Acta virologica **61**: 56 – 65, 2017 doi:10.4149/av_2017_01_56

Analysis of A549 cell proteome alteration in response to recombinant influenza A virus nucleoprotein and its interaction with cellular proteins, a preliminary study

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Received April 19, 2016; revised July 29, 2016; accepted January 18, 2017

Summary. – Influenza A virus undergoes frequent changes of antigenicity and contributes to seasonal epidemics or unpredictable pandemics. Nucleoprotein, encoded by gene segment 5, is an internal protein of the virus and is conserved among strains of different host origins. In the current study, we analyzed the differentially expressed proteins in A549 cells transiently transfected with the recombinant nucleoprotein of influenza A virus by 2D gel electrophoresis. The resolved protein spots on gel were identified by MALDI-TOF/Mass spectrometry analysis. The majority of the host proteins detected to be differentially abundant in recombinant nucleoprotein-expressing cells as compared to vector-transfected cells are the proteins of metabolic pathways, glycolytic enzymes, molecular chaperones and cytoskeletal proteins. We further demonstrated the interaction of virus nucleoprotein with some of the identified host cellular proteins. *In vitro* binding assay carried out using the purified recombinant nucleoprotein (pET29a+NP-His) and A549 cell lysate confirmed the interaction between nucleoprotein and host proteins, such as alpha enolase 1, pyruvate kinase and β-actin. The preliminary data of our study provides the information on virus nucleoprotein interaction with proteins involved in glycolysis. However, studies are ongoing to understand the significance of these interactions in modulating the host factors during virus replication.

Keywords: influenza A virus; A549 cells; nucleoprotein; alpha enolase 1; pyruvate kinase

Introduction

Influenza A virus, a member of Orthomyxoviridae family, exhibits many well-exemplified mechanisms to evade the innate and adaptive immune responses (Fernandez-Sesma *et al.*, 2006; van de Sandt *et al.,* 2012). It is a highly mutable virus and changes its antigenic properties frequently, contributing to seasonal epidemics and unpredictable pandemics. Incorporation of point mutations in the viral genome by error-prone RNA-dependent RNA polymerase, especially in genes encoding surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and the reassortment of genomic RNA segments of strains originating from different hosts lead to the emergence of a novel strain (Desselberger *et al.*, 1978). Therefore, the available vaccines and drugs fail to provide the immunity at a global scale. Newly emerged influenza virus strains account for significant mortality and morbidity worldwide, making it a public health threat. Encountering another influenza pandemic is inevitable due to its segmented genetic makeup; however, it is hard to predict the time, host origin and the severity of infection. These uncertainties prompt for the identification of new molecular targets both of virus-encoded or cellular factors to develop effective anti-influenza drugs active across species. Influenza A viral genome comprises eight negative sense RNA segments that together encode for 17 proteins, including the recently discovered 7 proteins (Reviewed by Vasin *et al.*,

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Abbreviations: MALDI-TOF = matrix-assisted laser desorption ionization-time of flight; MS = mass spectrometry; NP = nucleoprotein

2014). Nucleoprotein (NP), encoded by RNA segment 5, is a 56 kDa multifunctional protein, whose primary function is to encapsidate the segmented viral genome. Together with the viral RNA and the three RNA polymerase subunits (PA, PB1 and PB2), NP forms the viral ribonucleoprotein complexes (vRNPs) (Pons *et al.*, 1969). NP shuttles between the nucleus and the cytoplasm, interacting during virus replication with a large number of cellular proteins (Portela *et al.*, 2002) to regulate multiple functions. It facilitates viral RNA transcription, replication and genome packaging.

Currently available antiviral drugs, including drugs under development, are designed largely to target the viral surface proteins, neuraminidase (NA) and ion channel (M2) (Davies *et al.*, 1964; Moscona, 2005; Samson *et al.*, 2013). Two major classes of FDA-approved anti-influenza viral drugs are adamantine-based ion channel (M2) blockers (Amantadine and Rimantadine) and NA inhibitors (Oseltamivir, Zanamivir, and Peramivir). However, none of the inhibitors was shown to provide complete protection against the newly emerged viral strains. Viral envelope proteins, HA and NA, are under pressure of changing to escape the host immune response as they are the first proteins exposed to the neutralizing antibodies. In addition, virus develops various strategies to overcome the therapeutic interventions to establish a successful infection in the host. On the contrary, NP, one of the internal proteins of influenza virus, is highly conserved across the strains originating from different hosts (Shu *et al.*, 1993; Morens *et al.*, 2013). It plays a critical role in genome stabilization as well. A recent study by Kukol and Hughes reported a high degree of conservation among NP sequences using more than 4,400 sequences on host-based characterization (Kukol and Hughes, 2014). Due to conserved amino acid sequences of NP among different strains, it can be a potential target to develop cross-strain protective anti-influenza viral drugs.

In the current study, we sought to analyze the differential expression of host proteins induced by influenza A viral NP. A549 cells were transiently transfected with the recombinant influenza A virus NP (pcDNA3.1 + NP) or empty vector. Protein lysates recovered from transfected cells were subjected to 2D gel electrophoresis. The resolved proteins on gels were excised and identified by matrix-assisted laser desorption ionization-time of flight/Mass Spectrometry (MALDI-TOF/MS) analysis. Molecular chaperones, proteins involved in metabolic pathways, glycolytic enzymes and cytoskeletal elements were identified to be differentially expressed in response to recombinant viral NP. Preliminary experiments were carried out to investigate the interaction of NP with few identified host proteins by *in vitro* binding assay. For the host and viral protein interaction studies, recombinant NP was generated in pET29a+ vector, expressed and purified. Our experiments demonstrated the interaction of NP with host proteins alpha enolase 1, pyruvate kinase and β-actin. All the three proteins were reported to be incorporated into mature influenza virions (Shaw *et al.*, 2010). Nonetheless, it is the first evidence that influenza A virus NP interacts with glycolytic enzymes alpha enolase and pyruvate kinase.

Materials and Methods

Cells and virus. A549 cell line was obtained from National Centre for Cell Science, Pune, India for propagation of influenza A virus. The cell line was maintained in Ham's Nutrient Mixture F12 supplemented with 10% FBS and Penicillin-Streptomycin solution. Cells were maintained at 37°C in a 5% CO_2 incubator. Influenza A virus, H1N1 seasonal strain isolated from a clinical sample was propagated in A549 cells in the laboratory and used in this study.

Generation of recombinant viral nucleoprotein. Total RNA was isolated from virus-infected A549 cells and a fraction of RNA was reverse transcribed using MMuLV reverse transcriptase and random hexamer (pdn6). Complementary DNA was amplified using NP gene-specific primers (FP: 5′-ATATGAATTCAC CTGGCGTCCCAAGGCAC-3', RP: 5'-ATATCTCGAGTTAATTG TCGTACTCCTCTGC-3'). Amplified product was purified and cloned into eukaryotic expression vector pCDNA3.1+ driven by CMV promoter between *Eco*RI and *Xho*I restriction sites. For *in vitro* binding studies, NP-encoding viral gene was cloned into bacterial expression vector pET29a+ between the same restriction sites. However, the stop codon was removed from the reverse primer to generate the amplicon to be cloned into pET vector. Recombinant NP constructs pCDNA3.1+NP and pET29a+NP-His were confirmed by sequencing.

Transfection and protein recovery. A549 cells were transiently transfected with the recombinant viral NP and mock-transfected with the empty vector pCDNA3.1+ with Lipofectamine LTX reagent (Invitrogen, Life sciences) using the standard protocol. For proteomic analysis, at 48 h post transfection, cells were washed with 1xTBS (pH 7.6) and incubated on ice by adding 100 µl of 2D extraction buffer (7 mol/l Urea, 2 mol/l Thiourea, 2% CHAPS, 20 mmol/l Tris-HCl (pH 7.6), 5 mmol/l DTT, 1 mmol/l PMSF, 1 mmol/l Sodium orthovanadate, 20 units/ml DNases, 0.25 mg/ml RNases, 0.2% Bio-Lyte® 3/10 and 1xProtease inhibitor cocktail) for 30 min. Whole cell lysates were collected and protein concentration was determined.

2D gel electrophoresis. Protein lysate of 169 µg was mixed with rehydration buffer (8 mol/l urea, 2% CHAPS, 50 mmol/l DTT, 0.2% Bio-Lyte® 3/10 ampholyte, 0.001% Bromophenol Blue) to a final volume of 200 µl. Lysates were loaded onto IPG strips of 7 cm of a linear broad pH range of 3–10 and subjected to isoelectric focusing (IEF). Following IEF, IPG strips were reduced with equilibration buffer I (375 mmol/l Tris-HCl (pH 8.8), 6 mol/l urea, 2% SDS) containing 2% DTT for 15 min followed by alkylation with equilibration buffer II containing 2.5% iodoacetamide for 15 min. IPG strips with separated proteins were subjected to second dimension SDS-PAGE and stained with Gel Code safe blue (Thermo Scientific

Co, USA). Selected spots were excised using 2D gel spot cutter (Bio-Rad, USA) and subjected to tryptic digestion.

MALDI-TOF/MS analysis. Tryptic digested peptides were loaded onto 384 OptiTOF- MALDI plate (Applied Biosystems) by mixing 0.5 µl of the sample with 0.5 µl of 1% α-Cyano-4-hydroxycinnamic acid (CHA). Spots were air dried and plates were loaded onto proteome analyser SCIEX TOF/TOF 5800 (Applied Biosystems). The peptides identified by MALDI-TOF/MS were further subjected to MS/MS fragmentation. TOF/TOF TM series Explorer TM Software was used for acquisition and processing of MS/MS spectra. The identified peptides were searched against MASCOT database using Protein Pilot software (AB SCIEX) connected to its server.

Expression and purification of the recombinant viral nucleoprotein (pET29a+NP-His). Recombinant plasmid of viral NP with correct DNA sequence was transformed into BL21 (DE3) pLysS cells and spread on LB plates containing 100 µg/ml kanamycin. Plates were incubated overnight at 37°C. Next day, a single colony was inoculated in 2 ml LB with antibiotics and incubated overnight at 37°C. One percent of the primary culture was used to inoculate 10 ml LB with antibiotic and allowed to grow till the optical density at 600 nm reached 0.5 to 0.6. Upon reaching the desired OD, cells harboring the recombinant construct were induced with 1.0 mol/l isopropyl-beta-D-thiogalactopyranoside (IPTG) at 32°C. Following overnight induction, cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 200 µl of lysis buffer (1 mg/ml lysozyme, 2 mol/l DTT, 1 mmol/l PMSF, 1 U/ml of DNase prepared in 1xPBS) followed by sonication until a clear and non-viscous solution was obtained. The cell lysate was clarified by centrifugation at 14,000 rpm for 20 min at 4°C. The recombinant protein was purified using metal chelate affinity chromatography under native conditions. For purification of the protein, bacterial cell culture harboring recombinant plasmid was scaled up to 100 ml and cell lysate was prepared as described above. Prior to use, Ni-NTA resin was washed thrice with 1x PBS (pH 7.4). Fifty µl of Ni-NTA resin was incubated with 150 µl of cell lysate made up to 500 µl with binding buffer (2 mmol/l DTT, 1 mmol/l PMSF, 20 mmol/l imidazole in 1x PBS) for 6 h at 4°C. Ni-NTA resin with bound recombinant viral NP was washed thrice with binding buffer. Bound protein was eluted with 50 µl of elution buffer (50 mmol/l sodium phosphate buffer (pH 8.0), 300 mmol/l NaCl and imidazole). Elution was repeated with elution buffer containing increasing concentrations of imidazole (250 mmol/l and 400 mmol/l). Eluted protein was stored at -80°C for further use.

*In vitro binding assay***.** To check the interaction of virus NP with some proteins identified by proteomic analysis, *in vitro* binding assay was carried out using recombinant NP. In brief, BL21 cells were transformed with recombinant NP (pET29a+NP-His) and induced with 1.0 mmol/l IPTG overnight at 32°C. Bacterial cell lysates were prepared and incubated with Ni-NTA beads for 6 h at 4°C. Ni-NTA beads with bound His-tagged recombinant NP were washed with 1xPBS. Then, the beads were incubated overnight with 1 mg of A549 cell lysate. To confirm the specific interaction between viral NP and host proteins, an unrelated purified bacterial protein of 28 kDa was used as a nonspecific control. Next day, Ni-NTA beads were washed, the bound protein complexes were eluted and subjected to SDS-PAGE followed by western blot analysis using antibodies to

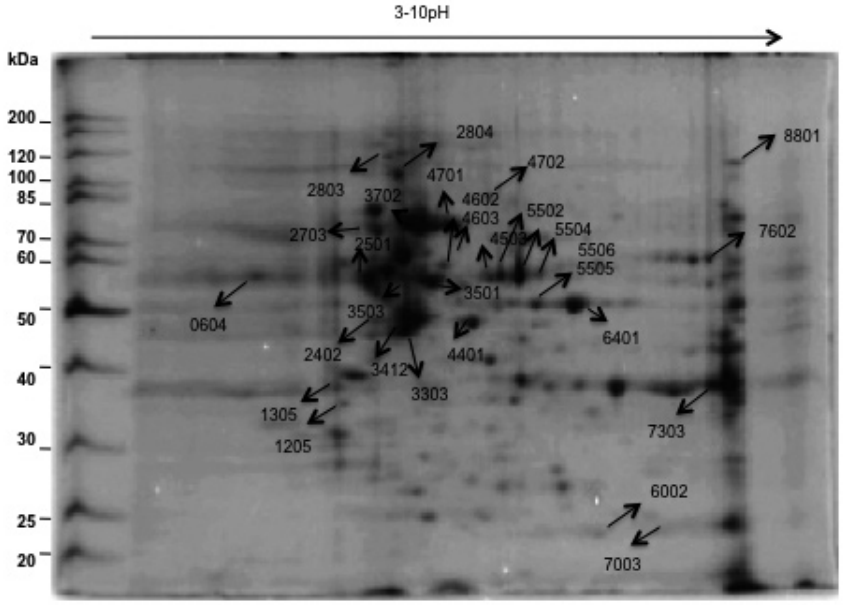

Fig. 1

Representative 2D gel with resolved protein spots of recombinant viral NP-expressing cells

A549 cells were transiently transfected with recombinant viral NP and mock-transfected with empty vector pcDNA3.1(+). At 48 h post transfection, cell lysates were prepared, subjected to IEF using 3-10 pH range IPG strips followed by 2D gel electrophoresis. Gels were stained with Gel code blue safe. PAGE gel with MS analyzed spots marked and numbered.

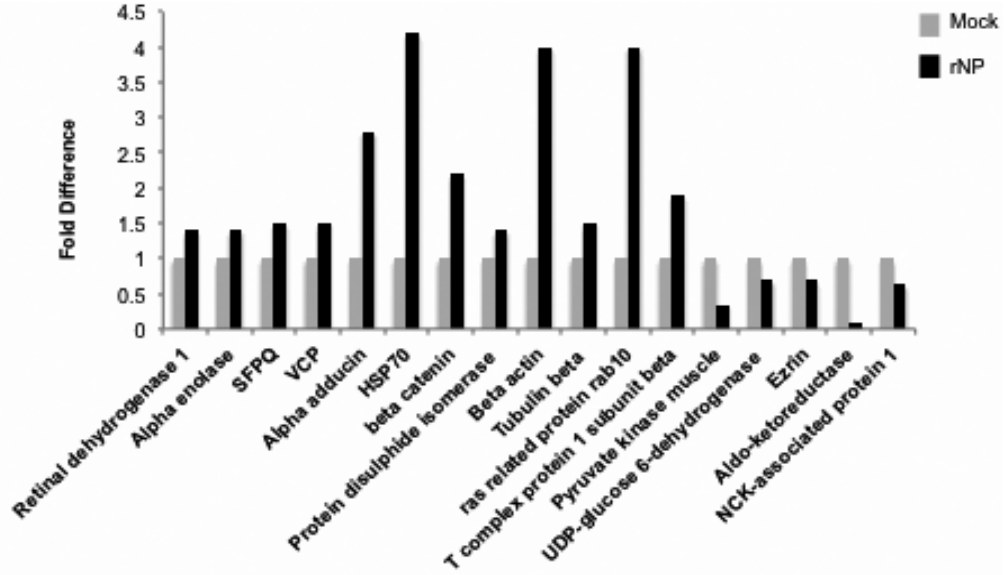

Fig. 2

Graphical representation of MALDI-TOF/MS data showing differential expression of proteins identified in recombinant viral nucleoprotein *vs.* mock transfected cells

alpha enolase 1, pyruvate kinase and β-actin. BL21 bacterial cells lysate with plain Ni-NTA beads without the recombinant protein was also included as a control.

Results

Analysis of differentially expressed A549 cell proteome

Virus NP-encoding gene segment was successfully cloned into eukaryotic expression vector pcDNA3.1 (+) for proteomic analysis and into pET29a+ vector for *in vitro* binding assay. Recombinant protein expression in A549 cells was confirmed by IFA staining using anti-influenza A virus NP antibody (Merck Millipore, Chemicon). To identify the differentially regulated host proteins induced by influenza A viral NP, cells were transfected with the recombinant NP construct (pCDNA3.1+NP) or mock-transfected with empty vector. Equal amounts of protein lysates recovered from transfected cells were analyzed on 2D gels. Representative 2D gel showing resolved protein spots marked is shown (Fig. 1). A total of 32 spots with a significant difference in fold intensity between recombinant protein and mock transfected cell proteome as determined by PD Quest software were selected for analysis. Protein spots were excised from gels and identified by MALDI-TOF/MS analysis. Protein hits were obtained for all the spots. However, only 20 spots had significant Mascot score, the statistical score associated with the match. Expected molecular weight and pI of each protein spot was matched with the obtained results. The fold difference of each spot between mock and recombinant NP-expressing cells was plotted as column graph (Fig. 2). The majority of proteins identified to be differentially abundant are the components of metabolic pathways, glycolysis, molecular chaperones, cytoskeletal remodelling elements and actin filament binding proteins. Mass of each protein detected with Mascot score and the number of peptides matched are shown (Table 1, 2). Twelve proteins were found to be up-regulated with significant score. Retinal dehydrogenase, alpha enolase, alpha adducin isoform c, HSP70, TCP1, β-actin etc., are the proteins up-regulated in response to NP (Table 1); while pyruvate kinase, ezrin, aldoketoreductase, GFAP are out of eight proteins noted to be down-regulated as compared to mock transfected cell proteome (Table 2).

Expression and purification of His-tagged recombinant viral nucleoprotein

To investigate the viral NP interaction with some of the differentially expressed host proteins, recombinant viral protein was purified and in *vitro* binding assay was carried out using A549 cell lysates. The bacterial BL21 cells containing recombinant viral NP construct (pET29a+ NP-His) were grown and the expression was induced with 1.0 mmol/l IPTG. The effect of induction temperature on the level of recombinant protein expression was optimized by incubating the cultures at temperature ranging from 28 to 32°C and allowed to grow overnight. Of total protein content,

Table 1. MALDI-TOF/MS data: Up-regulated proteins in A549 cells transfected with recombinant viral nucleoprotein

Spot No.	Protein identified	Accession ID	Molecular weight	pI	Mascot score	No. of peptides matched
5504	Retinal dehydrogenase 1	gi|2136117	55454	6.3	470	36
6401	Alpha enolase isoform 1	gi|4503571	47481	7.01	550	38
8801	Splicing factor proline/glutamine-rich (SFPQ)	gi|119627826	66420	9.45	155	18
2804	Transitional Endoplasmic Reticulum ATPase/ valosin containing protein	gi|6005942	89950	5.14	429	48
2803	Alpha adducin isoform c	gi|29826323	70397	5.6	24	20
3702	HSP70	gi|62897129	70294	5.48	90	16
2703	Unnamed protein product (highly similar to beta catenin)	gi|194389640	78097	5.53	30	9
4602	Protein disulphide isomerase family A, member 3 isoform CRA_a	gi|119597640	54454	5.98	237	19
3412	Beta actin variant/ACTB protein	gi|15277503	42080	5.29	222	16
2501	Tubulin beta	gi|57209813	48135	4.78	308	31
7003	Hypothetical protein (ras related protein rab10)	gi|12052826	22683	8.58	22	11
4503	T complex protein 1 subunit beta	gi|5453603	57766	6.01	156	20

high-level expression of the desired protein of 56 kDa was obtained from cells cultured overnight at 32°C (Fig. 3a). Cells were disrupted by sonication followed by centrifugation to separate the soluble fraction from the insoluble one. The recombinant protein content was comparatively equal in both the fractions – pellet and supernatant. Localization of the recombinant protein in both fractions was analyzed by SDS-PAGE (Fig. 3b). Small-scale protein purification was carried out with supernatants by affinity chromatography. For protein purification, initially 100 ml culture was taken. Binding of the recombinant protein to Ni-NTA resin was optimized using different concentrations of imidazole (10 mmol/l, 20 mmol/l, and 30 mmol/l). Also, elution of bound protein was optimized with elution buffer containing varying concentration of imidazole ranging from 200 mmol/l to 400 mmol/l. Optimal yield of ~56 kDa purified NP was obtained with the binding buffer and elution buffer containing 20 mmol/l and 250 mmol/l imidazole, respectively (Fig. 3c). Purified NP identity was confirmed by western blot analysis using anti-His (Fig. 3d) and anti-influenza viral NP antibodies (Fig. 3e).

Nucleoprotein interaction with host proteins identified by proteomic analysis

Interestingly, alpha enolase 1, pyruvate kinase, β-actin and aldoketoreductase that were identified to be differentially abundant in cells expressing recombinant viral NP in our study had been reported to be incorporated into mature influenza virion by proteomic analysis (Shaw *et al.*, 2010).

Table 2. MALDI-TOF/MS data: Down-regulated proteins in A549 cells expressing recombinant viral nucleoprotein

Spot No.	Protein identified	Accession ID	Molecular weight	pI	Mascot score	No. of peptides matched
7602	Pyruvate Kinase muscle	gi|31416989	58512	7.96	333	42
6604	UDP-glucose 6-dehydrogenase isoform1	gi|296040443	55674	6.73	130	18
0604	Tubulin alpha 1b chain isoform 2	gi|18204869	46797	4.94	71	8
4702	Ezrin	gi|28948869	69484	5.94	116	29
4701	Unnamed protein product (highly similar to homo sapiens albumin)	gi|158258947	71218	5.92	44	12
7303	Aldo-ketoreductase family 1 member c3	gi|24497583	37229	8.06	170	23
4401	NCK-associated protein 1	gi|119631362	43731	6.18	26	7
2402	Glial fibrillary acidic protein	gi|119571952	49776	5.42	39	10

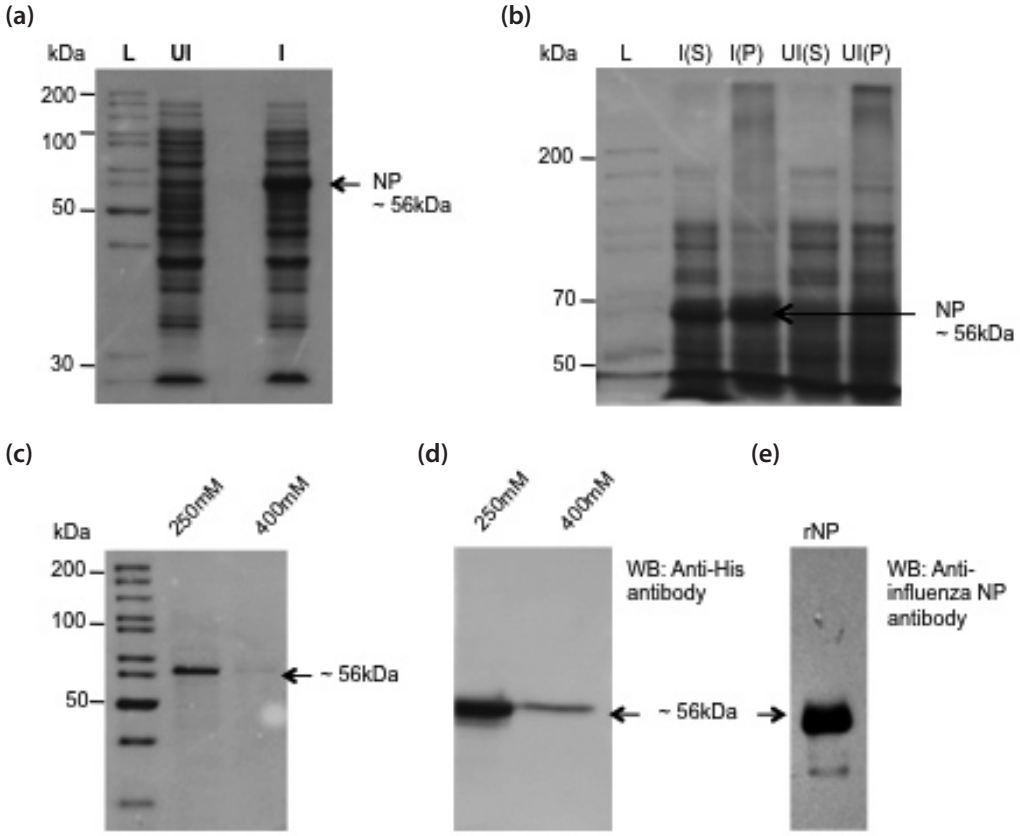

Fig. 3

Recombinant viral nucleoprotein expression and purification

NP-encoding viral gene segment was cloned into a pET29a+ vector. BL21 bacterial cells were transformed with recombinant NP (rNP) of influenza virus; NP was expressed, purified by affinity chromatography and eluted **(a)** SDS-PAGE analysis of bacterial cell cultures grown in LB medium showing increased expression of the recombinant protein following overnight IPTG induction (I) in comparison with uninduced (UI) culture **(b)** Localization of recombinant protein; an equal amount of recombinant protein in supernatant and pellet of induced (I(S), I(P)) in comparison with uninduced cultures (UI(S), UI(P)) **(c)** Eluted recombinant protein resolved on SDS-PAGE using buffer with different concentrations of imidazole **(d)** Confirmation of recombinant viral NP of 56 kDa by western blot (WB) analysis using anti-His antibody **(e)** anti-influenza NP antibody.

However, the mechanism through which the cellular proteins get incorporated in to the mature virion is not clear. Rationally, cellular proteins need to be associated with the viral components at the time of assembly to be packaged into the mature virion. Multiple subunits of NP encapsidate the eight segments of negative sense RNA genome independently to form ribonucleoprotein complexes. Hence, the incorporation of cellular proteins into the mature virion perhaps is through interaction with NP or matrix (M1) protein that forms the inner lining of viral envelope. An attempt was made to investigate the interaction of viral NP with the above identified proteins by *in vitro* binding assay using recombinant NP (pET29a+NP-His) bound to Ni-NTA resin and A549 cell lysates. Eluted protein complexes from Ni-NTA resin were analyzed by anti-alpha enolase 1, anti-pyruvate kinase, anti-β-actin, anti-viral NP and anti-His antibodies. *In vitro* binding assay confirmed our preliminary hypothesis of interaction of host proteins under test with the recombinant NP (Fig. 4a). However, no such interaction was noted with the unrelated bacterial protein, a nonspecific control used in the assay. Anti-His antibody confirmed the expression of purified recombinant viral NP of ~56 kDa and an unrelated control protein of ~28 kDa in the eluted immune complexes (Fig. 4b). Anti-influenza NP antibody further confirmed the recombinant viral NP expression. Endogenous expression of alpha-enolase 1, pyruvate kinase and β-actin in A549 cell lysates recovered after the formation of immune complexes was shown by western blot analysis using specific antibodies (Fig. 4c). The significance of host proteins being carried by influenza virus into the new susceptible host and the interaction of NP with alpha enolase and pyruvate kinase is an important aspect to be explored.

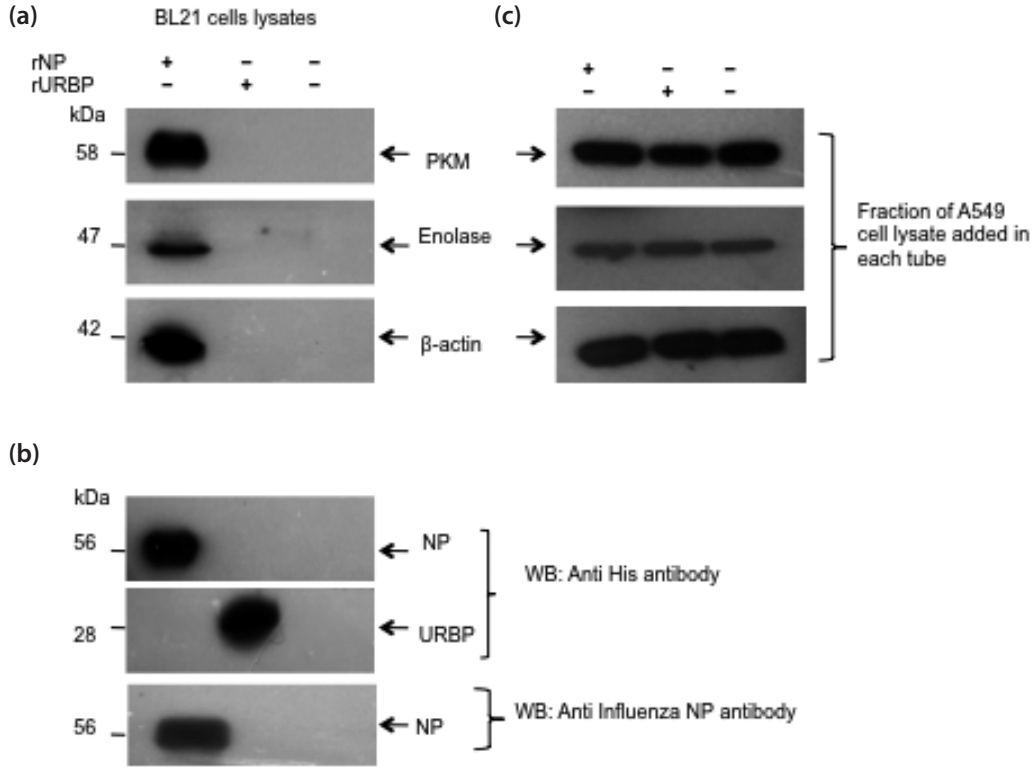

Fig. 4

***In vitro* binding assay**

Cell lysates prepared from BL21 cells expressing recombinant viral NP or unrelated bacterial protein (URBP) with His-tag were incubated with Ni-NTA beads and allowed to immobilize the recombinant protein onto the resin. Beads with bound proteins were washed and further incubated overnight with equal amount of total protein recovered from A549 cells. Next day, protein lysates were centrifuged to pellet down the beads. Protein complexes bound to the beads were eluted and subjected to western blot (WB) analysis using specific antibodies **(a)** Western blot showing pyruvate kinase, alpha enolase 1 and β-actin complexed with the recombinant NP of influenza A virus but not with the unrelated bacterial protein. No such band was seen in control; bacterial lysate without any recombinant protein **(b)** Western blot showing 56 kDa recombinant NP and 28 kDa recombinant non-specific bacterial protein as analyzed by anti-His antibody and anti-influenza NP antibody **(c)** Confirmation of endogenous levels of pyruvate kinase, alpha enolase and β-actin in a fraction of total A549 protein lysate recovered from each tube by centrifugation following overnight incubation with recombinant protein bound to Ni-NTA beads (virus NP, URBP, no protein).

Discussion

In the recent past, internal proteins of influenza A virus such as NP and non-structural protein (NS1) are gaining much importance as an alternate drug targets. NP, due to its conserved protein sequences among strains originating from different hosts was depicted as a potential drug target (Babar *et al.*, 2015). Therefore, the identification of host proteins regulated specifically in response to NP and knowledge on its interaction with host proteins could be an important consideration to design new anti-influenza viral therapeutics. With this point in mind, we evaluated the altered A549 cell proteome induced by influenza A virus recombinant NP by 2D gel electrophoresis coupled with MALDI-TOF/MS analysis. Considerable number of studies earlier reported the differential regulation of host proteins induced by influenza A viral strains. Interestingly, the protein profile in our study is strikingly similar to the published influenza A virus-infected cell proteome. Alpha-enolase 1, tubulin β, protein disulfide isomerase, splicing factor proline/glutamine-rich (SFPQ) proteins had been reported to be regulated in response to H1N1 strain (Zhao *et al.*, 2013). The differential abundance of aldoketoreductase, alpha enolase 1, UDP-glucose-6-dehydrogenase in response to H5N1 strain (Liu *et al.*, 2012), T-complex protein 1 in H3N2 infected cells (Wu *et al.*, 2013) and deregulation cell cycle proteins and lipid metabolism by H1N1 2009 pandemic strain (Dove *et al.*, 2012) were demonstrated. These reports collectively suggest the usage of common host factors by influenza A viral strains of different host origins.

The above mentioned studies were carried out using a complete virus to investigate the altered proteome

of infected cell. Very few studies had demonstrated the impact of a particular viral protein independent of other viral components. Host cellular protein PACT, an essential co-factor for IFN stimulation, had been identified to be an interacting partner to influenza A viral recombinant NS1 protein, a known inhibitor of innate and adaptive immunity by quantitative proteomic analysis. This interaction was shown to block PACT/RIG1-mediated IFN I activation and serve the virus to escape the host immune response (Tawaratsumida *et al.*, 2014). Host response to RNA polymerase complex of H5N1 avian strain had also been demonstrated by proteomic analysis and its association with mitochondrial proteins is presumed to be the regulator of apoptosis in host cell (Bradel-Tretheway *et al.*, 2016). Here, we had shown the differential expression of host proteins induced by influenza A viral NP alone.

Generally, enveloped viruses including influenza A virus bud from the host cell plasma membrane and are released into extracellular environment. During this process, some cell surface proteins are packaged into virus particles. Other than influenza A virus, packaging of cellular proteins was accounted for in other viruses, to specify few: human CMV (Varnum *et al.*, 2004), HIV (Cen *et al.,* 2004, Linde *et al.*, 2013), EBV (Johannsen *et al.*, 2004). Host proteins packaged into influenza A virus were grouped into several categories including proteins of metabolic pathways, glycolysis, annexins, and cytoskeletal elements (Shaw *et al.*, 2010). Our preliminary studies demonstrated the interaction of alpha enolase 1, pyruvate kinase and β-actin with the recombinant NP (pET29a+NP-His construct) by *in vitro* binding assay. It proposes the possible role of NP in packaging of the host proteins into mature virus particle; or the described interactions may regulate an alternate function during infection. The association of cytoskeletal elements actin and tubulin with influenza virus matrix protein (M1) and NP has been reported earlier (Avalos *et al.*, 1997), but the interaction of NP with alpha enolase 1 and pyruvate is found to be novel and needs further confirmation.

Alpha enolase is a key glycolytic enzyme expressed in the cytoplasm and on cell surface as well. In addition to its fundamental glycolytic function, it plays a significant role in several biological and pathological processes. So does pyruvate kinase, another key player in glycolysis. Enolase expression was correlated with tumor cell proliferation (Song *et al.*, 2014) and plays a significant role in tumor invasion by modulating oncogenes transcription control (Fu *et al.*, 2015). Similarly, localization of pyruvate kinase, a rate-limiting glycolytic enzyme in the nucleus was correlated with cell proliferation (Gao *et al.*, 2012). Alpha enolase had also been described as a neurotrophic factor (Takei *et al.*, 1991), a heat-shock protein (HSP48) (Iida and Yahara, 1985) and a hypoxic stress protein (Aaronson *et al.*, 1995). Likewise, pyruvate kinase has been described as a regulator of growth and apoptosis (Spoden *et al.*, 2009) and an immunomodulator in tumor cells (Zhang *et al.*, 2010). Oncogenic viruses are known to control the cell proliferation and DNA damage through modulating glucose metabolic pathways (Noch and Khalili, 2012), but increasing evidence on differential regulation of glycolytic enzymes and other metabolic enzymes in infected cells specify their importance in virus life cycle. Few studies reported the secretion of high level of alpha enolase in dengue-infected hepatic cells (Higa *et al.*, 2014), differential expression of enolase in response to chikungunya virus (Thio *et al.*, 2013) and of pyruvate kinase to bovine herpes simplex 1 virus (Guo *et al.*, 2015). In spite of the fact that the differential expression of glycolytic and other metabolic enzymes in response to viruses including influenza A virus had been accounted for in the literature, neither their interaction with viral proteins nor their functional significance in virus replication is clearly known. One study demonstrated the essential role of enolase and pyruvate kinase in transcription of Sendai virus genome (Ogino *et al.*, 2001).

Based on our preliminary data, we hypothesize that alpha enolase and pyruvate kinase may facilitate the virus assembly and packaging through their interaction with ribonucleoprotein complex. Secondly, when a mature virus infects another susceptible host, the two virion associated proteins, enolase and pyruvate kinase, are released into the host cell and may regulate the initial stages of infection or modulate the host cell glycolysis to create favorable environment for the virus to establish the infection. Likewise, other incorporated proteins may play a regulatory role during infection. Packaging of cellular proteins into virus particles was thought to be accidental, but recent studies demonstrated the vital role of incorporated host proteins for establishing new infection in a susceptible host. Ubiquitin, a regulatory protein incorporated into mature influenza virion was shown to play a crucial role in the internalization of virus during subsequent infection (Banerjee *et al.*, 2014). Similarly, incorporated annexin V counteracts the anti-influenza viral activity by disrupting γ IFN signaling and promotes the virus replication in the new host (Berri *et al.*, 2014). Packaging of glycolytic enzymes alpha enolase and pyruvate kinase into mature virion, perhaps through viral NP interaction, is expected to have a significant role in virus infections.

To conclude, incorporation of alpha enolase and pyruvate kinase into mature virion as demonstrated by Shaw et al. (2010), differential expression and interaction with NP as shown by us together indicate their potential role in virus life cycle. Further studies on implications of identified host proteins in response to influenza A virus infection with special reference to the conserved internal NP will possibly help to design and develop novel interventional strategies to combat the infection by different strains of influenza A virus.

Acknowledgement. This study was supported by Indian Council of Medical Research (No. VIR/15/2011-ECD-I), UPOE II (Project ID-56), and DST-PURSE grant, New Delhi. We acknowledge the Mass spectrometry facility of Advanced Instrumentation Research Facility at JNU, New Delhi, India.

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