Identification of H5N1 Virus Binding Proteins on Human Neuronal SH-SY5Y Cells

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ABSTRACT

After the infection, avian influenza A (H5N1) virus has been reported in several brain regions of many mammalian species that exhibit neurodegenerative change, including the substantia nigra and the hippocampus. Till date molecular mechanism that governs the neuroinvasiveness of H5N1 virus in human remain elusive. Therefore, highly pathogenic H5N1 virus strain A/Thailand/NK165/05 was chosen to infect human neuronal SH-SY5Y cells. We found that the virus particles abounded in the cytoplasm and neurites of infected cells as early as 12 h post-infection (p.i.) and increased in a time-dependent manner consistent with a rapid progression of cytopathological change. The highest viral titers were observed at 72 h p.i. with the maximum cytopathic effect. Then, we performed a virus overlay protein binding assay (VOPBA) coupled with LC-MS/MS and identified receptor for activated C kinase 1 (RACK1) and prohibitin-1 (PHB1) as H5N1 binding proteins on these neuronal cell membranes. Using antibodies targeting RACK1 and PHB1, western blot and immunofluorescence colocalization analyses demonstrated that infection decreased expression of RACK1 and PHB1. Pre-treatment with antibodies directed against these two proteins was able to reduce virus accumulation inside the cells. This is the first report demonstrating that RACK1 and PHB1 could be involved in H5N1 infection in human neuronal cells.

Key Words: Avain influenza virus, Neuronal cells, Neurotropic virus, RACK1, PHB1

Introduction

The influenza A virus belongs to the family *Orthomyxoviridae*. The virus contains eight single stranded RNA segments of negative polarity. Hemagglutinin (HA) and neuraminidase (NA) are their major antigenic determinants. Subtype classification is based on these two proteins. At present, at least 17 different HA and 9 NA antigens have been described (Tong et al., 2012). The HA protein is responsible for virus attachment and subsequent fusion of the virus to host cell membranes. It also plays roles in viral entry, particle formation and budding (Horimoto and Kawaoka, 2001).

Influenza A viruses are one of the most common infectious pathogens in humans. Circumstantial evidence has linked subsequent neurological manifestations in post-encephalitic Parkinson's disease to influenza A virus outbreaks (Takahashi et al., 1995). Recently, one of the most deleterious influenza pandemics has been engendered by the highly pathogenic avian influenza A (H5N1) virus. Neurotropism of certain strains of H5N1 virus has been reported (Jang et al., 2009). In 2004, the first outbreak of H5N1 virus in Thailand was found in poultry populations (OIE, 2011). At that time, the first human case succumbing to this virus was also reported. The H5N1 human cases were documented with live-viruses found predominantly in lung tissue and most patients succumbed to acute respiratory distress (Chotpitayasunondh et al., 2005; de Jong et al., 2005; de Jong and Hien, 2006). After systemic infection, the virus appears to infect multiple organ systems, including the brain (Ng and To, 2007). Yet, the neurotropism of H5N1 has remained unclear and needs to be explored.

Several studies have led to an awareness of the potential of H5N1 virus to cause neurological disorders in humans (de Jong et al., 2005). At autopsy, the virus has been

reported in several brain regions with elevated levels of pro-inflammatory cytokines in the CSF (Gu et al., 2007; Maines et al., 2005). In general, acute inflammation is beneficial to infected host in that it limits the survival and proliferation of invading pathogens and promotes tissue regeneration. In case of the brain, infection by the neurotropic viruses could compromise neural integrity and survival with subsequent proliferation and spread of infectious virus particles. Moreover, prolonged and excessive inflammation induced by the infection could exacerbate neurodegenerative processes.

Like other viruses, the initial step of H5N1 virus infection comprises of binding to the target cells via the interaction of viral proteins with specific receptor molecules on the host cell membranes. At present, our knowledge regarding H5N1 virus interacting proteins on human brain cells is still limited. Using the human neuronal SH-SY5Y cell line, this study aimed to examine neuronal susceptibility to highly pathogenic H5N1 virus and to identify H5N1-binding proteins that might play a role in virus infection and spreading in the central nervous system.

Results

Susceptibility of SH-SY5Y cells to H5N1 virus infection

In this set of experiments, cytopathic effect (CPE) of H5N1on SH-SY5Y cells was examined. Cells were infected with H5N1 virus strain NK165 at MOI of 1 for 6, 12, 24, 48 and 72 h. Then, the morphology of infected cells was observed and photographed under a phase-contrast microscope and compared with that of their time-matched, mock-infected controls. Results demonstrated that H5N1 virus infection caused cells to progress from typical neuronal morphology with neurite extension to become rounded. By 48 h p.i., infected cells formed small aggregates with cell debris in the supernatant. By 72 h p.i., only cellular debris remained in the infected cultures (Fig. 1). CPE at each time point p.i. was also scored as described previously (Moore et al, 1981) and results were shown in Table 1. The viral titer in cell culture supernatant and the viability of infected cells at each time point p.i. were determined simultaneously. We detected a very low viral titer in the supernatant at 12 h p.i.. The titer was increased exponentially over time (Fig. 2). After 24 h p.i., the viability of infected cells gradually declined. By 72 h p.i., all infected cells were dead. These results demonstrate that H5N1 infection and, perhaps recurrent infection of progeny viruses, causes widespread death of SH-SY5Y cells.

Immunofluorescence assay using a mouse monoclonal anti-H5N1 specific HA showed that virus particles were found in the cytoplasm of infected cells as early as 12 h p.i.. By 24 h p.i., all cells in cultures were infected and fluorescence intensity in each infected cells was increased compared with that of infected cells at 12 h p.i.. By 72 h p.i., all infected cells were dead, as evidenced by DAPI nuclear staining pattern (Fig. 3).

These results suggest that morphological changes observed in H5N1-infected SH-SY5Y cells may be caused by the replication of virus inside the cells.

H5N1-binding proteins on SH-SY5Y cells

To identify membrane proteins that could be involved in internalization of H5N1 viruses into SH-SY5Y cells, cell membrane-enriched samples were prepared, separated on 10% SDS-PAGE and stained with Coomasie brilliant blue. Duplicate gels were transferred to PVDF membranes, before incubating with H5N1 virus at 4°C or at room temperature. Membranes incubated without the virus at both temperatures were served as negative controls of the assay. The positions of virus-protein interaction were detected using a mouse monoclonal anti-H5N1 specific HA followed by an appropriate HRP-conjugated secondary antibody (Fig. 4). There were three bands that were excised from the duplicate gels running in parallel and processed for LC-MS/MS analysis. The first two bands with approximately molecular weights of 36-38 kDa and 34-35 kDa appeared when membranes were incubated with the viruses either at 4°C (Fig. 4C) or at room temperature (Fig. 4D). The third band with approximately molecular weights of 28-30 kDa was selected, because it was a major band appeared when membranes were incubated with the viruses at room temperature (Fig. 4D). Data generated from LC-MS/MS analysis were searched against the SwissPort database for protein identification using the Mascot software. Results of candidate H5N1 binding proteins were shown in Table 2. The targets were selected based on the following criteria. Each identified protein belongs to *Homo sapiens* species. The molecular mass of candidate protein resides within a range of relative protein molecular weight of a cut band. Candidate proteins must have

the highest percentage of the peptide match. Candidate proteins must be membrane proteins. Therefore, we identified receptor of the activated protein kinase C (RACK1) and prohibitin 1 (PHB1) as H5N1 binding proteins on SH-SY5Y cell membranes.

Expressions of RACK1 and PHB1 in mocked and H5N1 infected SH-SY5Y cells

To confirm expressions of RACK1 and PHB1 in mocked infected SH-SY5Y cells, we performed immunofluorescence assay using antibodies specific to either RACK1 or PHB1. Results demonstrated that both RACK1 and PHB1 were expressed constitutively in SH-SY5Y cells (Fig. 5). We also observed strong fluorescence intensity of RACK1 compared with that of PHB1, suggesting that SH-SY5Y cells expressed RACK1 more than PHB1. Then, we further examined expressions of RACK1 and PHB1 in H5N1-infected SH-SY5Y cells at 12 and 24 h p.i.. Results demonstrated that infection reduced expressions of RACK1 and PHB1 in a time dependent manner (Fig. 6, 7). Western blot analysis performed at 24 h p.i. also confirmed that H5N1 infection downregulated expressions of RACK1 and PHB1 (Fig. 8). These results suggest that both RACK1 and PHB1 may be involved in the entry of H5N1 viruses into SH-SY5Y cells.

Antibodies pre-treatment reduces H5N1 virus infection in SH-SY5Y cells

To examine the involvement of RACK1 and PHB1 in the entry of H5N1 viruses into SH-SY5Y cells, we performed antibody mediated infection inhibition assay. Cells were treated with 10 µg and 20 µg of antibody directed against either RACK1 or PHB1 before infection with H5N1 viruses at MOI of 1. This assay was evaluated at 24 h p.i. by immunofluorescence staining using a mouse monoclonal antibody directed against H5N1

specific HA and an appropriate AlexaFlour conjugated anti mouse IgG (H+L) antibody. Results showed that pre-treatment with antibody against either RACK1 or PHB1 reduced fluorescence intensity of HA-specific signals inside the infected cells in a concentration-dependent manner (Fig. 9, 10). These results suggest that antibody pre-treatment may disrupt the entry of H5N1 viruses into SH-SY5Y cells by interfering with the interaction between virus particles and RACK1 (or PHB1).

Discussion

As the first part of an ongoing study of pathogenesis of H5N1 virus in the human central nervous system, we demonstrate that highly pathogenic avian influenza H5N1 virus induces a rapid cytopathological change, the production of progeny viruses and subsequently pronounced death of neuronal SH-SY5Y cells, thus indicating that human neuronal cells are susceptible to H5N1 virus infection. Although there are several studies showing that H5N1 virus induces apoptosis in cell culture as well as in animal models (Bissel et al., 2012; Tran et al., 2013) and that H5N1-induced apoptosis in human monocyte-derived macrophages involves gene associated with retinoid and interferon induced mortality-19 (GRIM-19), TNF-related apoptosis-inducing ligand (TRAIL) and caspase-10 (Ekchariyawat et al., 2012, 2013), molecular mechanisms of H5N1-induced cell death in human neuronal cells still remain elusive.

In this given study, we also performed VOPBA in conjunction with LC-MS/MS and identified RACK1 as H5N1 virus binding protein in SH-SY5Y cells. RACK1 is an evolutionarily conserved intracellular protein of the tryptophan, aspartic acid repeat (WD repeat) protein family. It plays essential roles in basic cellular activities, including in many aspects of neuronal cell function (reviewed in [Adams et al., 2011]). RACK1 is known to anchor activated protein kinase C (PKC) at the cell membrane and can be isolated from the membrane-enriched samples (Mochly-Rosen, 1995; Rodriguez et al., 1999). RACK1 has been identified in the yeast two-hybrid system as protein interacting with the M1 protein of the avain influenza A virus (Reinhardt and Wolff, 2000). The M1 protein is a major structural protein of the virus playing essential roles during replication, virion formation and budding (Fujiyoshi et al., 1994; Bui et al., 1996; Watanabe et al.,

1996). Functional phosphorylation of M1 protein is known to be regulated by PKC and RACK1 of host cells (Reinhardt and Wolff, 2000). Recently, it has been shown that M1 protein-RACK1 interaction is indispensible for the release of influenza A virus particles (Demirov et al., 2012). Thus, a down-regulation of RACK1 in H5N1 infected SH-SY5Y cells as reported herein may reflect a host cell defence mechanism to reduce the release of virus particles. However, it remains unknown if expression of RACK1 is linked to programmed cell death of host cells to dampen H5N1 virus infection. Based on our finding that pre-treatment with anti-RACK1 antibody reduces H5N1 infection in SH-SY5Y cells, we therefore speculate that RACK1 may be involved in the internalization of H5N1 virus. Alternatively, it is possible that pre-treatment with anti-RACK1 antibody may interfere with RACK1-mediated M1 phosphorylation by PKC, thus disrupting replication, assembly, budding and spreading of the virus in infected cultures.

In addition to RACK1, we identified PHB1 as H5N1 virus binding protein in neuronal SH-SY5Y cells. PHB1 is a highly conserved and ubiquitously expressed multifunctional protein of PHB domain protein family, which play essential roles in cell signaling and in mitochondrial functions (reviewed in [Mishra et al., 2010]). PHBs have been reported to be involved in cell survival (Ross et al., 2008), the regulation of apoptosis (Merkwirth et al., 2008) and protection against oxidative stress (Theiss et al., 2007). While PHB2, a homolog of PHB1 with different molecular mass, is identified as a receptor mediating the entry of dengue virus serotype 2 (DENV-2) into both *Aedes aegypti* and *Aedes albopictus* mosquito cell lines (Kuadkitkan et al., 2010), PHB1 is recently shown to act as receptor protein for Chikungunya virus by interacting with E2 protein at the cell surface of microglial cells (Wintachai et al., 2012). Together with our

study, these findings are indicative of a novel role of PHBs as viral receptor, consistent with its cell surface and endosome/phagosome localization in addition to mitochondria and nucleus (Garin et al, 2001; Yuruki et al., 2012). Accordingly as predicted, interfering with expression of PHB1 at the cell surface using anti-PHB1 antibody reduced the internalization of H5N1 virus. It is tempting to speculate that reduced expression of PHB1, as with RACK1, is associated with the death of neuronal cells in an attempt to limit virus spread during H5N1 infection. Indeed, a neuroprotective role of PHB1 has been shown recently by several studies both in cell culture and animal models, thus further highlighting our speculation. These authors have demonstrated that cellular stresses induced by neurotoxin injection (Park et al., 2010), oxygen-glucose deprivation and transient ischemia (Zhou et al., 2012) up-regulated expression of PHB1 in neuronal cells and PHB1 gene silencing enhances neurotoxicity of 6-hydroxydopamine and glutamate induced cell death in neuronal cell cultures (Park et al., 2010; Zhou et al., 2012). Moreover, a significant decrease in PHB expression has also been reported and linked to mitochondrial damage in peripheral blood mononuclear cells of HIV-infected patients (Ciccosanti et al., 2010).

Interestingly, when VOPBA was performed at room temperature as compared with 4ºC, we observed more protein bands of different molecular weights including those of RACK1 and PHB1 (Fig. 4). It is therefore suggested that additional sets of proteins expressed on neuronal cell membranes may be involved in H5N1 virus binding and subsequent entry into the cells and such interactions may be regulated in a temperature-dependent manner. This is further supported by the fact that a complete inhibition of infection cannot be achieved using anti-RACK1 and anti-PHB1 antibodies. Thus, further

studies are required to determine viral proteins that interact with RACK1 and PHB1 in neuronal cells as well as signaling cascades downstream of RACK1 and PHB1 that may be involved in neuronal infection of H5N1 virus.

In this report, we have demonstrated a strong neurotropism of highly pathogenic avian influenza H5N1 virus in human neuronal cells and are the first to report that RACK1 and PHB1 are neuronal proteins that interact with H5N1 virus. Further studies on H5N1 virus infection using neuronal cells in which expressions of both RACK1 and PHB1 are suppressed should provide new insights into host-virus interactions that regulate H5N1 neuropathogenesis. In addition, these analyses should be useful for the development of new and specific therapeutic agents against highly pathogenic avian influenza outbreaks in humans.

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Materials and methods

All procedures involved with infection and handling of live avian influenza A (H5N1) virus strain A/Thailand/NK165/2005 were performed exclusively in a biosafety level 3+ facility at the Faculty of Medicine, Chulalongkorn University, Thailand.

Virus propagation and hemagglutination titration

Stocks of H5N1 (NK165) virus were kindly provided by the Center of Excellence in Clinical Virology, Chulalongkorn University, Thailand. The virus was propagated in 12-day-old embryonated chicken eggs at 35°C for 3 days. Then, the eggs were stored overnight at 4°C before harvesting the virus-containing allantoic fluids. Virus titre was determined by hemagglutination (HA) assay. Briefly, the allantoic fluids were centrifuged at 10,000 x g for 10 min to obtain clear supernatant containing the viruses. First, 25 µl of supernatant was mixed with 25 µl of PBS in a V-shaped 96-well plate and serially diluted 2-fold. Then, 50 µl of 1% chicken red blood cells was added to each well and gently agitated. The plate was incubated for 30 min at room temperature. The last dilution showing complete agglutination of the red blood cells was counted and expressed as HA unit (Mochalova et al., 2003; Kistner et al., 2007).

Cell culture and infection

Human neuronal SH-SY5Y cells (ATCC, VA) were cultured in DMEM-F12 medium (HyClone, UT) supplemented with 10% FBS (Gibco, MD), 100 U/ml of penicillin and 100 µg/ml of streptomycin at a humidified 37°C in 5% CO_2 atmosphere.

Then, cells were infected with H5N virus at a multiplicity of infection (MOI) of 1. After 1 h absorption, cells were washed with PBS and the cultures were continued in 10% FBS supplemented medium at 37°C under standard cell culture conditions. Mock-infected cells served as negative controls. Cell-free supernatants from infected and mock-infected cultures were collected at 0, 6, 12, 24, 48 and 72 h p.i. to determine the titre of progeny viruses by HA assay.

Cell viability assay

To assess cell viability, the number of viable cells at different time points p.i. was determined using trypan blue dye exclusion method. The assay is based on the ability of intact viable cells to exclude trypan blue dye. Briefly, H5N1-infected SH-SY5Y and mock-infected cells at 2 x 10^5 cells/well were cultured in 6-well plates. At pre-determined time points after infection, the cells were washed once with PBS and trypsinized to collect cell pellets by centrifugation at 300 x g for 5 min followed by re-suspending the pellets in PBS. Subsequently, 0.2 ml of the suspension was mixed with an equal volume of 0.4% trypan blue (Sigma-Aldrich, MO), incubated for 5 min and observed under a microscope to count total cell and stained cell numbers using a hemocytometer.

CPE assay

The cytopathic effect (CPE) manifested by multinucleated giant cells, cell shrinkage and foci of cell destruction in SH-SY5Y cultures following H5N1 virus infection at various time points were photographed using a phase-contrast microscope and scored as previously described: 0, no CPE; +/-, enlargement of some cells in

monolayer; 1+, 1 t-25% CPE; 2+, 25-50% CPE; 3+, 50-75% CPE; 4+, 75-100% CPE (Moore et al., 1981).

Indirect immunofluorescence and co-localization assay

Mock-infected and H5N1 (MOI of 1 at 37°C for 1 h) infected SH-SY5Y cells on coverslips were washed with FBS-free medium 3 times to remove uninternalized viruses. Then, fresh medium with 10% FBS was added and cultured for different time points as indicated in each experiment. The cover slips were washed twice with PBS, fixed with absolute methanol at room temperature for 20 min and left air dried for 30 min. Subsequently, cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature, washed twice with PBS and blocked with 5% normal goat serum in 0.03% Triton X-100/PBS for 2 h at room temperature before incubating with mouse monoclonal anti-H5N1 HA antibody (1:500; ProSci Inc., CA) at 4°C overnight. After washing with 0.03% Triton X-100/PBS, the cells were incubated with goat anti-mouse IgG (H+L) AlexaFlour 594-conjugated (1:5000; Invitrogen, CA) for 1 h at room temperature in the dark. For colocalization experiments, infected and mock-infected cells on cover slips stained with anti-H5N1 HA were blocked with 5% normal goat serum in 0.03% Triton X-100/PBS for 2 h at room temperature before incubating with a mouse monoclonal ant-RACK1 antibody (1:500; Santa Cruz, CA) or a goat polyclonal anti-PHB1 antibody (1:500; Santa Cruz, CA). After washing with 0.03% Triton X-100/PBS, the cells were incubated with an appropriate secondary antibody; a goat anti-mouse IgG (H+L) AlexaFlour 488-conjugated (1:5000; Invitrogen, CA), a donkey anti-goat IgG (H+L) AlexaFlour 488-conjugated (1:5000; Invitrogen, CA) or a donkey anti-goat IgG

(H+L) AlexaFlour 546-conjugated (1:5000; Invitrogen, CA) for 1 h at room temperature in the dark. Finally, the cells were washed six times with 0.03% Triton X-100 in PBS and then mounted with Prolong® Antifade reagent with DAPI (Invitrogen, CA) onto glass slides. The stained cells were visualized using an Olympus inverted fluorescence microscope with DP2-BSW application software (Olympus Imaging America Inc., PA).

Preparation of cell membrane extracts

Membrane proteins were extracted as previously described (Sakoonwatanyoo et al, 2006). Briefly, SH-SY5Y cells were washed twice with TBS. Cell pellets in TBS were collected by centrifugation at 1200 x g at 4°C for 4 min, re-suspended in 1 ml of ice-cold buffer containing 100 mM of NaCl, 20 mM of Tris-HCl (pH 8), 2 mM of $MgCl_2$, 1 mM of EDTA, 0.2% Triton X-100 and protease inhibitor cocktail (Thermo Scientific Pierce, IL) and lysed by vigorous vortex. After centrifugation at 600 x g for 3 min at 4°C, the supernatant was collected and centrifuged at 6,000 x g for 5 min at 4°C. Then, the supernatant was centrifuged at 20,800 x g for 10 min at 4°C to collect the membrane protein pellets, which were re-suspended in ice-cold modified buffer M. The membrane protein concentration was quantified by BCA assay kit (Thermo Scientific Pierce, IL).

Virus overlay protein binding assay (VOPBA)

Cell membrane proteins (100 ug) was loaded onto 10% SDS-PAGE together with molecular weight protein markers, separated according to molecular mass and transferred to PVDF membranes. The membranes were blocked for non-specific binding with 5% skim milk in TBS for 2 h with constant agitation followed by incubation with H5N1 virus

diluted in 1% skim milk in TBS and subsequently incubated for 2 h. The amount of the virus used in the experiment was $1x10^7$ pfu/cm^2 of the membrane area. For comparison, virus incubation step was performed at 4°C and at room temperature. Membrane proteins on PVDF membranes that were incubated without virus at 4°C and at room temperature were served as negative control of the assay. After washing three times with TBS (5 min each), the membranes were incubated with a mouse monoclonal anti H5N1 HA antibody (1:200; ProSci Inc., CA) made up in 5% skim milk in TBS with constant agitation at room temperature for 2 h. After incubation, the membranes were washed three times (5 min each) and then incubated with peroxidase-conjugated rabbit anti-mouse IgG antibody (1:3000; Thermo Scientific Pierce, IL) in 5% skim milk in TBS with constant agitation at room temperature for 1h. Finally, after washing three times in TBS (5 min each), the signal was generated using ECL-Plus Western Blotting Substrate (Thermo Scientific Pierce, IL) and directly exposed to CL-XPosure film (Thermo Scientific Pierce, IL). Then, corresponding protein bands on parallel SDS gels stained by the Coomassie brilliant blue R-250 were cut and the membrane proteins interacting with H5N1 virus were identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Finnigan LTQ linear ion trap mass spectrometer; Genome Institute, BIOTEC, Thailand). Data was searched against the SwissPort database for protein identification using the Mascot software (http://wwwmatrixscience.com) as described elsewhere.

Western blot analysis

Mock-infected and H5N1 (MOI of 1 at 37°C for 1 h) infected SH-SY5Y cells were harvested at 24 h p.i. by rinsing once with PBS before adding trypsin. The cell

pellets were collected by centrifugation at 300 x g for 5 min and re-suspended in ice-cold RIPA buffer (150 mM of NaCl, 50 mM of Tris-HCl (pH 8), 0.5% DOC, 1 mM of EDTA, 0.2% Triton X-100, 1% SDS) and protease inhibitor cocktail (Thermo Scientific Pierce, IL). The pellets were incubated for 10 min on ice and briefly sonicated to ensure complete lysis, followed by centrifugation at 14,000 x g for 10 min at 4°C to collect protein in the supernatant. Protein concentration was quantified by BCA assay (Thermo Scientific Pierce, IL). Whole cell proteins were separated by SDS-PAGE, and then transferred to PVDF membranes by electroblotting. The membranes were subsequently blocked with 5% skim milk in TBS for 1 h at room temperature followed by incubation with either a mouse monoclonal anti-RACK1 antibody (1:500; Santa Cruz, CA) or a goat polyclonal anti-PHB 1 antibody (1:200; Santa Cruz, CA) diluted in 5% skim milk in TBS overnight at 4°C. The blots were washed three times with TBS and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After washing three times in TBS with 0.1% Tween 20, the signal was generated by ECL-Plus Western Blotting Substrate (Thermo Scientific Pierce, IL) and exposed to CL-XPosure film (Thermo Scientific Pierce, IL).

Antibody mediated inhibition of infection assay

SH-SY5Y cells were grown on the cover slips in 24-well plates and treated with a mouse monoclonal anti-RACK1 antibody or a goat polyclonal anti-PHB1 antibody (Santa Cruz, CA) at 37 °C for 1 h prior to infection. After incubation, the cells were infected with H5N1 virus at MOI of 1 at 37°C for 1 h. The cells were washed with FBS-free medium 3 times to remove uninternalized virus and fresh medium with 10% FBS was

added. Subsequently the role of RACK1 or PHB1 in mediating H5N1 virus internalization was identified by indirect immunofluoresence colocalization with a mouse monoclonal antibody directed against H5N1 HA protein as described above.

Statistical analysis

Data were presented as mean ± S.E.M. from at least three independent experiments performed in triplicate in order to confirm the reproducibility of the results. Statistical analysis of quantitative data of significance was performed by paired *t* test with a probability value of $p < 0.05$ considered to be statistically significant.

Competing interests

Authors declare no competing interests.

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Tables

Table 1. CPE scores of H5N1-infected SH-SY5Y cells at various time points.

CPE Scoring	**0**	**+/-**	**1+**	**2+**	**3+**	**4+**
Mock infected cells in all conditions	√					
6 h post-infection	√					
12 h post-infection		√				
24 h post-infection			√			
48 h post-infection					√	
72 h post-infection						√

0, no CPE; +/-, enlargement of some cells in monolayer; 1+, 1-25% CPE; 2+, 25-50% CPE; 3+, 50-75% CPE; 4+, 75-100% CPE, as described previously [56].

Table 2. Mascot search results of protein bands cut from electrophoretic gels analysed by LC-MS/MS. These bands correspond to the positions of H5N1 binding proteins obtained from virus overlay protein binding assay (Fig. 4).

Band (kDa)	Accession no.	Protein name	Location[a]	Function/structure in which protein is involved[b]	Mol mass (Da)	% Coverage
1 (~36-38)	NP_002128.1	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	N, C	pre-mRNA processing, mRNA metabolism and transport	36005.7	60.70
	NP_060979.2	Leucine rich repeat containing 59	ER, Mi, M	Protein-protein interactions and have different functions and cellular locations	34930.2	37.13
2 (~34-35)	sp|O42249.1	Guanine nucleotide-binding protein subunit beta-2-like 1 (Receptor of activated protein kinase C) (RACK1)*	C, M	Intracellular receptor to anchor the activated PKC to the cytoskeleton, adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly	35076.5	84.86
	sp|P40926	MDHM_HUMAN Malate dehydrogenase, mitochondrial precursor	Mi	L-malate dehydrogenase activity	35531.1	56.21
	sp|Q16836	HCDH_HUMAN Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor (Short chain 3-hydroxyacyl-CoA)	Mi	Plays an essential role in the mitochondrial beta-oxidation of short chain fatty acids	34277.2	10.51
	NP_009204.1	Prohibitin 2	Mi, C, N	A mediator of transcriptional repression by nuclear hormone receptors via recruitment of histone deacetylases	33296.1	10.51
3 (~28-30)	NP_002625.1	Prohibitin 1*	Mi, N, M	Inhibits DNA synthesis, It has a role in regulating proliferation, as yet it is unclear if the protein or the mRNA exhibits this effect	29803.9	70.96
	NP_004729.1	VAMP-associated protein B/C	G, ER, M	Vesicle trafficking	27228.2	30.40
	sp|Q9Y5M8	SRPRB_HUMAN Signal recognition particle receptor subunit beta (SR-beta) (Protein APMCF1)	C, ER, M	Mediate the membrane association of SR-alpha	29702.0	29.90
	sp|P05141	ADT2_HUMAN ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2)	Mi, M	Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane	32895.0	22.10

[a] Locations of the protein are indicated as follows: C, cytoplasmic (including intracellular vesicles); M, plasma membrane or integral to plasma membrane; N, nuclear; ER, endoplasmic reticulum; G, Golgi apparatus; Mi, mitochondrion

[b] Data from NCBI Protein Databases.

*The identified candidate protein results were selected based on the following criteria: 1) each identified protein had a *Homo sapiens* species; 2) their molecular mass covered a relative protein molecular weight range of each cutting gel, when compared with the protein pre-stained marker; 3) these identified proteins had high % coverage of the peptide match and 4) the subcellular localization of identified proteins, particularly cell surface proteins.

Figure Legends

Fig. 1. H5N1 infection induces CPE in neuronal SH-SY5Y cells. Cells were infected with H5N1 strain A/Thailand/NK165/05 virus at MOI of 1. Representative photomicrographs showed progressive morphological changes at 6, 12, 24, 48 and 72 h p.i. compared with that of mock-infected cells. Original photomicrographs were taken at 200x.

Fig. 2. The viability of SH-SY5Y cells and virus production following H5N1 virus infection. Monolayers of SH-SY5Y cells were infected with H5N1 at MOI of 1 and cell-free supernatants were collected for HA assay. The viability of infected and mock-infected cells was determined by trypan blue dye exclusion method. All data are presented as mean ± S.E.M. from three independent experiments performed in triplicate. A solid line indicates the viability of infected cells. A dashed line indicates viral titer in the media.

Fig. 3. Expression of H5N1 specific HA in infected SH-SY5Y cells. Cells on glass cover slips were infected with H5N1 virus at MOI of 1. The infected and mock-infected cells were examined for the localization of H5N1 specific HA using a mouse monoclonal anti-H5N1 HA and goat anti mouse IgG (H+L) AlexaFlour 594-conjugated antibody (red). The nuclei were counterstained with DAPI (blue). Original photomicrographs were taken at 400x.

Fig. 4. Virus overlay protein binding assay shows H5N1 binding to membrane proteins from SH-SY5Y cells. Membrane proteins were extracted, separated on 10% SDS-PAGE and stained with Coomasie brilliant blue R-250 (A). Duplicate gels were transferred to PVDF membranes, before incubating with H5N1 virus at 4°C (C) or at room temperature (D). Membranes incubated without H5N1 at 4°C or at room temperature were served as negative controls (B). Virus binding protein bands were visualized using a mouse monoclonal anti-H5N1 specific HA. M represents the molecular weight protein markers.

Fig. 5. Immunofluorescence staining of RACK1 and PHB1 proteins in mock-infected SH-SY5Ycells. An arrows indicates strongger fluorescent signals of RACK1in the neurites compared with that of PHB1. Original photomicrographs were taken at 600x.

Fig. 6. H5N1 infection decreases expression of RACK1in SH-SY5Y cells. Fluorescence signals of RACK1 (green) and HA (red) in H5N1 infected cells at 12 h p.i. were compared with that of 24 h p.i.. The nuclei were counterstained with DAPI (blue). Original photomicrographs were taken at 400x.

Fig. 7. H5N1 infection decreases expression of PHB1 in SH-SY5Y cells. Fluorescence signals of PHB1 (green) and HA (red) in H5N1 infected cells at 12 h p.i. were compared with that of 24 h p.i.. The nuclei were counterstained with DAPI (blue). Original photomicrographs were taken at 400x.

Fig. 8. Western blot (A) and densitometric analysis (B) of RACK1 and PHB1 expression in mock and H5N1-infected SH-SY5Y cells at 24 h p.i.. The values are mean ± SEM of three replicates.*, $p < 0.05$.

Fig. 9. Pre-treatment with anti-RACK1 antibody reduces the internalization of H5N1 into SH-SY5Y cells. The fluorescence signals (red) of H5N1-specific HA protein in antibody pre-treatment groups were examined under a fluorescence microscope at 24 h p.i. compared with that of mock and H5N1 infected cells in the absence of antibody pre-treatment. The nuclei were counterstained with DAPI (blue). Original photomicrographs were taken at 400x.

Fig. 10. Pre-treatment with anti-PHB1 antibody reduces the internalization of H5N1 into SH-SY5Y cells. The fluorescence signals (red) of H5N1-specific HA protein in antibody pre-treatment groups were examined under a fluorescence microscope at 24 h p.i. compared with that of mock and H5N1 infected cells in the absence of antibody pre-treatment. The nuclei were counterstained with DAPI (blue). Original photomicrographs were taken at 400x.