form of the glycoprotein as previously described by Li et al. (1999). This study shows that the H5N1 HaNaM-pseudotyped HIV vector can be safely handled in lower biosafety environments and the trypsin-dependent feature of this system will allow it to be conditionally activated and easily used in many cell types for screening of specific compounds against influenza virus membrane proteins.

The influenza virus membrane proteins HA, NA and M2 play key roles to initiate virus replication. HA protein binds to sialic acid receptors and mediates fusion of viral membrane to endosomal membrane after endocytosis (Klenk and Rott, 1988; Skehel and Wiley, 2000), M2 is a small transmembrane protein with an ion channel activity which regulates the pH inside the virion during viral entry into cells. The NA has been shown to play an important role both in facilitating virus release and contributing to the initiation of virus infection (Matrosovich et al., 2004; Ohuchi et al., 2006). Even though the functional role of each membrane protein has been well documented, how these proteins collaborate together to maximize influenza infection is still not fully understood. In this study, results showed that, although M2 itself did not affect HA-pseudotyped HIV vector production, co-expression of M2 with NA significantly increased vector particle production, as compared to NA expression alone. This suggests an as yet undefined interaction between M2 and NA functions which greatly enhances virus production that may be important for influenza morphogenesis and possibly influenza-induced pathophysiology *in vivo*. Therefore, it is interesting to further investigate the mechanism underlying this synergistic effect between M2 and NA proteins.

The NA inhibitor oseltamivir was shown to efficiently inhibit the production and infectivity of HaNaM-pseudotyped HIV vector (Fig. 4). Consistently, vector pseudotyped with HA alone showed very low transduction level (Fig. 2). It is clear from these data that NA not only facilitated the release of the VLPs, but also directly affected their transduction ability. It is possible that, when NA activity was inhibited, the sialic acid was not removed from progeny

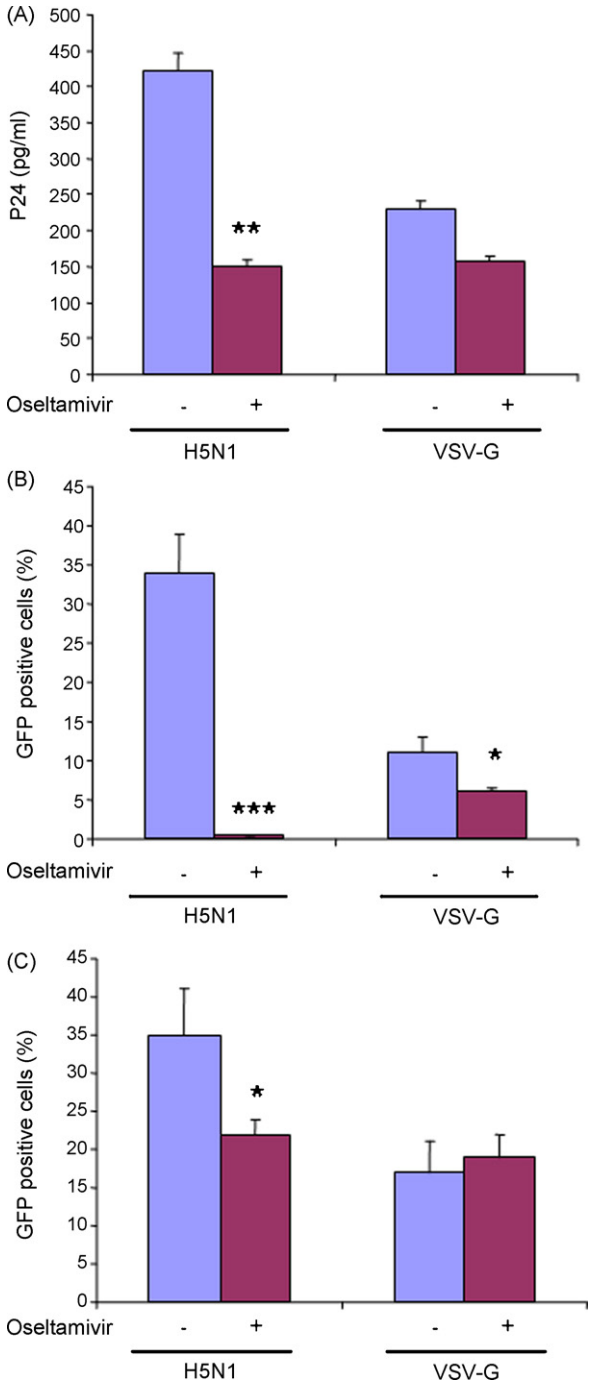

Fig. 4. NA inhibitor oseltamivir interferes with HaNaM-pseudotyped VLP production and its transduction. (A) HIV vectors were co-transfected with H5N1 HA, NA and M2 protein expressors or VSV-G protein expressor in 293T cells. After 12 h, the transfected cells were washed with serum-free medium and fresh DMEM added with or without 100 nM oseltamivir. At 48 h post-transfection, pseudotyped VLPs were pelleted by ultracentrifugation, and viral production was quantified by HIV-1 p24 antigen detection ELISA. (B) Equal amounts of HaNaM-pseudotyped VLPs from oseltamivir-treated or non-treated VLP producing cells were used to infect 293T cells and GFP-positive cells were quantified by FACS analysis. (C) Equal amounts of HaNaM- or VSV-G-pseudotyped VLPs produced in cells that were not treated with oseltamivir were incubated with 293T cells in the absence or presence of 100 nM oseltamivir for 2 h. The infection medium was replaced by fresh medium and incubated for 48 h. GFP-positive cells were quantified by FACS analysis. The results represent the mean ± standard deviations of three independent replicates. *, statistically significant ($p < 0.05$); **, very statistically significant ($p < 0.01$); ***, extremely statistically significant ($p < 0.0001$).

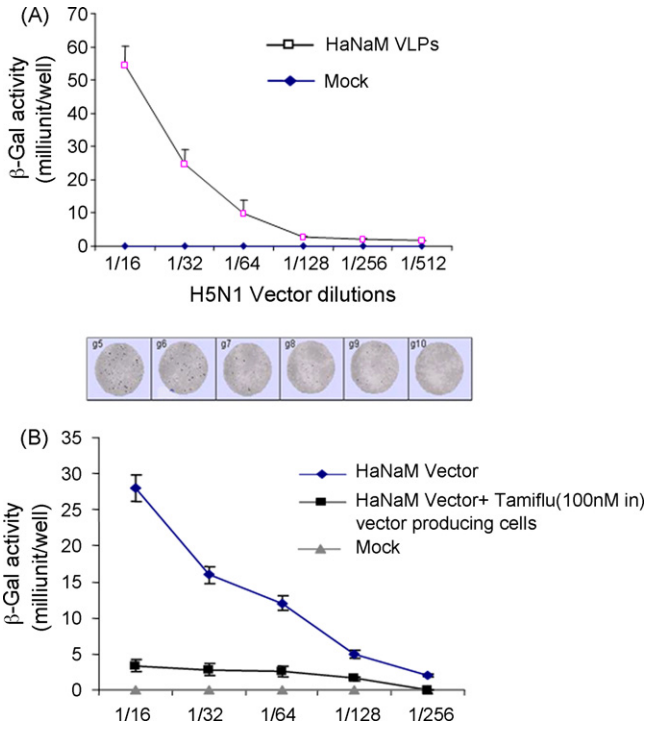

Fig. 5. Adaptation of HaNaM-pseudotyped vector transduction to a 96-well microplate assay. HIV vectors encoding for LacZ reporter gene were co-transfected with H5N1 HA/NA/M2 expressors or VSV-G expression plasmid in 293T cells. The supernatant containing HaHaM-pseudotyped VLPs were treated with trypsin (4 μg/ml) for 1 h at 37 °C before ultracentrifugation. (A) Two-fold serial dilutions of VLP stock were used to transduce 293T cells in 96-well plates. After 48 h, the β-Gal activity in cell lysates (upper panel) for each well was measured. Also, the β-Gal positive cells in the culture was stained by X-gal staining (lower panel). (B) Effect of oseltamivir on HaNaM-pseudotyped VLPs transduction. Equal amounts of HaNaM-pseudotyped VLPs from oseltamivir-treated or non-treated VLP producing cells were serially diluted and incubated with 293T cells cultured in 96-well plate. After 48 h, the cells were lysed and the β-Gal activity was quantified by with an enhanced β-galactosidase assay kit. The results are representative for three independent experiments.

viral particles, which may result in virus aggregation and consequently disrupt their transduction efficiency. Moreover, our results also indicate that oseltamivir was able to directly interfere with viral entry of HaNaM-pseudotyped VLPs (Fig. 4C). These results are consistent with recent studies showing that NA is important for the initiation of influenza virus infection (Matrosovich et al., 2004; Ohuchi et al., 2006). Altogether, the results indicate that HaNaM-pseudotyped vector utilizes an entry mechanism very similar to the natural H5N1 influenza virus entry process.

Avian influenza HA is the most abundant surface protein of the virus and this viral protein has been an attractive target for the development of antiviral agents. However there is still no antiviral agent(s) active against this viral protein. Even though several specific anti-influenza compounds, including amantadine and oseltamivir, are available against NA and M2 proteins, the emergence of drug resistant strains has been reported (Suzuki et al., 2003; Kiso et al., 2004; Bright et al., 2005; Cheung et al., 2006). Thus, there is a strong need for developing new drugs against influenza viruses. This H5N1-pseudotyped vector transduction assay provides a cell-based and sensitive system for high throughput screening for compounds and/or polypeptides that can effectively inhibit H5N1 influenza virus entry in cell culture simply by measuring β-Gal activity using a β-Gal assay kit or by counting β-Gal positive cells with an Elispot Reader. Moreover, this H5N1-pseudotyped vector system clearly show the functional coordination among three viral membrane proteins and therefore, the

utilization of such H5N1 virus entry system will help to elucidate the mechanisms underlying the functional coordination of three viral member proteins and to identify and develop new classes of antiviral agents. Since this vector system can be pseudotyped with different HA/NA/M2 proteins derived from various avian influenza strains, it also has broad application to test the effect of antiviral agents on virus entry of different avian influenza viruses. In addition, this pseudotyped vector system can also be used as a neutralizing assay to evaluate the vaccine efficacy against different avian influenza viruses.

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