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Evidence for phosphorylation of human parainfluenza virus type 3 C protein: mutant C proteins exhibit variable inhibitory activities in vitro

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Abstract

The P mRNA of human parainfluenza virus type 3, like other members of the subfamily *Paramyxovirinae*, gives rise to several polypeptides, one amongst them, the C protein, which is involved in inhibition of viral RNA synthesis as well as counteracting the host interferon signaling pathway. As a further step towards characterizing the function of C protein we present evidence to demonstrate the phosphorylation of C protein. Evidence for this observation emerged from deletion mapping studies coupled with mass spectroscopy analysis confirming residues S7, S22, S47T48 and S81 residues as the phosphorylation sites within the NH₂-terminus of C protein. Here, we utilized a HPIV 3 minigenome replication assay and real time RT-PCR analysis to measure the relative RNA levels synthesized in the presence of mutant C proteins. Mutants S7A and S81A displayed low levels of RNA while mutant 5A that was devoid of all these phosphorylation sites exhibited high RNA level in comparison to wild type C during transcription. Interestingly, high levels of RNA were observed in the presence of S81A and mutant 5A during replication. Taken together, our results indicate that phosphorylation may differentially affect the inhibitory activity of C protein thereby regulating viral RNA synthesis.

Keywords

human parainfluenza virus type 3; C protein; phosphorylation; minigenome replication

1. Introduction

Human parainfluenza virus type 3 (HPIV 3) is a non-segmented, negative-sense RNA virus and a member of the subfamily *Paramyxovirinae*. The viral genome of 15,462 nucleotides is arranged co-linearly in the order, 3'-[leader (*le*)-N-P-M-F-HN-L-trailer (*tr*)]-5' and encapsidated by the nucleoprotein (N) to form a helical nucleocapsid, i.e. the N-RNA

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(Banerjee, et al., 1991). Transcription of the viral genome by the RNA polymerase complex composed of a large, multifunctional protein (L) and the phosphoprotein (P) leads to the synthesis of five monocistronic mRNAs and a unique P mRNA (Chanock, et al., 2001; Lamb and Kolakofsky, 2001).

The unedited P mRNA, which is an exact copy of the P gene, encodes the longest open reading frame that gives rise to P protein. However, the edited P mRNA generates two additional polypeptides, the V and PD proteins, following incorporation of one or two guanosine residues, respectively, within the “editing site”. The P, V and PD proteins therefore share a common NH₂-terminus but differ in their COOH-termini (Galinski, et al., 1992; Chanock, et al., 2001; Lamb and Kolakofsky, 2001; Wells and Malur, 2008).

Additionally, the P mRNA also synthesizes a basic polypeptide, the C protein, from an alternative AUG codon in the +1 open reading frame relative to P mRNA (Luk, et al., 1986; Galinski, et al., 1986). Besides HPIV 3, other viruses within the subfamily *Paramyxovirinae* including Sendai virus (SeV) and measles virus (MV) are also capable of synthesizing C proteins. Interestingly, SeV P mRNA synthesizes four C proteins (C', C, Y1 and Y2) compared to a single C protein synthesized by MV P mRNA (Chanock, et al., 2001; Lamb and Kolakofsky, 2001). The HPIV 3 and SeV C proteins display low homology amongst themselves but share similar structural profiles throughout their COOH-termini (Luk, et al., 1986; Spriggs and Collins, 1986).

The *Paramyxovirinae* C proteins have been a subject of intense research to study their role in virus replication. One such approach involving the generation of mutant viruses using “reverse genetics” demonstrated that C proteins play an important role not only in virus replication but also in counteracting the host interferon signaling pathway (Durbin, et al., 1999; Garcin, et al., 1997; Kurotani, et al., 1998; Patterson, et al., 2000; Escoffier, et al., 1999; see reviews: Gotoh, et al., 2001, 2002, Neumann, et al., 2002). A second approach which utilizes an in vitro system to address the role of C proteins and examine their interactions with other viral proteins has been a subject of continued investigation in several laboratories including our own. Studies on SeV C proteins demonstrated their ability to interact with L protein resulting in a down regulation of viral RNA synthesis (Curran, et al., 1992; Cadd, et al., 1996; Horikami, et al., 1997; Tapparel, et al., 1997). Furthermore, mutational studies of SeV and MV C proteins revealed a direct correlation with L binding and RNA inhibition (Grogan and Moyer, 2001; Kato, et al., 2004; Reutter, et al., 2001; Bankamp, et al., 2005).

Recent studies on HPIV 3 C protein, both in vitro and in vivo, demonstrated an inhibitory effect on RNA synthesis that was mediated by binding of C protein to L at a site independent from P-L binding site (Smallwood and Moyer, 2004). Moreover, studies from our laboratory, as well as work from others, have demonstrated that heterologous C proteins are capable of inhibiting transcription by about 50% of the level of transcription observed with homologous proteins (Smallwood and Moyer, 2004; Malur, et al., 2004). Additional studies aimed towards addressing the role of C protein in interferon signaling also demonstrated that C protein was capable of counteracting the host interferon signaling pathway by specifically inhibiting the phosphorylation of STAT 1 by utilizing a cell line that stably expressed the C protein (Malur, et al., 2005).

In our ongoing efforts aimed towards investigating the function of C protein, we noticed a prominent band identical to the size of C protein that was consistently immunoprecipitated from cell extracts upon subsequent labeling with [³²P]-orthophosphate. This preliminary observation along with an earlier study demonstrating phosphorylation of two specific SeV polypeptides, C' and C, (Hendricks, et al., 1993) prompted us to ascertain the phosphorylation status of the C protein and identify the amino acid residues capable of undergoing

phosphorylation. Here, using deletion mapping studies coupled with mass spectroscopy analyses we have successfully identified five amino acids S7, S22, S47, T48 and S81 located within the NH₂-terminal half of the HPIV 3 C protein capable of undergoing phosphorylation. Furthermore, we have utilized the in vitro HPIV 3 minigenome assay to measure RNA synthesis and evaluate the relative inhibitory activities of mutant C proteins. Results from our assays demonstrate that mutations within the phosphorylation sites of C protein exhibit variable inhibitory activities in vitro suggesting that phosphorylation of C protein may have a plausible role in regulating viral RNA synthesis.

2. Materials and methods

2.1 Cells, viruses and antibody

HeLa and Vero cells were cultured in Dulbecco's modified medium (DMEM) (Invitrogen, CA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, CA), 100 U/ml penicillin (Invitrogen, CA) and 100 U/ml streptomycin (Invitrogen, CA) and 2 mM glutamine (Invitrogen, CA). Recombinant vaccinia virus (vTF7-3) that expresses T7 RNA polymerase was grown in HeLa cells. HPIV 3 (HA-1, NIH 47885) was propagated in CV-1 cells as described previously (De, et al., 1991, 1993) and used in the infection of Vero cells at a multiplicity of infection of 3 (MOI 3) for 48 hrs at 37° C. A polyclonal antiserum to the full length C protein was generated using custom antibody services (Covance Inc., PA). The anti-FLAG antibody conjugated to agarose and monoclonal anti-FLAG antibody was purchased from Sigma (Sigma, MO).

2.2 Plasmids and site-directed mutagenesis

The FLAG-epitope tagged wild type HPIV 3 C (Cwt) and SeV C plasmids (SeV C) have been described earlier (Malur, et al., 2004). The HPIV 3 C mutants harboring point mutations or deletions were generated by utilizing oligonucleotides and Cwt plasmid as template in a PCR involving Expand polymerase (Roche Biochemicals, IN). The amplified fragments were cloned into Cwt plasmid and the presence of specific mutations was confirmed by dideoxy sequencing.

2.3 Metabolic labeling with [³⁵S]-methionine or [³²P]-orthophosphate and immunoprecipitation of proteins

Metabolic labeling and immunoprecipitation analysis was performed as described by Malur et al., (2002). Briefly, HeLa cells in a 6-well plate were infected with vTF7-3 at a MOI of 1. At 1 hr post-infection, cells were transfected with empty plasmid (Mock) or transfected with Cwt, SeV C or various mutant plasmids as indicated. At 12 hrs post-infection, the medium was replaced with 2 ml methionine or phosphate deficient DMEM, and incubated for another 2 hrs at 37° C. At 24 hrs post-infection, cells were labeled with 100 µCi of [³⁵S]-methionine (1175 Ci/mmol, NEN, MA) or 500 µCi of [³²P]-orthophosphate (NEN, MA) in 2 ml of methionine or phosphate deficient DMEM for an additional 6 hrs. Cells were washed with PBS, and lysed in 250 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP 40) containing protease and phosphatase inhibitor cocktail (Sigma, MO). Cell lysates were immunoprecipitated with anti-FLAG antibody (Sigma, MO) conjugated to agarose beads following the manufacturer's protocol. The immune complexes were washed with lysis buffer, denatured in SDS-polyacrylamide gel sample buffer and resolved by SDS-PAGE followed by autoradiography. Relevant portions of the gel were quantified by Typhoon imaging system (GE Healthcare, NJ). Additionally, the expression levels of C proteins from [³⁵S]-methionine cell lysates were monitored following SDS-PAGE and Western blot analysis with anti-FLAG antibody (Sigma, MO).

2.4 Okadaic acid treatment of HeLa cells

Vaccinia virus infection and transfection of HeLa cells with Mock or Cwt plasmid was performed as mentioned above except cells were incubated with either DMSO alone (NT) or in the presence of 10 nM or 100 nM of okadaic acid (OA) (Calbiochem, NJ) during depletion and subsequent labeling in the presence of 100 μ Ci of [35 S]-methionine and 500 μ Ci of [32 P]-orthophosphate. Cell lysates were subjected to immunoprecipitation, SDS-PAGE analysis and autoradiography as described earlier.

2.5 Immunoprecipitation of C protein from HPIV 3 infected cells

Vero cells were infected with HPIV 3 at a MOI 3 for 1 hr and incubated for an additional 48 hrs at 37° C. After depletion for 2 hrs, cells were labeled with 100 μ Ci of [35 S]-methionine and 500 μ Ci of [32 P]-orthophosphate. At 6 hrs post labeling, cells were harvested and lysed as previously mentioned. Following incubation with polyclonal anti-C antiserum and protein A Sepharose beads (Invitrogen, CA), the immunoprecipitated C protein was resolved by SDS-PAGE and visualized by autoradiography. The expression of C protein from HPIV 3 infected cells was determined by Western blot analysis with polyclonal anti-C antiserum.

2.6 MALDI-TOF analysis

Following SDS-PAGE analysis and staining with Coomassie brilliant blue R 250, protein bands were excised, washed and destained in 50% ethanol. Gel slices were then dehydrated in acetonitrile and dried in a SpeedVac (Thermo Scientific, MA). In gel proteolytic digestion was accomplished by adding 100 ng of trypsin in 50 mM ammonium bicarbonate solution and incubating overnight at room temperature to achieve complete digestion. The peptides extracted from two aliquots of 30 μ l solution of 50% acetonitrile and 5% formic acid were combined, evaporated and resuspended in 30 μ l of 1% acetic acid for LC-MS analysis.

Samples were analyzed on a Finnigan LCQ-Deca ion trap mass spectrometer (Thermo Scientific, MA) equipped with a Protana microelectrospray ion source. The LCQ-MS ion source was interfaced to a self-packed 7cm $\times$ 75 μ m internal diameter Phenomenex Jupiter C18 reversed-phase capillary chromatography column. Two microliters of the extract was injected and peptides were eluted from the column by an acetonitrile/0.05 M acetic acid gradient at a flow rate of 0.2 μ l/minute. The system was operated at source and capillary voltages of 2.5 kV and 43 V, respectively, at 160° C. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. This mode of analysis produced approximately 2500 collision induced dissociation (CID) spectra of ions ranging in abundance over several orders of magnitude.

The entire CID spectra were used to search against the NCBI non-redundant databases using the search program MASCOT. All matching spectra were verified by manual interpretation. Further interpretation was aided by additional searches using the programs SEQUEST and BLAST as needed.

2.7 HPIV 3 minigenome assay

The precise determination of genomic and anti-genomic RNA synthesis in the HPIV 3 minigenome assay in vitro was achieved following modification of a previously described protocol (Hoffman and Banerjee, 2000). First, the firefly luciferase reporter gene present within the pMG(-) plasmid was replaced by a gene encoding the enhanced green fluorescent protein (eGFP) to obtain the pMG(-eGFP) plasmid. Second, eGFP RNA levels were measured by real-time RT-PCR analysis following isolation of total RNA and micrococcal nuclease resistant RNA to determine transcription and replication, respectively.

For transfection of plasmids, confluent monolayers of HeLa cells in a six well plate were infected with vTF7-3, at a MOI of 3. After 1 hr at 37° C, 1.25 µg of the pMG(-eGFP) minigenome plasmid and support plasmids pN, (1.130µg), pP (1.380 µg), pL (0.350 µg) and 0.500 µg of Cwt or the indicated mutant C plasmids were transfected using Lipofectin (Invitrogen, CA) according to the manufacturer's instructions. The total amount of transfected DNA was kept constant by including empty plasmid DNA where applicable. After 5hrs, the transfection medium was replaced with 1ml of DMEM-10% fetal calf serum. At 28 hrs post-transfection, total RNA was extracted using the RNeasy mini kit (Qiagen, CA) following the manufacturer's instructions and eluted in RNase free water.

For isolation of micrococcal nuclease resistant RNA, cells were transfected as mentioned above and scraped in culture media followed by centrifugation at 1000× *g* to obtain a pellet. Cells were resuspended in 50 µl of S7 nuclease buffer (20 mM Tris-HCl, pH 8.0, 5 mM NaCl, 2.5 mM CaCl₂) containing 50U of micrococcal nuclease (Takara Bio Inc., Japan) and incubated at 37° C for 30 min. At the end of the incubation period, RNA was extracted using the RNeasy mini kit (Qiagen, CA) as per the manufacturer's protocol and eluted in RNase free water.

2.8 Real time-RT-PCR analysis

The cDNAs corresponding to either eGFP mRNA and glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA or anti-sense eGFP mRNA and GAPDH mRNA were prepared separately in a single RT reaction containing primers specific for each of these RNAs (eGFP mRNA primer: 5' TGTGATCGCGCTTCTCGTT 3', anti-sense eGFP primer: 5' TGCTGCTGCCCCGACAA, and GAPDH primer: 5' ACTTGATTTTGGAGGGATCTCGCTCC 3'). The reaction mixtures were incubated at 42° C for 50 minutes in the presence of Super Script II reverse transcriptase enzyme (Invitrogen, CA).

For PCR quantitation assay, individual cDNAs (100 ng) were combined with each of the two eGFP primers (300 nM) and one fluorogenic primer (5' CCACTACCTGAGCACCC 3', 300 nM) in a 96 well plate to a final volume of 50 µl containing TaqMan Universal Mastermix (Applied Biosystems, CA). A similar reaction involving two GAPDH specific primers (300 nM) and one fluorogenic primer (5' CAAGCTTCCCGTTCTCAGCC 3', 300 nM) was performed for the amplification of GAPDH cDNA. All amplifications were performed in duplicate using ABI 7000 Sequence Detection and subjected to the 7900 default program comprising an incubation step of 50° C for 2 minutes, followed by heating at 95° C for 10 minutes to activate the AmpliTaq polymerase. Subsequent amplification was carried out at alternating temperatures of 95° C for 15 seconds and 60° C for 1 minute for a total of 40 cycles. The sense and anti-sense eGFP RNA levels were determined on the basis of a standard curve and compared to the relative amounts of GAPDH mRNA (ΔC_t) present in each sample after assuming that both eGFP and GAPDH genes were amplified with similar efficiencies. RNA samples were considered positive only if threshold cycle (C_t) values were less than 40 ($\Delta C_t = C_{t_{eGFP_{avg}}} - C_{t_{GAPDH_{avg}}}$). Results from two independent experiments were compared and relative eGFP RNA levels were calculated as fold change ($2^{-\Delta\Delta C_t}$) and subsequently expressed as relative percent replicon RNA levels compared to the RNA levels in the absence of Cwt plasmid.

3. Results

3.1 Phosphorylation of HPIV 3 C protein in vitro and in vivo

Based on our preliminary findings we first sought to confirm the phosphorylation status of the HPIV 3 C protein in vitro. To achieve this objective, HeLa cells were infected with vaccinia virus and transfected in duplicate with FLAG tagged Cwt and SeV C plasmids. At 12 hrs post

transfection, the culture media was replaced with methionine free or phosphate free DMEM and cells were further incubated with DMEM containing [^{35}S]-methionine or [^{32}P]-orthophosphate. At the end of the incubation period, C proteins were immunoprecipitated with anti-FLAG antibody coupled to agarose beads and analyzed by SDS-PAGE followed by autoradiography. As seen from Fig. 1A, a band corresponding to the predicted size of HPIV 3 C protein was immunoprecipitated from [^{32}P]-orthophosphate labeled cell lysates (lane 2, top panel) and also readily visible in [^{35}S]-methionine labeled cell lysates (lane 2, bottom panel). A similar immunoprecipitation analysis with the control SeV C plasmid, revealed the presence of two SeV specific polypeptides, C' and C, (lane 3, top panel). On the other hand, four polypeptides C', C, Y1 and Y2 were immunoprecipitated from [^{35}S]-methionine labeled cell extracts (lane 3, bottom panel). The results from our immunoprecipitation analysis of SeV C proteins in vitro were in accordance with the earlier report that conclusively demonstrated the phosphorylation of C' and C polypeptides and that C' is expressed at about 10% the level of the C protein (Hendrick, et al., 1993). In contrast, no bands could be detected from mock transfected cells (Fig. 1A, lane 1, top and bottom panel). This result confirmed our preliminary observation indicating that HPIV 3 C protein was indeed phosphorylated in vitro.

To ascertain the phosphorylation status of the HPIV 3 C protein in vivo, Vero cells were infected with HPIV 3 and labeled with [^{35}S]-methionine and [^{32}P]-orthophosphate followed by immunoprecipitation with a polyclonal anti-C antibody. As anticipated, the C protein was efficiently immunoprecipitated from both [^{32}P]-orthophosphate and [^{35}S]-methionine labeled HPIV 3 infected cell extracts (Fig. 1B, lane 2, top panel and middle panel, respectively). Moreover, Western blot analysis of HPIV 3 infected cell extracts revealed a normal expression of the C protein under these conditions. Taken together our results indicated that C protein was not only phosphorylated in vitro but also present in a phosphorylated form in HPIV 3 infected cells.

We next wanted to examine whether inhibition of host cellular phosphatases would enhance phosphorylation turnover and stability of the phosphorylated C protein in vitro. To achieve this objective, vTF7-3 infected HeLa cells were mock transfected (Mock) or transfected with the Cwt plasmid and incubated with DMSO alone (NT) or incubated in the presence of 10 nM or 100 nM of okadaic acid (OA), a potent inhibitor of serine-threonine phosphatase 2A (PP2A) (Bialojan and Takai, 1998). Labeling of cells with [^{35}S]-methionine and [^{32}P]-orthophosphate was performed in the presence of OA and cell lysates were subjected to immunoprecipitation as before and C proteins were visualized by autoradiography. As can be seen from Fig. 1C, a diffuse band with an apparent size corresponding to the C protein was immunoprecipitated from cell extracts following incubation with 10 nM OA (lane 3, top panel). Interestingly, the intensity of this band was further enhanced upon incubation with 100 nM of OA (lane 4, top panel). A comparison of band intensities by PhosphorImage analysis subsequently revealed a 21% and 57% increase in the phosphorylation levels of C protein at 10 nM and 100 nM of OA, respectively, as compared to cells transfected with Cwt and incubated with DMSO alone (lane 2, top panel) or mock transfected cells incubated in the presence of 100 nM of OA (lane 1, top panel). As anticipated, synthesis of C protein from [^{35}S]-methionine labeled cell extracts remained relatively constant (Fig. 1C, lanes 2 to 4, middle panel) indicating that treatment with OA enhances the phosphorylation of C protein. A Western blot analysis of cell lysates utilizing anti-FLAG antibody additionally confirmed a normal expression of C protein (Fig. 1B, bottom panel). These results clearly suggested that a host cellular activity was involved in regulating the phosphorylation of C protein. Additional experiments involving deletion and mutation analyses were undertaken to dissect the phosphorylation sites with the C protein.

3.2 Phosphorylation resides at the NH₂-terminus of C protein

Plasmids harboring deletions within the NH₂- and COOH-termini of the C protein were generated using a PCR based approach and manipulations involving standard molecular techniques. vTF 7-3 infected HeLa cells were transfected in duplicate with Cwt or plasmids containing three NH₂-terminal deletions, NΔ2–25, NΔ2–50, NΔ2–100 and four COOH-terminal deletions, CΔ24, CΔ49, CΔ74 and CΔ99 (Fig. 2A). After labeling with [³⁵S]-methionine and [³²P]-orthophosphate followed by immunoprecipitation with anti-FLAG antibody C proteins were separated by SDS-PAGE and visualized by autoradiography. As can be seen from Fig. 2B, bands corresponding to the predicted sizes of Cwt and deletion mutants were readily observed in cell lysates labeled with [³⁵S]-methionine (lanes 2 to 9). Interestingly, apart from the band observed for Cwt (lane 11), two additional bands corresponding to mutants NΔ2-25 (lane 12) and NΔ2-50 (lane 13) were also present in cell lysates labeled with [³²P]-orthophosphate. However, no band corresponding to mutant NΔ2-100 (lane 14) could be detected under these conditions. It is noteworthy to mention here that we routinely observed the presence of a non-specific band co-migrating with mutant NΔ2-25 (lane 12) and were unable to obtain a better resolution despite several attempts to overcome this problem. On the other hand, a clearer picture emerged upon immunoprecipitation of COOH-terminal deletion mutants using anti-FLAG antibody revealing a different scenario. As can be observed from Fig. 2B, mutants CΔ24, CΔ49, CΔ74 and CΔ99 which retained their NH₂-termini were not only readily immunoprecipitated from cell lysates labeled with [³⁵S]-methionine (Fig. 2B, lanes 6–9) but bands corresponding to these polypeptides were easily discernible from cell lysates labeled with [³²P]-orthophosphate (Fig. 2B, lanes 15–18). Although, a non-specific band was also seen co-migrating with mutant CΔ24 (lane 15), the presence of C-specific polypeptides was more conspicuous for mutants, CΔ49, CΔ74 and CΔ99 (Fig. 2B, lanes 16–18). Taken together, the results obtained from immunoprecipitation studies using deletion mutants clearly indicated that phosphorylation was restricted to the NH₂-terminus of C protein. Further experiments involving mass spectroscopy analysis were undertaken to precisely identify the amino acids involved in phosphorylation.

3.3 S7, S22 and S81 are predominantly phosphorylated

Tryptic peptides corresponding to wild type and CΔ74 mutant proteins were injected on a C18 reverse phase column of LCQ-Deca system and eluted with an acetonitrile gradient. The CID spectra and the sequence coverage map which ranged from 58%–76% indicated that the peptides were derived from a fragment lacking the COOH-terminal portion (CΔ74 mutant) and a fragment corresponding to the full length of the C protein, respectively. Three definitive phosphorylation site assignments of S7, S22 and S81 within three polypeptides ⁴TIKSWILGK¹², ¹⁴NQEINQLISPRPSTSLNSYSAPTPK³⁸ and ⁷⁹IDSLGHHTNVQK⁹¹, with mean peptide mass [MS⁺] 1126.28, 2824.03 and 1557.61, respectively, were confirmed through informative CID spectra (Fig. 3). However, the spectrum was unable to assign a definitive phosphorylation site between the adjacent S47 and T48 residues present within a fourth polypeptide ⁴³KTTQSTQEPSNSAPPSVNQK⁶² (Fig. 3). Additional observations from mass chromatograms for each of the phosphorylated peptides indicated that S7 phosphorylation was present only within the truncated protein (mutant CΔ74), while S81 phosphorylation was more extensive in Cwt. Results obtained from mass spectroscopy analysis along with data obtained from immunoprecipitation studies confirmed the existence of three amino acid residues S7, S22 and S81 within the NH₂-terminus as predominant phosphorylation sites within the C protein (Fig. 3). Studies involving site-directed mutagenesis of the individual S7, S22 and S81 residues were then undertaken to examine the role of phosphorylation on HPIV 3 minigenome replication. In the absence of any conclusive evidence on the phosphorylation status of S47 and T48 residues, a combined mutagenesis of S47 and T48 residues was included for subsequent site-directed mutagenesis studies and minigenome replication assays.

3.4 Mutant C proteins exhibit variable phosphorylation levels

Based on confirmatory evidence obtained from MALDI-TOF analysis, we decided to mutate the phosphorylation sites within the C protein. Accordingly, three individual mutants, S7A, S22A, S81A, one double mutant, S47AT48A, and a fifth mutant, devoid of all the phosphorylation sites, mutant 5A, was also included for further studies. Individual transfections of mutant plasmids into HeLa cells were performed as before and [³⁵S]-methionine and [³²P]-orthophosphate labeled C proteins were visualized by autoradiography. As can be observed from Fig. 4A, [³²P] labeled C proteins corresponding to Cwt (lane 1, top panel) and mutants S7A, S22A, S47AT48A and S81A (lanes 3–6, top panel) were visible. Moreover, as expected, mutant 5A, was barely visible under these conditions (see Fig. 4A, lane 7, top panel). The relative phosphorylation level of various C proteins were determined following PhosphorImage analysis and expressed as percent change relative to the basal level of phosphorylation observed with wild type C protein (see Fig. 4B). Accordingly, an elevation in phosphorylation levels by as much as 78%, 66% and 59% were observed for mutants S7A, S22A and S81A, respectively, as compared to the Cwt (Fig. 4B). Surprisingly, mutant S47AT48A exhibited the highest (101%) extent of phosphorylation. On the other hand, mutant 5A exhibited a 20% reduction in the phosphorylation level as compared to the basal phosphorylation level of Cwt (see Fig. 4B). As observed from Fig. 4A, synthesis and expression of mutant C proteins were relatively constant under these conditions (middle and bottom panels). Further studies utilizing mutant C proteins in the HPIV 3 minigenome assay were undertaken to examine the effect of phosphorylation on RNA synthesis.

3.5 Mutant C proteins display variable inhibitory activities

The ability of mutant C proteins to inhibit RNA synthesis was finally assessed by the HPIV 3 minigenome replication assay following transfection of Cwt and mutant C plasmids. The generation of eGFP mRNA from pMG(-eGFP) minigenome plasmid (transcription) was measured after isolation of total RNA and quantification by real time RT-PCR analysis. The assay was performed in duplicate and results from two independent experiments were calculated as fold change ($2^{-\Delta\Delta C_t}$) and expressed as relative percent eGFP mRNA levels compared to RNA levels in the absence of Cwt. The eGFP mRNA level in the absence of Cwt was arbitrarily set at 100%. As expected, eGFP mRNA level in the presence of Cwt was 55% while lowest levels of eGFP mRNA were observed for mutants S7A (29.5%) and S81A (31%) as compared to Cwt (55%) suggesting that S7A and S81A mutants exhibited a greater inhibitory activity than Cwt (Fig. 5A). On the other hand, no significant changes in eGFP mRNA levels could be detected for mutants S22A (51%) and S47AT48A (57%), however, a significant increase in eGFP mRNA level was observed for mutant 5A in comparison to Cwt (70%) (Fig. 5A).

In order to investigate the effect of mutations on minigenome replication, micrococcal nuclease resistant RNA was isolated following transfection with Cwt and mutant plasmids and subjected to real time RT-PCR analysis. The levels of eGFP mRNA and anti-sense eGFP mRNA in the absence of Cwt were arbitrarily set at 100%. No significant differences in eGFP mRNA levels were observed for mutants S7A (40%) and S22A (48%), although a slight increase was observed for mutant S47AT48A (62%) indicating that mutants S7A, S22A and S47AT48A retained a similar extent of inhibition as Cwt (51%). In contrast, a drastic increase in eGFP mRNA level was seen for mutants S81A (92%) and 5A (70%). On the other hand, analysis of anti-sense eGFP mRNA levels for all mutants and Cwt were relatively lower than the corresponding levels of their respective eGFP mRNA and varying from 20%–40% (Fig. 5B). Taken together these results suggested that mutations within the phosphorylation sites of the C protein exerted variable inhibitory activities thereby differentially affecting viral RNA synthesis during transcription and replication processes.

4. Discussion

The *Paramyxovirinae* C proteins have uniquely evolved to perform various functions. Studies on a number of viruses within this family have highlighted the role of C proteins in regulating virus replication (Cadd, et al., 1996; Tapparel, et al., 1997; Grogan and Moyer, 2001; Sweetman, et al., 2001; Reutter, et al., 2001; Bankamp, et al., 2005) and their ability to evade immune responses by interfering with host cellular proteins involved in interferon signaling pathways (see reviews: Bose and Banerjee, 2003; Gotoh, et al., 2001, 2002). In addition, C proteins have been shown to regulate apoptosis (Itoh, et al., 1998; Koyama, et al., 2003) and associate with host factors during virus assembly and budding (Hasan, et al., 2000; Sugahara, et al., 2004; Devaux and Cattaneo, 2004; Sakaguchi, et al., 2005).

Current studies to ascertain the phosphorylation status of the HPIV 3 C protein were undertaken based on our preliminary in vitro study as well as an earlier report on the phosphorylation of SeV C protein (Hendricks, et al., 1993). Since cells utilize phosphorylation/dephosphorylation mechanisms to regulate the activity of several proteins (see reviews: Whitmarsh and Davis, 2000; Yang, et al., 2003; Millward, et al., 1999; Garcia, et al., 2000), it was of interest to examine the phosphorylation status of the C protein. Immunoprecipitation studies clearly demonstrated phosphorylation of the C protein both in vitro and in vivo (see Figs. 1A & B, top panel) and further experiments utilizing OA in our assays revealed a dose-dependent increase in the phosphorylation level of the C protein (Fig. 1C, top panel) consistent with the fact that OA was able to partially and totally inhibit activities of PP1A and PP2A, respectively, at this concentration (Bailoian and Takai, 1998). Interestingly, OA treatment was also shown to differentially affect the phosphorylation turnover rates at several serine and threonine residues for respiratory syncytial virus P protein in vivo and in vitro (Asenjo, et al., 2005; Asenjo, et al., 2006).

The phosphorylation status of the C protein was subsequently determined by MALDI-TOF analysis which conclusively identified three serine residues at positions 7, 22 and 81 to be phosphorylated. However, the phosphorylation status of the adjacent S47 and T48 residues could not be verified (Fig. 3). A search for the putative kinase(s) capable of phosphorylating S7, S22 and S81 residues revealed three putative protein kinases, namely, casein kinase I, with consensus sequence $[S_p/T_p]-X_{2-3}-[S/T]-X$ to phosphorylate the S7 residue. A second kinase, protein kinase C, with a consensus sequence $[S/T]-X-[R/K]$, was likely involved in the phosphorylation of S22, while S81 residue was a target for phosphorylation by the beta-adrenergic kinase (Onorato, et al., 1991) with a consensus sequence $[D/E]_n-[S/T]-X-X-X$ (Fig. 3). Although, experimental evidence leading to the involvement of these kinase(s) needs to be verified, it should be noted here that phosphorylation mediated regulation of several phosphoproteins from non-segmented, negative strand RNA viruses have been well documented (De and Banerjee, 1997).

We then examined the phosphorylation status of mutant the C proteins, and surprisingly, all four mutant C proteins were found to exhibit higher levels of phosphorylation compared to the basal level observed for Cwt. The enhancement in the phosphorylation levels for these mutants seems to indicate that mutation of an individual serine residue at a specific site(s) augments the net phosphorylation level of the remaining site(s) within the C protein. This prompted us to generate mutant 5A and examine its phosphorylation status. As expected, a significant reduction in the phosphorylation level was observed for mutant 5A suggesting that mutation of all these specific phosphorylation sites within the C protein renders it incapable of undergoing phosphorylation.

We finally investigated the effect of these mutations on the C protein's ability to inhibit viral replication by utilizing the HPIV 3 minigenome replication assay for precise quantification of

genomic and anti-genomic RNA. Accordingly, low levels of RNA were observed for mutants S7A and S81A as compared to mutant S22A and Cwt. On the contrary, no changes in RNA levels were observed for mutant S47AT48A despite the fact that this mutant exhibited the highest phosphorylation level compared to all other mutants and Cwt (see Figs. 4B and 5A). It is noteworthy to mention here that we have been unable to distinguish whether S47 or T48 residues are individually phosphorylated. However, a significant increase in RNA level observed for mutant 5A suggests that a loss of phosphorylation results in elevated RNA synthesis leading to a lower degree of inhibition by the C protein. Additional experiments to determine eGFP mRNA and anti-sense eGFP mRNA levels following S7 nuclease treatment yielded interesting results. A dramatic increase in eGFP mRNA was observed for mutant S81A during replication, contrary to the low level of RNA during transcription. This result seems to suggest that mutant S81A is able to differentially regulate viral RNA synthesis indicating that mutations within the C proteins exert greater inhibitory activity at the genomic promoter than on the anti-genomic promoter (Tapparel, et al., 1997). Although, the precise mechanism(s) underlying the inhibition by mutant C proteins remains to be elucidated, it is likely that phosphorylation induced structural changes may affect binding of C protein with the viral polymerase complex (L-P). Interestingly, the presence of C-specific binding site(s) for both SeV and HPIV 3 L proteins has been reported (Horikami, et al., 1997, Cevik, et al., 2003, Smallwood and Moyer, 2004). Moreover, in the case of SeV C proteins, in vitro studies involving mutagenesis of charged amino acids to alanine and subsequent generation of recombinant SeV's harboring these mutations demonstrated that down regulation of RNA synthesis and interferon antagonism was differentially regulated by amino acid residues present within the C protein (Kato, et al., 2004, 2007). Furthermore, in the case of MV, a number of naturally occurring mutations within the C protein have been shown to regulate both genome replication and transcription (Bankamp, et al., 2005).

It is tempting to speculate here that mutations within the phosphorylation sites of HPIV 3 C protein may have an impact on the regulation of host interferon responses. In this context, experiments involving generation of mutant HPIV 3's and characterization of their replication profiles as well as their role in interferon antagonism shall prove beneficial in understanding the mechanism(s) regulating viral RNA synthesis and host immune responses.

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Fig. 1.

Phosphorylation of HPIV 3 C protein in vitro. (A). HeLa cells were infected with vaccinia virus (vTF 7-3) and mock transfected (Mock) or transfected with FLAG-tagged Cwt and Sendai virus C (SeVC) plasmids. At 12 hrs post infection, cells were labeled with [^{32}P]-orthophosphate (top panel) and [^{35}S]-methionine (bottom panel). Cell lysates prepared at the end of labeling period were immunoprecipitated with anti-FLAG antibody coupled to agarose beads and immunoprecipitated C proteins were analyzed by SDS-PAGE followed by autoradiography. The HPIV 3 C protein is denoted by an arrow. The SeV polypeptides, C', C, Y1 and Y2 are denoted by arrowheads. (B). Phosphorylation of HPIV 3 C protein in vivo. Vero cells were mock infected (Mock) or infected with HPIV 3 at an MOI of 3 for 48 hrs and cells were labeled with [^{32}P]-orthophosphate (top panel) and [^{35}S]-methionine (middle panel). The C protein from virus infected cells was immunoprecipitated with a polyclonal anti-C antiserum and analyzed by SDS-PAGE and autoradiography. The expression of C protein from virus infected cell extract was determined by Western blot analysis (bottom panel). (C). Effect of okadaic acid (OA) treatment on phosphorylation of HPIV 3 C protein in vitro. vTF 7-3 infected HeLa cells were mock transfected (Mock) or transfected with FLAG-tagged Cwt plasmid as mentioned above and either non-treated (NT) or treated with the indicated amount of okadaic acid (OA) and labeled in the presence of [^{32}P]-orthophosphate (top panel) and [^{35}S]-methionine (middle panel) medium containing OA. Following immunoprecipitation and SDS-PAGE, the C protein was visualized by autoradiography. An aliquot of cell lysates was subjected to Western blot analysis using anti-FLAG monoclonal antibody (bottom panel).

Fig. 2. Phosphorylation residues at the amino terminus of HPIV 3 C protein. (A). Schematic representation of wild type (Cwt) and mutant C proteins used in immunoprecipitation studies. The phosphorylation sites within the C proteins (determined after MALDI-TOF analysis) are indicated by asterisks (figure not drawn to scale). (B). Vaccinia virus infected HeLa cells were mock transfected (Mock) or transfected with FLAG-tagged Cwt and indicated FLAG-tagged mutant C plasmids. [³²P]-orthophosphate and [³⁵S]-methionine labeling and immunoprecipitation was performed as mentioned in Fig. 1A. The positions of Cwt and mutant C proteins are indicated by arrowheads.

FIG. 3.

Summary of the phosphorylation sites of HPIV 3 C protein-Results of MALDI-TOF analysis. Phosphorylation sites (asterisk) within tryptic peptides (underlined) identified by MALDI-TOF analysis of HPIV 3 C protein are shown. The mean mass [MH⁺] and the putative kinase involved in the phosphorylation is indicated under each peptide. (CK I, casein kinase I, PK-C, protein kinase C, BARK- beta adrenergic receptor kinase).

Fig. 4.

(A) Phosphorylation status of mutant C proteins. vTF7-3 infected HeLa cells were mock transfected (Mock) or transfected with FLAG-tagged Cwt and indicated FLAG-tagged mutant C plasmids. Following labeling with [^{32}P]-orthophosphate (top panel) and [^{35}S]-methionine (middle panel) the immunoprecipitated C proteins were resolved by SDS-PAGE and visualized by autoradiography. The expression of C protein was determined by Western blot analysis with anti-FLAG antibody (bottom panel). The positions of Cwt and mutant C proteins are indicated by arrows. (B). Relevant portions of the gel from Fig. 4A (top panel) were subjected to PhosphorImage analysis and band intensities were expressed as percent phosphorylation with respect to Cwt.

Fig. 5.

(A) Activity of mutant C proteins in HPIV 3 minigenome assay. vTF7-3 infected HeLa cells in duplicate were transfected with minigenome plasmid, pMG(-eGFP), and support plasmids pN, pP, pL along with either empty vector control (+L-C), Cwt or mutant C plasmids. The eGFP mRNA (transcription) was measured after extracting total RNA at 28 hrs post transfection and anti-sense primer mentioned in Materials and Methods. For PCR quantitation, individual cDNAs were combined with the corresponding primers and amplification was performed using ABI 7000 Sequence Detection using the 7900 default program comprising of 50°-2 min, 95° C-10 min, 95° C-15 sec, 60° C-1 min. Standard curve and values were compared to the relative amounts of GAPDH mRNA. Results from two independent experiments were

compared and relative eGFP mRNA levels were calculated as fold change ($2^{-\Delta\Delta C_t}$) and expressed as percent compared to eGFP mRNA levels in the absence of Cwt. (B). For analyzing HPIV 3 minigenome replication a similar transfection protocol was utilized and RNA extracted post micrococcal nuclease treatment was subjected to similar conditions of cDNA synthesis using eGFP mRNA and anti-sense eGFP primers and real time RT-PCR analysis. Error bars represent S.D. between duplicate samples.