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Rapid Communication

CDK/ERK-mediated phosphorylation of the human influenza A virus NS1 protein at threonine-215

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ABSTRACT

Posttranslational modification of viral proteins by cellular enzymes is a feature of many virus replication strategies. Here, we report that during infection the multifunctional human influenza A virus NS1 protein is phosphorylated at threonine-215. Substitution of alanine for threonine at this position reduced early viral propagation, an effect apparently unrelated to NS1 antagonizing host interferon responses or activating phosphoinositide 3-kinase signaling. *In vitro*, a subset of cellular proline-directed kinases, including cyclin dependent kinases (CDKs) and extracellular signal-regulated kinases (ERKs), potently phosphorylated NS1 protein at threonine-215. Our data suggest that CDK/ERK-mediated phosphorylation of NS1 at threonine-215 is important for efficient virus replication.

Introduction

Reversible phosphorylation of serine, threonine, and tyrosine residues is a well-documented method by which cellular kinases can control the structure, function, and subcellular localization of protein substrates [reviewed in Cohen, 2000]. As with numerous host-cell processes, many viruses have evolved strategies to take advantage of this normal regulatory mechanism, thereby enhancing their own replication. The influenza A virus NS1 protein is a multifunctional virulence factor that promotes virus propagation by modulating viral RNA metabolism, host innate immune responses, and several cell signaling pathways [reviewed in Hale et al., 2008]. Early work indicated that during infection NS1 is phosphorylated at two distinct residues, which are likely to be threonines (Privalsky and Penhoet, 1978; 1981). In this study, we sought to determine the biological significance of NS1 protein phosphorylation, and to identify the host-cell signaling pathways involved.

Results and discussion

NS1 protein is phosphorylated during infection

To confirm that NS1 is phosphorylated, we infected a human lung epithelial cell-line (A549) with the human influenza virus A/Udorn/72 (Ud). After 12 h, cells were metabolically labelled with either [^{32}P]-orthophosphate or [^{35}S]-methionine, and cell lysates were subjected to immunoprecipitation using an NS1-specific antiserum. A [^{32}P]-orthophosphate labelled band corresponding to NS1 was clearly detectable in immunoprecipitates from virus-infected (but not mock-infected) cell lysates (Fig. 1a). Interestingly, analysis of immunoprecipitates from [^{35}S]-methionine labelled lysates revealed at least two distinct polypeptide species corresponding to NS1. The major [^{35}S]-methionine labelled NS1 polypeptide had fastest mobility, whilst a minor species with slower mobility was also observed (Fig. 1a). Strikingly, the mobility of the minor [^{35}S]-methionine labelled species corresponded exactly with that of [^{32}P]-orthophosphate labelled NS1 (Fig. 1a). We conclude that only a subpopulation of the total NS1 protein synthesised at any given time during infection is phosphorylated.

Identification of NS1 phosphorylation sites by mass spectrometry (MS)

A permissive, non-transformed human lung fibroblast cell-line (MRC-5) was infected with the human influenza virus strains A/WSN/33 (WSN) or Ud, and total-cell lysates were prepared after 12 h. MRC-5 cells were used as viral protein expression is easily detectable over host-cell background, and the WSN/NS1 polypeptide (unlike Ud/NS1) readily separates from other viral proteins (particularly M1) by SDS-PAGE. Gel slices corresponding to NS1 were excised and subjected to in-gel tryptic digestion and MS. Analysis of the resulting mass spectra showed that our data covered 85% of the WSN/NS1 protein (Fig. 1b),

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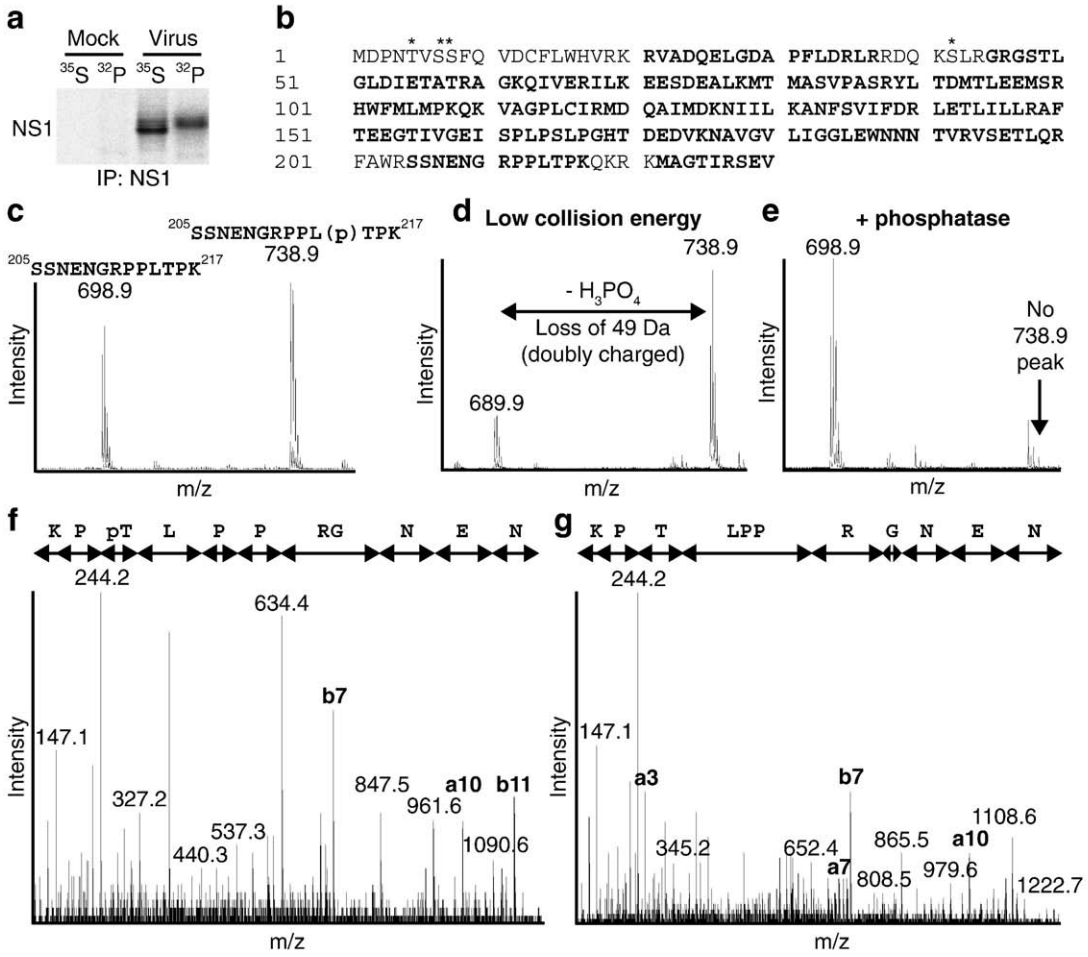

Fig. 1. Thr-215 of NS1 is phosphorylated during infection. (a) NS1 protein was immunoprecipitated from [^{35}S]-methionine or [^{32}P]-orthophosphate labelled A549 cells infected for 12 h (or mock) with rUd virus (5 PFU/cell). SDS-PAGE followed by autoradiography was used to visualize labelled polypeptides. (b) MRC-5 cells were infected with rWSN (5 PFU/cell) for 12 h and total-cell polypeptides were separated by SDS-PAGE. Figure shows peptide coverage (bold) obtained after nanoLC-ESI MS/MS of the tryptic peptide mixture corresponding to WSN/NS1 protein. Asterisks denote serine, threonine, and tyrosine residues not covered. (c) Doubly charged signals corresponding to the phosphorylated and non-phosphorylated WSN/NS1 peptide SSNENGRPPL(p)TPK. The phospho-peptide was isolated (d) and subjected to low energy collision-induced dissociation resulting in the neutral loss of H_3PO_4. (e) Phosphatase treatment results in disappearance of the phospho-peptide signal. Comparison of high energy collision-induced dissociation of the phospho-peptide (f) and the non/de-phosphorylated peptide (g). Analysis of the fragmentation patterns determined the site of phosphorylation as Thr-215. The *y* ion series is labelled along with the prominent *b* and *a* ions.

but only 75% of the Ud/NS1 protein (Supplementary Figure S1). However, between the two NS1 proteins our data included all common serines, threonines, and tyrosines except Thr-5, Ser-7, Ser-8, and Ser-42. Using an automated MASCOT algorithm to search for NS1 peptides containing possible phosphorylated residues, a single phospho-peptide was identified for each NS1 protein (WSN/NS1, ^{205}SSNENGRPPLTPK^{217} with a Mascot Ion Score of 60; Ud/NS1, ^{201}FAWGSSNENGRPPLTPK^{217} with a Mascot Ion Score of 73) (Fig. 1c and Supplementary Fig. S1). Phosphorylation of the WSN/NS1 peptide was confirmed by the diagnostic loss of 98 Da (i.e. loss of 49 Da from the doubly charged ion) seen in the MS/MS spectrum at low collision energies (Fig. 1d), and by disappearance of the doubly-charged 738.9 signal upon phosphatase treatment (Fig. 1e). Similar results were obtained with Ud/NS1 (Supplementary Fig. S1). No equivalent phospho-peptide was observed in tryptic digests of gel slices from mock-infected cell-lysates (data not shown). The length difference between the WSN and Ud NS1 phospho-peptides is a result of strain-dependent sequence variation that leads to differential trypsin cleavage. This supports the notion that the observed phospho-peptides derive from the virus-specific NS1 protein, rather than the host-cell.

To determine the phosphorylated residue on these NS1-derived peptides, the tryptic peptide mixture of WSN/NS1 was subjected to high collision energy MS/MS. Interpretation of the fragmentation pattern obtained (Fig. 1f) and comparison with that obtained for the non-phosphorylated peptide (Fig. 1g) identified the site of phosphorylation to be Thr-215. Similar results were obtained using the Ud/NS1 tryptic peptide mixture (data not shown). Interestingly, Thr-215 is considered to be a marker for human NS1 proteins, as proline is present at this position in most avian viruses (Finkelstein et al., 2007). This observation may explain the previously reported strain-specificity regarding phosphorylation of NS1 proteins from human, but not avian, influenza viruses (Petri et al., 1982), and implies potentially important differences in how these viruses may interact with their hosts.

Characterization of a recombinant influenza A virus expressing NS1 with a T215A amino-acid substitution

Reverse genetics was used to engineer a recombinant Ud virus that expressed NS1 with a T215A amino-acid substitution. The rUd/NS1-T215A virus was clearly attenuated, as it formed small plaques in

Madin–Darby canine kidney (MDCK) cells (Fig. 2a). Furthermore, the mutant virus grew to infectious titres ~100-fold lower than WT virus during multi-cycle growth analysis in A549 cells (Fig. 2b). In MDCK cells, multi-cycle replication was also significantly (~100-fold) slower, but surprisingly the mutant virus eventually reached final titres similar to WT (Supplementary Fig. S2). The defect in virus propagation in human A549 cells is not solely related to viral spread, as rUd/NS1-T215A also grew to titres ~10-fold lower than WT virus during single-cycle growth analysis (Fig. 2c). Consistent with the single-cycle growth kinetics, the mutant virus clearly exhibited a reduction in viral protein synthesis compared to WT at ~6 h post-infection (Fig. 2d). It was also apparent that NS1-T215A protein was slightly delayed in its ability to localize into discrete intra-nuclear foci during infection (Fig. 2e and Supplementary Fig. S2). Metabolic labelling of infected cells with [^{32}P]-orthophosphate showed that mutant NS1 protein was still phosphorylated (Fig. 2f), demonstrating that Thr-215 is not the only site of phosphorylation. Given that our MS analysis did not cover the entire NS1 protein (Fig. 1b), it is possible that NS1 is also phosphorylated at one of the residues not covered by our data. This would be consistent with original studies, in which phosphorylation was identified on at least two separate NS1 tryptic peptides (Privalsky and Penhoet, 1981).

Although a major function of NS1 is to antagonize host innate immune responses (Garcia-Sastre et al., 1998; Noah et al., 2003), the ability of rUd/NS1-T215A virus to control interferon (IFN) production by infected cells did not differ significantly from that of WT virus (Fig. 3a). Furthermore, rUd/NS1-T215A was attenuated in A549 cells specifically engineered to be deficient in IRF-3, a cellular transcription factor essential for both IFN production and many other IFN-independent antiviral effects (Hilton et al., 2006) (Fig. 3b and c). Recently, it was reported that residue 215 of avian influenza virus NS1 proteins influences the ability of NS1 to hyperactivate human

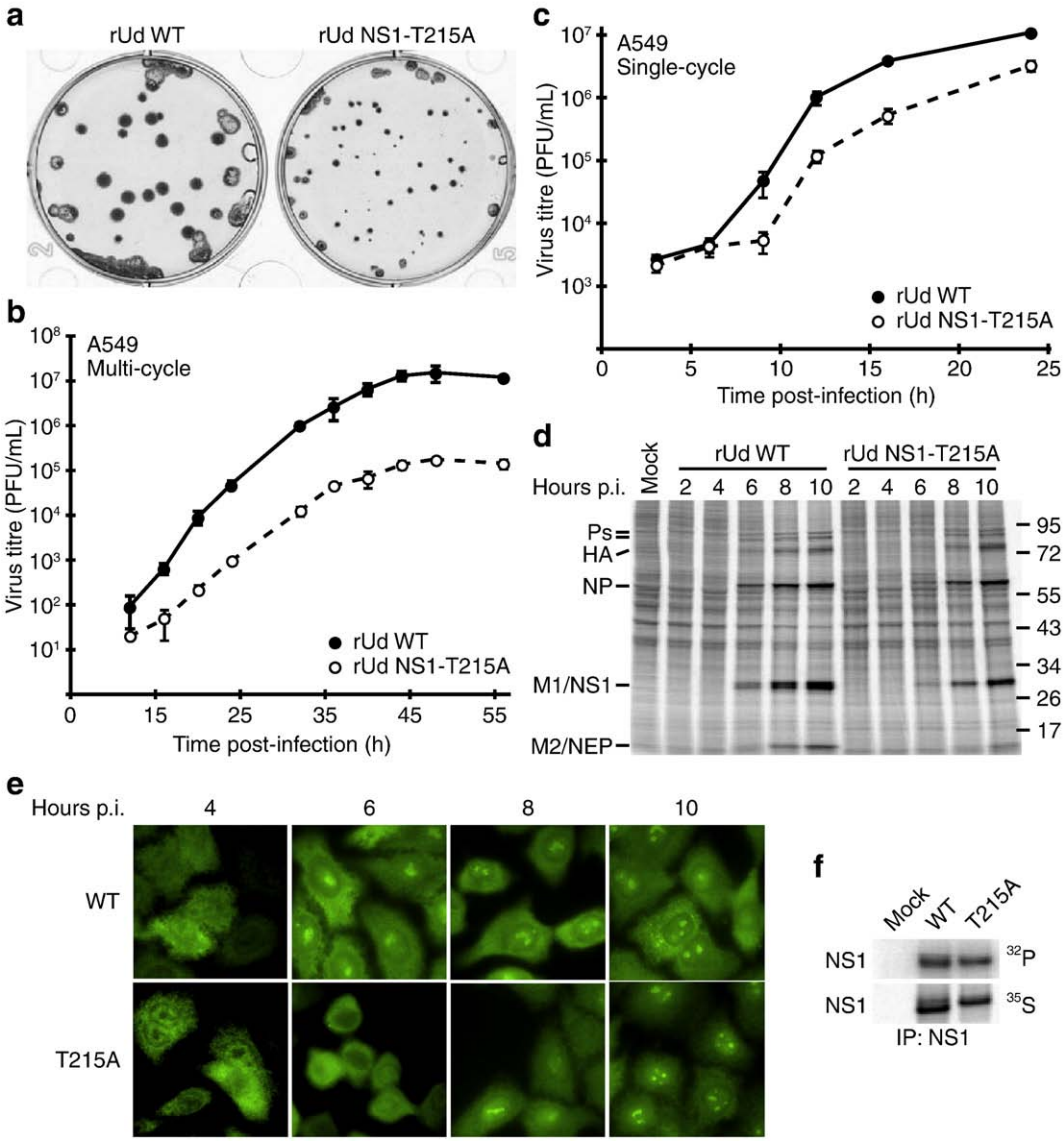

Fig. 2. A recombinant human influenza A virus expressing NS1 protein with a T215A amino-acid substitution is attenuated in tissue-culture. (a) Plaque phenotype of rUd WT and rUd NS1-T215A in MDCK cells. (b) Multi-cycle growth analysis (~0.001 PFU/cell) of rUd WT and rUd NS1-T215A in A549 cells. (c) Single-cycle growth analysis (~3 PFU/cell, trypsin absent) of rUd WT and rUd NS1-T215A in A549 cells. For both (b) and (c), titres represent mean values from three replicates. Error bars represent standard deviation. (d) Protein synthesis profiles of rUd WT and rUd NS1-T215A in A549 cells. Monolayers were infected at an MOI ~3 PFU/cell. 1 h prior to harvest, cells were labelled with [^{35}S]-methionine. Proteins were separated by SDS-PAGE and detected by autoradiography. Approximate mobilities of viral proteins are indicated to the left. The asterisk refers to a viral protein likely to be either M2 or NEP. Molecular weight markers are indicated to the right. (e) Intracellular localization of NS1 during rUd WT and rUd NS1-T215A infection (5 PFU/cell) of A549 cells. Cells were fixed at the times indicated and immunostained for NS1 protein. (f) Immunoprecipitation of NS1 from [^{35}S]-methionine and [^{32}P]-orthophosphate labelled A549 cells infected with rUd WT and rUd NS1-T215A carried out as described for Fig. 1a.

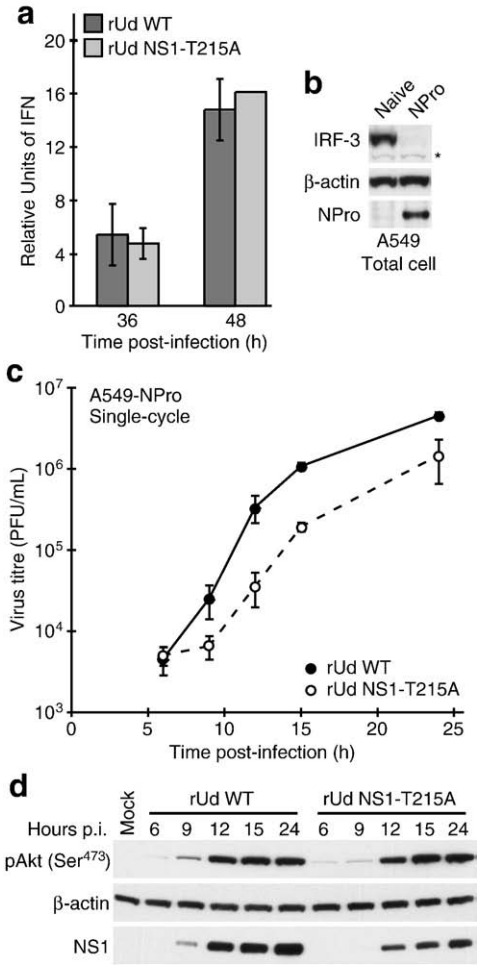

Fig. 3. Attenuation of rUd NS1-T215A is independent of IFN and PI3K. (a) Induction of IFN by rUd WT and rUd NS1-T215A in A549 cells. Monolayers were infected at an MOI of ~3 PFU/cell and supernatants were harvested at the times indicated. IFN in the supernatants was estimated using a biological CPE-reduction assay. Bars represent mean relative units of IFN from three replicates. Error bars represent standard deviation. (b) IRF-3 is degraded in A549 cells expressing BVDV/NPro. Polypeptides from naive or BVDV/NPro-expressing A549 total-cell lysates were separated by SDS-PAGE and transferred to PVDF membrane. IRF-3 and β-actin were detected using specific antibodies. N-terminally V5-tagged BVDV/NPro was detected using an anti-V5 antibody. The asterisk corresponds to a non-specific cross-reacting protein that confirms equal sample loading. (c) Single-cycle growth analysis (MOI ~ 3 PFU/cell, trypsin absent) of rUd WT and rUd NS1-T215A in A549-NPro cells. (d) Phosphorylation of Akt/PKB during infection of A549 cells with rUd WT and rUd NS1-T215A. Lysates were prepared at the times indicated post-infection (MOI ~ 3 PFU/cell) and subjected to SDS-PAGE followed by transfer to PVDF membrane. NS1 and phospho-Akt/PKB (Ser-473) were detected using specific antibodies. β-actin served as a loading control.

phosphoinositide 3-kinase (PI3K) signaling (Heikkinen et al., 2008). However, both WT rUd and rUd/NS1-T215A were similarly capable of inducing Akt/PKB phosphorylation in A549 cells (Fig. 3d), suggesting that mutation of this residue (at least for human influenza viruses) does not cause an intrinsic defect in PI3K-activating capability. We conclude that phosphorylation of Thr-215 must be required for another, as yet undefined, function of human NS1 proteins.

Identification of cellular kinases that phosphorylate NS1 in vitro

Analysis of the motif surrounding Thr-215 in NS1 revealed that proline at position +1 is highly conserved amongst most influenza A virus strains sequenced to date. The CMGC (*CDK*, *MAPK*, *GSK3*, *CLK*) superfamily of proline-directed kinases consists of 61 different human kinases, which preferentially phosphorylate serine/threonine residues that are followed by a proline. We therefore screened a panel of representative human CMGC kinases for their ability to phosphorylate purified recombinant full-length GST-tagged Ud/NS1 protein *in vitro*. As shown in Fig. 4a, neither the stress-activated protein kinases (p38α/SAPK2A, p38β/SAPK2B, and p38γ/SAPK3: also known as mitogen-activated protein kinases) nor the c-Jun N-terminal kinases (JNK1 and JNK2) were able to phosphorylate recombinant NS1 protein. However, it was clear that a representative cyclin-dependent kinase (CDK5/p35) and a representative extracellular signal-regulated kinase (ERK2) could phosphorylate Ud/NS1 (Fig. 4a). Furthermore, these two kinases (as well as CDK1/cyclin B and CDK2/cyclin A) phosphorylated a 6His-tagged Ud/NS1 protein that lacked the N-terminal 72 amino-acids (RNA-binding domain) of NS1 (Fig. 4b and c). Substitution of alanine for threonine at position 215 of NS1 effectively eliminated phosphorylation by both the CDKs and ERK2 (Fig. 4b and c), indicating that Thr-215 is the likely phosphorylation site in NS1 for these kinases.

Previous studies on the primary sequence specificities of proline-directed kinases revealed that ERK1/2 preferentially phosphorylate serine/threonine-proline motifs that also contain a proline residue at the −2 position, as well as leucine at the −1 position (Songyang et al., 1996). This is consistent with motifs identified within *bona fide* ERK1/2 substrates *in vivo* (Songyang et al., 1996), as well as NS1 protein around Thr-215 (Fig. 1b). Furthermore, proline at the −2 position is selected for by CDKs, and this group of kinases also prefer lysine/arginine residues at positions +2 and +4 (Songyang et al., 1996). Again, this is in full agreement with the sequence surrounding Thr-215 in human influenza A virus NS1 proteins. Given that NS1 phosphorylation was reported to occur in the nuclei of infected cells (Privalsky and Penhoet, 1981), it is

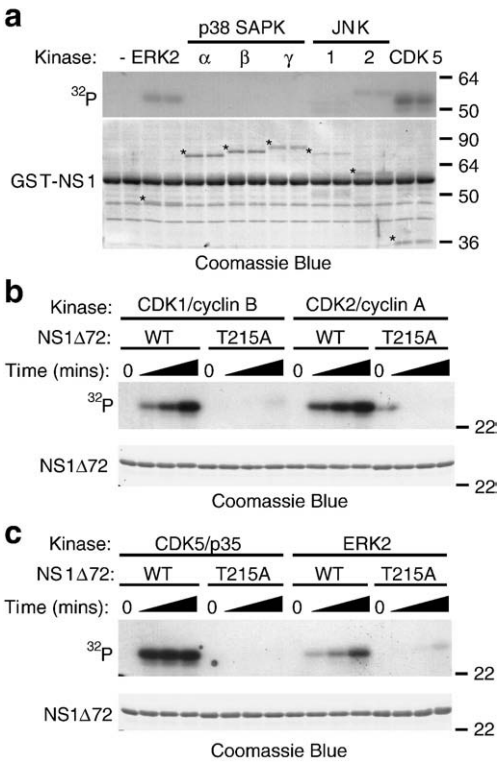

Fig. 4. CDKs and ERK phosphorylate Thr-215 of NS1 *in vitro*. (a) Screening of CMGC family kinases for phosphorylation of full-length NS1 protein *in vitro*. 100 ng of each indicated kinase was incubated with 1.5 μg GST-Ud/NS1 and analyzed for substrate phosphorylation as described in Materials and methods. Asterisks indicate input kinases. CDK1 and CDK2 (b), and CDK5 and ERK2 (c) phosphorylate the effector domain of Ud/NS1, but not a mutant protein with the T215A amino-acid substitution. Kinase assays were performed using 25 ng of the CDKs and 100 ng of ERK2 incubated with 2 μg of substrate for 3, 10, or 30 min. Substrates were 6His-tagged Ud/NS1Δ72 and Ud/NS1Δ72-T215A.

worth noting that both ERKs and CDKs are predominantly active in the nucleus (Cardoso et al., 1993; Chen et al., 1992). These observations further substantiate the possibility that NS1 is a true biological substrate for both ERK and CDK kinases.

We found that ERK2 has a relatively low initial rate of NS1 phosphorylation *in vitro* (33 U/mg). In contrast, the CDKs (particularly CDK5) were very efficient NS1 kinases (CDK1, 56 U/mg; CDK2, 138 U/mg; CDK5, >400 U/mg). Furthermore, high-stringency motif analysis of the Ud/NS1 amino-acid sequence using Scansite 2.0 (Obenauer et al., 2003) strongly predicts Thr-215 phosphorylation by CDKs. Interestingly, this algorithm also predicts that two other NS1 residues can be phosphorylated: Ser-42, possibly by cellular protein kinase C isoforms (PKCs); and Thr-49, possibly by Akt/PKB. In the present study we were unable to ascertain whether Ser-42 is phosphorylated *in vivo* as our MS data did not cover this residue. Although our data did cover Thr-49, we did not detect phosphorylation at this site. However, it may be that phosphorylation of a second site is temporally regulated. Given that NS1 itself binds and activates PI3K (the upstream regulator of Akt/PKB) (Hale et al., 2006), the potential Akt/PKB-mediated phosphorylation of Thr-49 is intriguing.

Concluding remarks

We have identified Thr-215 as an *in vivo* phosphorylation site of human influenza A virus NS1 proteins, and shown that members of the CDK and ERK kinase families can phosphorylate this residue *in vitro*. Although clearly important for efficient viral replication in tissue-culture, the precise biological function of Thr-215 phosphorylation is unknown. Indeed, future studies will have to determine whether phosphorylation of NS1 protein *in vivo* is actually mediated by a single specific kinase rather than all members of the ERK and CDK families.

It is uncertain whether NS1 protein acts as a pseudosubstrate to limit the activities of ERKs/CDKs, or is modified in order to carry out a specific function. In this latter regard, it is tempting to speculate that posttranslational modification of NS1 is one of the factors that determines its remarkably multifunctional nature (Hale et al., 2008). Phosphorylation of full-length NS1 protein derived from infected cell-lysates was reported to be wholly refractile to alkaline phosphatase treatment (Privalsky and Penhoet, 1978; 1981). However, NS1 tryptic phospho-peptides are sensitive to phosphatases, therefore in the context of full-length NS1 protein it may be that phosphorylated Thr-215 is not surface available. This could be caused by phosphorylation-induced structural changes in this region of NS1 (which appears to be intrinsically disordered (Hale et al., 2008a)), or masking of this site by a viral or cellular binding partner that is specifically recruited to phospho-NS1.

The data presented here provide additional insights into the strain-specific interactions of NS1 with host-cell enzymes. Such studies re-emphasize the multiple roles that cellular kinases have in influenza virus biology, and highlight the need for future studies on other potential NS1 posttranslational modifications.

Materials and methods

Cells, viruses, and IFN

Cells were grown as monolayers in DMEM supplemented with 10% FBS. Methods to generate cell-lines with targeted degradation of IRF-3 have been detailed elsewhere (Hilton et al., 2006). WT and mutant influenza viruses (Ud and WSN) were generated by reverse genetics from cDNAs kindly provided by Prof. Robert Lamb (Northwestern University, USA), and propagated/titrated/verified as described previously (Jackson et al., 2008). Threonine-215 in Ud/NS1 was changed to alanine by PCR mutagenesis of the relevant NS segment cDNA (pHH21 vector). Although NS1 and NS2/NEP are both encoded by vRNA segment 8, no amino-acid changes were introduced into NS2/NEP. IFN in supernatants from infected monolayers was estimated using a modified biological EMCV cytopathic effect (CPE) reduction assay in A549 cells.

Metabolic labelling, immunoanalyses, and SDS-PAGE

Cells in 6-well plates were starved for 30 min in phosphate- or methionine-free medium prior to metabolic labelling with [^{35}S]-methionine (50 μCi/well) or [^{32}P]-orthophosphate (500 μCi/well) as appropriate. Immunofluorescence, immunoprecipitation, plaque staining, SDS-PAGE, and immunoblot analysis were carried out as before (Hale et al., 2006). Protease and phosphatase inhibitors (Roche) were added to all lysis buffers. Anti-V5 antibody was purchased from Serotec. The phospho-Akt/PKB (Ser-473) antibody was from Cell Signaling Technology. The IRF-3 antibody was from Abcam, and the β-actin antibody was from Sigma. Generation of a rabbit anti-NS1 polyclonal antiserum has been discussed previously (Hale et al., 2008b).

Mass spectrometry

Protein bands were digested using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) (Shevchenko et al., 1996). Briefly, gel cubes were destained with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37 °C. Peptides were extracted with 10% formic acid and concentrated using a SpeedVac (ThermoSavant). Peptides were then separated using an UltiMate nanoLC (LC Packings, Amsterdam) equipped with a PepMap C18 trap and column, using a 60 min gradient of increasing acetonitrile concentration containing 0.1% formic acid (5–35% acetonitrile in 35 min, 35–50% in a further 20 min, and 95% acetonitrile to clean the column). The eluent was sprayed into a Q-Star Pulsar XL tandem mass spectrometer (Applied Biosystems, Foster City, CA) and analyzed in Information Dependent Acquisition (IDA) mode, performing 1 s of MS followed by 3 s MS/MS analyses of the 2 most intense peaks seen in MS. These masses were then excluded from analysis for the next 60 s. The experiment was carried out using a standard rolling collision energy setting and again at a higher fixed collision energy. MS/MS data for doubly- and triply-charged precursor ions was converted to centroid data, without smoothing, using the Analyst QS1.1 mascot .dll data import filter with default settings. The MS/MS data file generated was analyzed using the Mascot 2.1 search engine (Matrix Science, London, UK) against UniRef100 database (January 2008), containing 5,241,852 sequences. No species restriction was applied. The data were searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification, and methionine oxidation and phosphorylation on serine, threonine or tyrosine selected as a variable modification.

Phosphatase treatment of tryptic peptides

Peptides were concentrated to dryness and resuspended in 50 mM ammonium bicarbonate buffer supplemented with 2 mM $MnCl_2$. λ protein phosphatase (133 units, NEB, Hitchin, UK) was added to the peptide mix and incubated overnight at 30 °C. Resulting peptides were acidified and analyzed by nanoLC-ESI-MS/MS as described above.

Kinase assays

GST-Ud/NS1 and 6His-tagged Ud/NS1Δ72 were expressed and purified by methods previously described (Hale et al., 2008a; Hale et al., 2006). Recombinant active CDK1/cyclin B and CDK2/cyclin A were from Biomol, CDK5/p35 from Invitrogen, ERK2 from Proqinase, JNK1α1 and JNK2α2 from Upstate, and p38/SAPKs from the University of Dundee, UK. Kinases were used at 1 μg/mL or 4 μg/mL to phosphorylate 60–80 μg/mL substrate for various times in 50 mM

Tris pH 7.5, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 10 mM Mg-acetate and 0.1 mM [γ^{32}P]-ATP at 30 °C. The reactions were stopped with SDS, proteins separated by SDS-PAGE, and gels analyzed by Coomassie Blue staining and autoradiography.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.10.002.

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