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Short communication

Analysis of rotavirus non-structural protein NSP5 by mass spectrometry reveals a complex phosphorylation pattern

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ABSTRACT

Genomic replication and partial assembly of Rotavirus takes place in cytoplasmic viral structures called viroplasms. NSP5 is a viral phosphoprotein localized in viroplasms and its expression is imperative for viral cycle progress. During infection three isoforms of NSP5 can be observed by SDS-PAGE (26, 28 and 33–35 kDa) and previous reports suggested that they differ in their phosphorylation patterns. In this study we obtained NSP5 from infected cells and by mass spectrometry we were able to identify nine phosphorylation sites. We detected that in all the isoforms the same residues can be found either phosphorylated or unmodified. Quantitative analysis showed that the 28 kDa isoform has a higher phosphorylation level than the 26 kDa isoform suggesting that migration properties depend on the total number of phosphorylated residues. Moreover, we identified two not previously described modifications for this protein: an N-acetylation in Serine-2 and an intramolecular disulfide bond in a highly conserved motif, CXXC which is located between two charged alpha-helix motifs.

Rotavirus, a member of the *Reoviridae* family, is the major cause of viral gastroenteritis in young children (Parashar et al., 2003). Its genome is made up of 11 segments of dsRNA. After entering the cell, it loses the outer protein layer and the resulting double-layered particles promote transcription of the viral mRNA (Estes, 2001). Genome replication (dsRNA synthesis using mRNA as template), assortment and partial assembly of new particles occurs in cytoplasmic viral structures called viroplasms. These are composed of the structural proteins VP1, VP2, VP3 and VP6 and the non-structural proteins NSP5 and NSP2 (Gallegos and Patton, 1989). These non-structural proteins are able to aggregate and co-localize when expressed in cells (Fabbretti et al., 1999). Previous data indicate that NSP5 and ssRNA compete for the interaction with NSP2 (Jiang et al., 2006).

Three NSP5 isoforms are detected in SDS-PAGE assays (26, 28 and 35 kDa) and all of them are phosphorylated in serine residues (Afrikanova et al., 1996; Blackhall et al., 1997). Treatment of immunoprecipitated NSP5 with phosphatases eliminates the presence of hyperphosphorylated (28–35 kDa) isoforms and increases the amount of the 26 kDa band, suggesting a correlation between its degree of phosphorylation and its mobility properties; importantly, the 26 kDa isoform retains some phosphate groups after treatment (Afrikanova et al., 1996; Blackhall et al., 1997; Poncet et al., 1997). No polyphosphates have been found attached to NSP5 (Afrikanova et al., 1996). Experimental data suggest that NSP5 phosphorylation is enhanced by the presence of NSP2 (Afrikanova et al., 1998).

NSP5 has ATPase (Bar-Magen et al., 2007) and autophosphorylation activity (Vende et al., 2002) but cellular kinases seem to be required for NSP5 hyperphosphorylation (Campagna et al., 2007; Eichwald et al., 2002). In fact, in vitro, NSP5 is phosphorylated by members of the casein kinase (CK) family (Eichwald et al., 2002, 2004) and treatment of infected cells with PKC inhibitors decreases the amount of hyperphosphorylated isoforms (Blackhall et al., 1998) and increases translation of viral proteins (Chnaiderman et al., 2002). On the other hand, phosphatase inhibitors increase the intensity of heavy forms of NSP5, suggesting a balance of kinase and phosphatase activities in NSP5 phosphorylation (Blackhall et al., 1998; Sen et al., 2006).

Since the exact sites of NSP5 phosphorylation have not been identified, we analyzed the 26, 28, and 35 kDa isoforms of NSP5 by mass spectrometry (MS) to identify post-translational modifications which occurred during infection.

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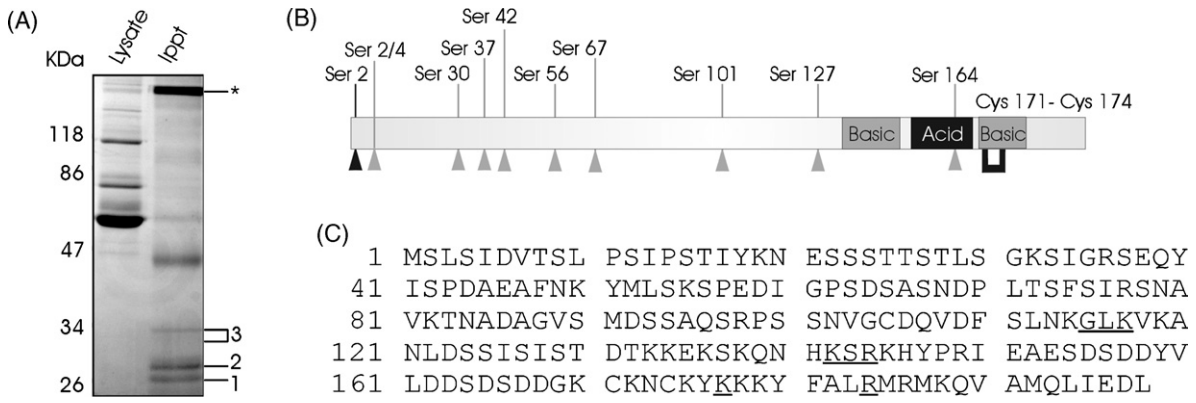

Fig. 1. NSP5 modifications and protein coverage. (A) SDS-PAGE including the immunoprecipitation (Ippt) and lysate used for immunoprecipitation (Lysate). Bands used for MS analysis (1, 2 and 3) and the non-reduced antibody (*) is indicated on the right side. Additional bands correspond to other proteins previously described as interacting with NSP5. (B) Scheme showing NSP5 post-translational modifications identified in NSP5 isoforms: black triangle, acetylation; gray triangles, phosphorylation; U, disulfide bond. (C) Coverage of NSP5 was 95% (189/198), using different proteases. Non-mapped residues are underlined. Coverage was the same for 26, 28 and 35 kDa isoforms.

1. NSP5 purification and analysis

MA104 cells were infected with rotavirus SA11-4F (G1P[6]) at a multiplicity of infection of 20. Extracts were prepared in immunoprecipitation buffer (50 mM Tris pH 8, 125 mM NaCl, 30 mM KCl, 20 mM EDTA, 0,1% Triton X-100, 0,05% SDS and 2 μg/mL aprotinin), and NSP5 was isolated by immunoprecipitation with a guinea pig anti-NSP5 polyclonal antibody (Vende et al., 2002). We performed the isolation at 6 h post-infection, when all the isoforms are evident (Blackhall et al., 1998). Different isoforms were cut from non-reducing polyacrylamide gels stained with Coomassie Blue (Fig. 1A) and each isoform was digested in gel with either trypsin, chymotrypsin, trypsin + chymotrypsin or Glu-C. It is important to note that phosphate groups are stable during these treatments. The excised protein bands were washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The gel pieces were reswollen in 15 μL of 5 mM ammonium bicarbonate containing 50 ng trypsin (Promega) and incubated at 37 °C, overnight. In the case of chymotrypsin, excised protein bands were incubated with 70 ng of enzyme (Boheringer) in 15 μL of 5 mM ammonium bicarbonate for 20 h at 26 °C. For Glu-C digestion, the gel slices were incubated in 10 μL of 0,03 μg/μL Glu-C in 5 mM ammonium bicarbonate solution for 20 h at 26 °C. When multiple enzymes were used, the slices were incubated with chymotrypsin for 18 h at 26 °C and then incubated with trypsin for 6 h at 37 °C. In all cases the peptides were extracted using 10 μL of 0.5% (v/v) trifluoroacetic acid (TFA) in acetonitrile, the samples were sonicated for 5 min, and the separated liquid was dried under vacuum. The samples were reconstituted in 5 μL of 0.1% (v/v) TFA and 6% (v/v) acetonitrile in water. Then, the peptide mixtures were analyzed by mass spectrometry using nanoLC ESI MS/MS with a Q-Tof Ultima instrument equipped with a CapLC (Micromass-Waters, Manchester, UK) as described (Bauer and Krause, 2005). To perform MS/MS experiments, automatic MS to MS/MS function switching (survey scanning) was employed. For identification of phosphorylated NSP5 peptides, the processed MS/MS spectra (MassLynx version 4.0 software) were compared with the theoretical fragment ions of NSP5 peptides using the MASCOT server version 2.0 (Matrix Science Ltd., London, UK). Most of the analyses were done against sequence database Swissprot, allowing 3–5 misscuts and mass tolerance of 0.07 Da; in some cases, like chymotrypsin and trypsin/chymotrypsin cuts, more misscuts were allowed. For identification of peptide sequences associated to specific masses, we included in the analysis different theoretical post-translational modifications, namely: acetylations, methylations, different types of phosphorylations, and different types of O-glycosylation. Additionally, possible modifications during isolation procedure like methionine oxidation and cystein alkylation (propionamide) were included in the analysis. All modified peptides detected during this analysis are described in Supplementary Table 1.

2. NSP5 phosphorylation sites

MS analysis revealed nine serine phosphorylated sites distributed across the whole protein (Fig. 1B) present in all NSP5 isoforms, except for serines 30 and 37, which were not phosphorylated in the 35 kDa protein (Table 1). The MS/MS spectra interpretations for each phosphorylation site are shown in Fig. 3 and in Supplementary Figs. 1–7. Using different enzymatic digestions almost 95% of the NSP5 sequence was covered by MS experiments, leaving only one possible phosphorylation position (Ser-143) not analyzed (Fig. 1C). The presence of multiple phosphorylation sites is in agreement with previous results showing several spots in 2D gels of a tryptic digestion of 28 kDa isoform (Blackhall et al., 1997). Previous results show divergent results regarding the presence of phosphothreonine residues in NSP5 (Afrikanova et al., 1996; Blackhall et al., 1997). In our analysis we did not found any phosphorylated threonine or tyrosine residues, despite the high sensibility of MS method and the stability of these O-phosphorylated residues (Reinders and Sickmann, 2005).

Unexpectedly, we did not find any phosphorylation position exclusive for a specific isoform, indicating that electrophoretic

Table 1
Phosphorylated serines identified in NSP5.

Position[a]	Isoform			Enzyme[b]	%Phosphorylation[c]	
	26	28	35		26	28
2 or 4	+	+	+	G,T	nc	nc
30	+	+	–	T	1.9	2.6
37	+	+	–	T,C	nc	nc
42	+	+	+	T	8.4	20.0
56	+	+	+	T	nc	nc
67	+	+	+	T	4.7	6.1
101	+	+	+	T,C	61.5	80.0
127	+	+	+	T	0.7	4.0
164	+	+	+	C	73.3	83.3

nc: not calculated.
[a] Refers to SA11 sequence (AAK15267).
[b] Peptides found by digestion with chymotrypsin (C), Glu-C (G) and trypsin (T).
[c] Calculated by the following formula: 100 × Pho/(Pho + NoPho) where "Pho" means intensity of phosphorylated peptide and "NoPho" means intensity of non-phosphorylated peptide.

migration is not related to specific phosphorylation events. To clarify this issue, we determined the degree of phosphorylation at individual sites by measuring relative peak intensity ratios of the phosphorylated and non-phosphorylated peptides (Table 1). Calculation of the degree of phosphorylation for individual phosphorylation sites of NSP5 isoforms was based on direct comparison of signal intensities of extracted ion chromatograms measured for each digestion experiment as recently described (Seidler et al., 2009). The mass window for all extracted ion chromatograms was 0.15 Da. Signal intensities were taken from peak maxima of phosphorylated and non-phophorylated peptide ions. For calculation of intensity ratios all major charge states were considered. Quantification was not possible for the 35 kDa isoform due to the interference of co-immunoprecipitated NSP2. Most peptides showed low phosphorylation levels and higher ratios were observed for every residue in the 28 kDa protein compared to the 26 kDa isoform. This increase was also detected at different times of infection (Supplemental Figure 8).

In spite of possible non-detected phosphorylation sites (notably Ser-143), our results suggest that NSP5 migration is driven by total phosphorylation levels rather than phosphorylations in specific residues. In this scenario, it is not possible to explain isoform formation by a sequential phosphorylation of NSP5. Moreover, considering that MS analysis is done with protein fragments and that each NSP5 isoform is composed of a mixture of variants with differential isoelectric points (Blackhall et al., 1997), each isoform may include molecules with many phosphorylation patterns.

Previous reports using recombinant protein suggested that region 130–180 of NSP5 (specifically serines 154, 156, 164 and 166) is the hyperphosphorylation substrate (Eichwald et al., 2002); in our analysis we found only Ser-164 phosphorylated and no substantial difference in phosphorylation ratio was observed between the 26 and 28 kDa isoforms. It is feasible that deletion mutants previously used affected the protein folding and exposed non-functional phosphorylation substrates.

Serine 164 is in a highly acidic region that has been previously implicated in the interaction with a basic groove of NSP2 (Jiang et al., 2006). Our results showed that this residue has the highest phosphorylation level (Table 1) and this modification could improve NSP5 affinity for NSP2 by increasing the negative charges. Interestingly, serine 164 is highly conserved and group C NSP5 has a glutamate residue in this position (Fig. 2), which can be considered a conservative mutation compared to a phosphorylated serine.

Another model proposes that NSP2–NSP5 interaction drives phosphorylation of serine 67 by CK1, inducing hyperphosphorylation (Eichwald et al., 2004), but this does not fit with findings showing hyperphosphorylation of the non-soluble form of NSP5 in the absence of serine 67 (Sen et al., 2006). Additionally, it has been shown that depletion of CK1α diminishes the formation of both 28 and 35 kDa isoforms (Campagna et al., 2007). Our data showed low levels of phosphorylated Ser-67 in 26 and 28 kDa isoforms, suggesting that there is no direct correlation between this event and the first mobility shift. A possible mechanism to explain these results is that phosphorylated Ser-67 is a transient state of the protein that acts like an activator inducing other phosphorylation events; this intermediate state could be target of phosphatases that regulates Ser-67 phosphorylation levels (Blackhall et al., 1998). In any case, conservation of this serine between group A and group C NSP5 (despite the low similarity between them in the whole protein, Fig. 2) suggests an important role for this residue.

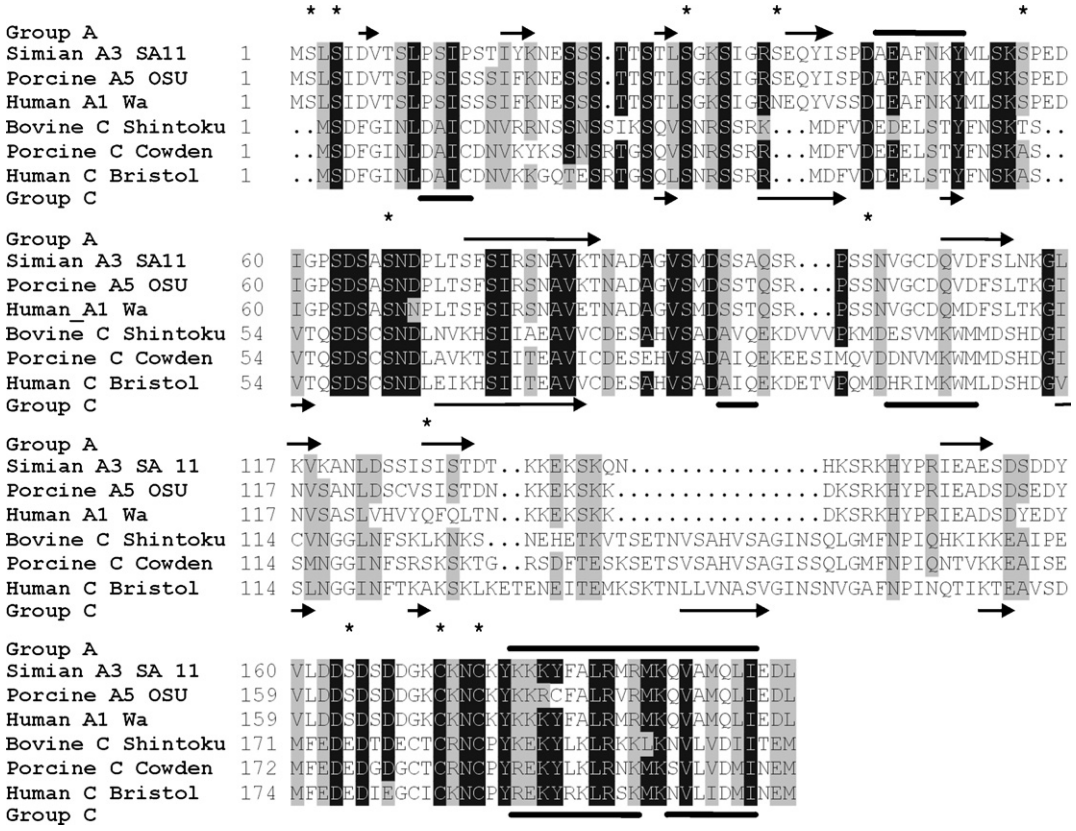

Fig. 2. Sequence alignment of group A and C NSP5. Identical and similar residues are highlighted in black and gray, respectively. The asterisks indicate modified residues. Sequences are from Genbank: Simian A3 SA11 (AAK15267), Porcine A5 OSU (P19715), Human A1 Wa (V01191), Bovine C Shintoku (P34718), Porcine C Cowden (P36358) and Human C Bristol (Q00682). The secondary structure was predicted using JPRED (Cuff et al., 1998) using SA11 for group A and Bristol for group C. Structure prediction of group A is in the upper side of the alignment and the structure prediction of group C is in the lower side of the alignment. Arrows mean beta sheet and lines mean alpha helix. Similarity of NSP5 between group A and C is around 20%.

3. Additional NSP5 post-translational modifications

Mass spectrometry demands good knowledge of the sequence and modifications of the analyzed protein, to recognize corresponding sequences of an identified mass. In our analysis we included several theoretical modifications (acetylation, methylation, different amino acid phosphorylations and many types of O-glycosylations) to increase the protein coverage. It has been reported that the NSP5 from strain OSU is O-glycosylated with a N-acetylglucosamine residue (NAG) (Gonzalez and Burrone, 1991); in our analysis, using strain SA11-4F, we included the possible presence of NAG residues, however, no peptides with this modification were found.

Fig. 3A shows the MS/MS spectrum of a peptide ^{2}SLSIDVTSLPSIPSTIYK^{19}; the b ions correspond to N-terminal fragment ions. The presence of a b2 signal with an increase of 42 Da correspond to the N-terminal acetylation in serine 2. Additionally, we observed b ions (b3 to b6) with decreased mass of 56 Da which correspond to acetylated N-terminal fragment ions (+42) and showing a neutral loss of phosphoric acid (−98 Da), indicating that peptides were acetylated and phosphorylated.

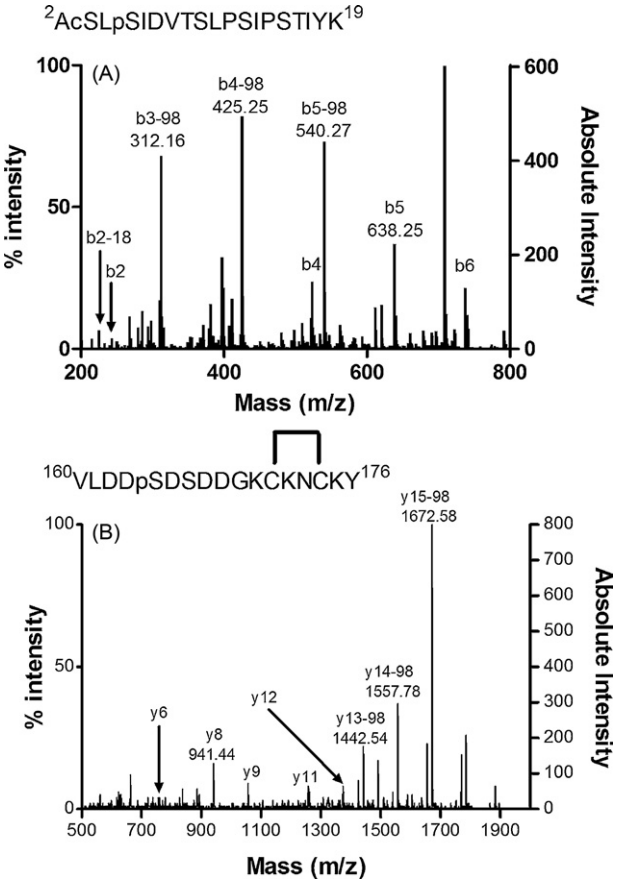

Fig. 3. Identification of N-terminal acetylation and disulfide bond. (A) MS/MS spectra of the tryptic peptide ^{2}SLSIDVTSLPSIPSTIYK^{19} with an m/z of 1021.99. Relevant ions are labeled according to nomenclature proposed by Biemann and Scoble (1987) in which the b ions correspond to N-terminal fragment ions. The increase of 43 Da from the predicted mass in b2 corresponds to an N-terminal acetylation in serine 2. The acetylated and phosphorylated ions b2-55 to b6-55 present a decreased mass of 55 (−98 Da of the phosphorylation + 43 Da acetylation). (B) MS/MS spectra of the chymotryptic peptide ^{160}VLDDSDSDDGKCKNCKY^{176} with an m/z of 661.58 (y ions correspond to C-terminal fragment ions). The y6 to y12 ions showing a mass decrease of 2 Da indicate the formation of a disulfide bond between cysteine 171 and 174. The presence of ions y13-100 to y15-100 with a decreased mass of 100 also shows a neutral loss of 98 Da (phosphorylation in serine 164) and 2 Da (disulfide bond).

However, the presence of only weak acetylated b2-98 signal prevent a clear assignment of phosphorylation to either serine 2 or 4. N-terminal acetylation occurs co-translationally when 20–50 amino acids are extruding from the ribosome, after methionine removal. This is a very common covalent modification that can be involved in the protection of proteins from degradation and regulation of their biological functions, stability, and interactions with other proteins and/or specific peptide receptors (Polevoda and Sherman, 2000). Also in this case, high conservation of the Ser-2 (Fig. 2) suggests a possible conserved function.

Fig. 3B shows the fragment ion spectrum of the ^{160}VLDDSDSDDGKCKNCKY^{176} peptide; y ions correspond to C-terminal fragment ions. Ions y6 to y12 reveal a mass decrease of 2 Da, indicating the formation of an intramolecular disulfide bond between cysteines 171 and 174. The presence of C-terminal fragment ions y13 to y15 which show a neutral loss of 98 Da, indicating that position y13 (Ser-164) is phosphorylated. Remarkably, the intramolecular disulfide bond was observed in all isoforms (Supplementary Table 1).

We cannot rule out the possibility of the disulfide bond being formed during the protein isolation. However, our purification procedure induces alkylation of free cysteines and this is reflected by the fact that the only remaining free cysteine (Cys-105) was found highly alkylated (Supplementary Table 1), up to 90%. Cysteines 171 and 174 were never found either alkylated or reduced (in any isoform) which strongly suggests that this modification was present prior to purification.

Since this structure includes only two amino acids between the cysteines, it is probably bending the protein backbone forming a typical 14-membered disulfide ring (Ren et al., 1998). Given that this structure is in the limit between an acidic and a basic region (Fig. 1B), it can be proposed that it could be stabilizing a specific domain.

Most of cytosolic proteins do not form disulfide bonds due to the reducing activity of thiol-disulfide oxidoreductases which oxidizes its own CXXC motif (Rietsch and Beckwith, 1998). Interestingly, the disulfide bond in NSP5 is present in a CXXC motif which could facilitate its stability in the cytosolic environment. In thiol-disulfide oxidoreductases the CXXC motif is usually followed by an α-helix structure (Iqbalsyah et al., 2006); previous predictions of secondary structure have shown an α-helix motif close to the C-terminal region of the disulfide bond in NSP5 (Sen et al., 2007; Torres-Vega et al., 2000).

It has been previously described that changes in residues that separate the two cysteines dramatically influence redox potentials and pK_a values of cysteines in CXXC motifs, altering their ability to form disulfide bonds (Chivers et al., 1996, 1997). Remarkably, for NSP5, not only the α-helix structure, but also the cysteines that participate in the disulfide bond and the residues between them are highly conserved between groups A and C (Fig. 2). We conclude that the disulfide bond is highly conserved and suggest that it is relevant for the protein function. Noteworthy, both cysteines are not needed for NSP5 multimerization (Torres-Vega et al., 2000).

In this study we analyzed for the first time post-translational modifications of NSP5 by mass spectrometry in the context of viral infection. Our results differ with some previous reports probably due to the source of protein for analysis: in this study we used protein from SA11-4F infected cells compared to other authors that used either recombinant partial proteins or cells infected with other viral strains. We did not detect any O-linked carbohydrate in the protein, but we cannot rule out that the serine 143 is glycosylated, since we were not able to cover it in our experiments.

We showed that NSP5 phosphorylation follows a complex pattern in which several sites are modified and may contribute to shift its electophoretical migration. Even though that the degree of phosphorylation differs in each isoform, no isoform-specific phos-

phorylation site could be identified. These results suggest that the mobility shift of NSP5 is produced by the increase of phosphorylation rates of many residues rather than a specific phosphorylated residue. This lead to the hypothesis that each isoform of NSP5 is composed by many variants that possess almost the same number of phosphorylations distributed in different sites. In addition, we identified an intramolecular disulfide bond in the context of a highly conserved oxydoreductase motif and an N-terminal acetylation. These findings raise new questions regarding the NSP5 structure and function in the context of the rotavirus infecting cycle.

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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.virusres.2009.12.006.

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