

Definition of the cellular interactome of the highly pathogenic avian influenza H5N1 virus: identification of human cellular regulators of viral entry, assembly, and egress

F Kien *, HL Ma, R Bruzzone, LLM Poon, B Nal †

KEY MESSAGES

1. Novel H5N1-human host protein-protein interactions were identified using a genome-wide yeast two-hybrid screen.
2. Annexin A6 interacts with the cytoplasmic tail of influenza A M2 protein.
3. Annexin A6 negatively regulates influenza A infection.
4. Annexin A6 impairs influenza A virus budding and release.

Hong Kong Med J 2016;22(Suppl 4):S10-2

RFCID project number: 09080892

^{1,2,3} F Kien, ^{1,4} HL Ma, ^{1,5} R Bruzzone, ³ LLM Poon, ^{1,6} B Nal

¹ HKU-Pasteur Research Pole, Hong Kong

² Ksilink, French-German Advanced Translational Centre, Strasbourg, France

³ Centre of Influenza Research, School of Public Health, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China

⁴ Emory University Vaccine Research Center, Atlanta, Georgia, USA

⁵ Department of Cell Biology and Infection, Institut Pasteur, Paris, France

⁶ Brunel University London, Division of Biosciences, College of Health and Life Sciences, Uxbridge, United Kingdom

* Principal applicant: francois.kien@ksilink.com

† Corresponding author: beatrice.nal-rogier@brunel.ac.uk

Introduction

Influenza A virus causes acute infection of the respiratory tract in humans. H1N1 and H3N2 viral strains are responsible for seasonal epidemics. Symptoms include fever, headache, chills, myalgia, dry cough and sore throat. Influenza is usually a self-limiting disease with recovery within a week in healthy individuals. Nonetheless, risk of serious illness affects young children, the elderly and patients with underlying health conditions. Due to antigenic variation of the virus, vaccination only enables incomplete protection. Circulation of influenza viruses in the animal reservoir causes additional threat. Because of rapid adaptation of viruses to environmental pressures, drugs directed against viral components are likely to induce selection of drug-resistant strains.¹ Our strategy is to characterise cellular pathways used by viruses in the human host and identify potential novel cellular targets for drug development.

The spread of H5N1 avian influenza in South East Asia in the mid 2000s, its extreme virulence in poultry and the threat of a deadly pandemic in humans prompted us to work on this virus. We used a yeast-two hybrid approach to screen the entire human genome for factors that interact with H5N1 viral structural proteins. We had previously successfully used this technology for virus-host interaction studies on the severe acute respiratory syndrome coronavirus.^{2,3} Six highly relevant

interactions were identified for H5, M1, and M2. Pilot biochemical confirmation of interactions and gene silencing studies prompted us to primarily focus on human annexin A6 (AnxA6). The yeast-two hybrid assay had revealed that AnxA6 binds the cytosolic tail of the viral M2 proton channel.

M2 is an essential protein for the virus. It has a proton channel activity with key roles during virus entry and uncoating, transport of viral haemagglutinin to the cell surface and assembly, budding and release of progeny virions at the surface of infected cells.⁴ There, M2 is known to accumulate at the edges of cholesterol-enriched viral budding platforms and induce a negative membrane curvature at the neck of the bud, responsible for scission and release of viral particles.⁵

AnxA6 belongs to the annexin family of human proteins. It plays important roles in membrane trafficking and cell signalling in mammalian cells. Its overexpression has been shown to cause accumulation of cholesterol in late endosomes and alter transport and recycling of cellular cargos.⁶

In this study we confirmed interaction between influenza M2 and human AnxA6 and demonstrated that AnxA6 restricts release of progeny viruses from infected cells.

Methods

This study was conducted from October 2009 to September 2011. To confirm the interaction

identified in yeast (Hybrigenics, Paris, France), both biochemical and microscopy assays were performed. To determine whether AnxA6 is essential for infection both AnxA6 depletion and AnxA6 overexpression studies were analysed. Steps of virus life cycle regulated by AnxA6 were identified using a comprehensive and systematic dissection of the viral life cycle. Impact of AnxA6 expression modulation on infection was studied using the previously mentioned AnxA6 depletion and overexpression protocols together with virus life cycle step-specific assays such as transmission electron microscopy to study virus budding and morphology.

Results

We hypothesised that cellular factors interacting with influenza M2 54-residue cytoplasmic tail (CT) could either help or restrict processes involving M2. We identified a novel interaction between the M2 CT of a highly pathogenic avian influenza strain (H5N1) and human AnxA6 in yeast. This interaction was confirmed *in vitro* using GST pull-down assay with M2 CT of the laboratory adapted influenza strain A/WSN/33 (H1N1). We found that a GST-M2 CT fusion protein interacts and pulls down AnxA6 from total human cell lysate, demonstrating that this interaction is not an artefact observed in the yeast system and the interaction is shared among different viral subtypes. Physical interaction was further confirmed in human virus-infected cells using a reciprocal co-immunoprecipitation assay of recombinant myc-AnxA6 and viral M2 protein. Co-localisation studies using confocal fluorescent microscopy showed a partial co-localisation of viral M2 and recombinant GFP-AnxA6 in distinct puncta-like areas at or close to the plasma membrane of virus-infected human cells, suggesting that this interaction occurs close to the virus assembly and budding site.

To define the role and impact of M2-AnxA6 interaction on the viral replication cycle, we used two complementary approaches and studied the effect of AnxA6 depletion or overexpression on the production of viral particles. AnxA6 depletion significantly increased virus production, while overexpression reduced the titre of virus progeny, suggesting a negative regulatory role for AnxA6 during influenza A virus infection. This was observed with different virus strains and subtypes of human seasonal influenza viruses, A/WSN/33 (H1N1), A/HK/1/68 (H3N2), and A/HK/54/98 (H1N1), suggesting a common hijacked cellular pathway by influenza A viruses.

Using AnxA6 depletion or overexpression protocols combined with virus life cycle step-specific assay, we showed that AnxA6 does not affect virus binding and entry, vRNA replication and viral mRNA transcription, viral polymerase complex

activity or NP nuclear export. On the contrary, we demonstrated that AnxA6 negatively modulates influenza virus infection by affecting virus budding as indicated by a drop in progeny virus PFU titre in supernatants and an increased number of virions at the cell surface of cells overexpressing AnxA6, which exhibit an elongated rather than spherical shape. This was observed by a semi-quantitative analysis of virus budding and morphology by transmission electron microscopy. These results demonstrate that AnxA6 overexpression results in defects in virus budding and release of infectious viral particles.

Discussion

This work identified AnxA6 as a novel cellular regulator that impairs virus budding and release stages of the lifecycle of influenza A virus. We proposed that AnxA6 acts as a restricting factor of influenza virus budding either by interacting with M2, which is able to mediate membrane scission,⁵ or by modulating cellular components such as cortical actin and cholesterol⁷ involved in viral budding, or both. Use of a recombinant mutant WSN influenza virus lacking M2 suggested that regulation of influenza virus production by AnxA6 is dependent on M2 and its interaction with AnxA6; this work has been published.⁸ Since then inhibition of influenza A virus production by AnxA6 has been further confirmed, and this effect linked to deregulation of cholesterol transport by AnxA6.⁹

Our study has enhanced the understanding of cellular factors requirements for influenza A virus assembly and budding and underscores the unique budding strategy implemented by this virus. Nevertheless, the detailed molecular mechanism underlying this AnxA6-mediated negative modulation of influenza virus budding awaits further investigation. It will be important to determine the functional correlation between M2-AnxA6 interaction and the defective viral budding phenotype. This research will help to delineate new strategies for the rational design of antiviral molecules.

Acknowledgements

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#09080892), University Grants Committee Area of Excellence Scheme (AoE/M-12/-06), French Ministry of Health, RESPARI Pasteur network, and the Li Ka Shing Foundation. We thank Dr Carlos Enrich (Departament de Biologia Celular, Facultat de Medicina, Universitat de Barcelona, Spain) for expert advice and helpful discussions and all the staff from HKU-Pasteur Research Pole for stimulating discussions. We acknowledge the Core Imaging

Facility and Electron Microscopy Unit of the Faculty of Medicine of the University of Hong Kong.

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