Impaired hyperphosphorylation of rotavirus NSP5 in cells depleted of casein kinase 1α is associated with the formation of viroplasms with altered morphology and a moderate decrease in virus replication

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The rotavirus (RV) non-structural protein 5, NSP5, is encoded by the smallest of the 11 genomic segments and localizes in ‘viroplasms’, cytoplasmic inclusion bodies in which viral RNA replication and packaging take place. NSP5 is essential for the replicative cycle of the virus because, in its absence, viroplasms are not formed and viral RNA replication and transcription do not occur. NSP5 is produced early in infection and undergoes a complex hyperphosphorylation process, leading to the formation of proteins differing in electrophoretic mobility. The role of hyperphosphorylation of NSP5 in the replicative cycle of rotavirus is unknown. Previous in vitro studies have suggested that the cellular kinase CK1α is responsible for the NSP5 hyperphosphorylation process. Here it is shown, by means of specific RNA interference, that in vivo, CK1α is the enzyme that initiates phosphorylation of NSP5. Lack of NSP5 hyperphosphorylation affected neither its interaction with the virus VP1 and NSP2 proteins normally found in viroplasms, nor the production of viral proteins. In contrast, the morphology of viroplasms was altered markedly in cells in which CK1α was depleted and a moderate decrease in the production of double-stranded RNA and infectious virus was observed. These data show that CK1α is the kinase that phosphorylates NSP5 in virus-infected cells and contribute to further understanding of the role of NSP5 in RV infection.

INTRODUCTION

Rotaviruses are non-enveloped, triple-layered particles (TLPs) that contain a genome of 11 segments of double-stranded RNA (dsRNA) encoding six structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6). Early viral morphogenesis is concentrated in discrete cytoplasmic inclusion bodies called ‘viroplasms’, in which VP1, VP2, VP3 and VP6 accumulate, together with two of the non-structural proteins, NSP2 and NSP5.

Both NSP2 and NSP5 are essential for virus replication. In cells in which NSP5 is depleted by specific RNA interference against NSP5 mRNA (Campagna et al., 2005; Lopez et al., 2005) or neutralized by anti-NSP5-specific intrabodies (Vascotto et al., 2004), there is no formation of viroplasms and no virus replication. Similar results were obtained by blocking NSP2 production using specific small interfering RNA (siRNA; Silvestri et al., 2004).

Rotavirus NSP5, which is encoded by RNA segment 11, is a protein of 196–198 aa (depending on virus strain) with a high proportion of serine (21%) and threonine (4.5%). NSP5 has been shown to interact with NSP2 and the viral polymerase VP1 (Afrikanova et al., 1998; Arnoldi et al., 2007). NSP5 undergoes post-translational modifications that include cytoplasmic O-glycosylation (Gonzalez & Burrone, 1991) and a complex pattern of hyperphosphorylation (Afrikanova et al., 1996; Blackhall et al., 1997). As a consequence, NSP5 from virus-infected cells appears, after SDS-PAGE, as two major bands of 26 and 28 kDa and a series of slower-migrating bands with apparent molecular masses between 30 and 34 kDa. It has been shown that the 26 kDa form of NSP5 is the precursor of the higher-molecular-mass forms, corresponding to species characterized by increasing levels of phosphorylation (Afrikanova et al., 1996; Blackhall et al., 1997). Several studies that have addressed the mechanism of NSP5 phosphorylation...
(Afrikanova et al., 1998; Eichwald et al., 2002, 2004a) suggested that this is a multi-step process that requires activation of the NSP5 substrate. When expressed alone, NSP5 is mostly non-phosphorylated, whilst interaction with NSP2 was shown to induce a phosphorylation cascade (Afrikanova et al., 1998), with phosphorylation of Ser 67 by cellular kinases as a necessary first step (Eichwald et al., 2004a). Based on in vitro phosphorylation assays, casein kinase 1α (CK1α) was proposed to be responsible for the phosphorylation of Ser 67 (Eichwald et al., 2004a). In addition to the interaction with NSP2, activation of the substrate activity of NSP5 using particular NSP5 deletion mutants was also possible. One such mutant (Δ2), lacking region 2 (aa 34–80), is not phosphorylated when expressed alone in vivo, but becomes hyperphosphorylated when co-expressed with Δ3, which lacks aa 81–130, a mutant that is phosphorylated per se and that can function as an activator of Δ2 phosphorylation (Eichwald et al., 2002, 2004a). The activation function of Δ3 was shown to depend on the phosphorylation of Ser 67. Mutants in which Ser 67 was mutated to Ala were unable to activate phosphorylation of Δ2 (Eichwald et al., 2004a). In contrast, a mutant of NSP5 with aspartic acid at position 67 (NSP5-S67D), which mimics a phosphorylated serine, becomes hyperphosphorylated when expressed alone. NSP5 phosphorylation requires dimerization, as demonstrated by the need for the C-terminal tail (a region known to promote dimerization) both in activator and substrate molecules (Eichwald et al., 2004a).

The CK1 kinases represent a separate group within the superfamilies of serine/threonine-specific protein kinases. Members of this family are characterized by being constitutively active as monomers that use ATP as the sole source of phosphate. In vertebrates, seven members of the CK1 family have been identified (Burzio et al., 2002), which lacks aa 81–130, a mutant that is phosphorylated per se and that can function as an activator of Δ2 phosphorylation (Eichwald et al., 2002, 2004a). The activation function of Δ3 was shown to depend on the phosphorylation of Ser 67. Mutants in which Ser 67 was mutated to Ala were unable to activate phosphorylation of Δ2 (Eichwald et al., 2004a). In contrast, a mutant of NSP5 with aspartic acid at position 67 (NSP5-S67D), which mimics a phosphorylated serine, becomes hyperphosphorylated when expressed alone. NSP5 phosphorylation requires dimerization, as demonstrated by the need for the C-terminal tail (a region known to promote dimerization) both in activator and substrate molecules (Eichwald et al., 2004a).

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METHODS

Cell culture and virus propagation. MA104 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS; Invitrogen), 2 mM L-glutamine and 50 µg gentamicin ml⁻¹ (Invitrogen).

Stably transfected cells (NSP5–EGFP) were cultured in medium supplemented with 500 µg genetin (G-418) ml⁻¹ (Invitrogen) as described previously (Afrikanova et al., 1996; Eichwald et al., 2004b).

Simian rotavirus strain SA11 (G3, P[6]) and porcine rotavirus strain OSU (G5, P[9]) were propagated in MA104 cells as described previously (Estes et al., 1979).

Viral titres were determined by plaque assay as described previously (Graham et al., 1987). Recombinant vaccinia virus vTF7.3 was propagated in HeLa cells as described previously (Fuerst et al., 1986).

siRNAs, plasmids and transfections. The siRNA against CK1α, which has been used previously (Hino et al., 2003; Liu et al., 2002), was chemically synthesized with a 3’ TT deoxynucleotide overhang on both strands and obtained as annealed duplexes (IBA). The sequence of the irrelevant siRNA (sirI) is CAGGUGUGUGCAAGUCCTT.

The plasmids pcDNA3-NSP5 and pcDNA3-NSP2 and the deletion mutants pcDNA3-SV5Δ2 and pcDNA3-A3 have been described previously (Eichwald et al., 2004b; Fabbretti et al., 1999). pcDNA3-NSP5/S67D (where serines at positions 63 and 65 are mutated to alanine and the serine at position 67 to aspartic acid), pcDNA3-NSP5/S67A (where only serine 67 is mutated to alanine) and pcDNA3-NSP5a (where all three serines in position 63, 65 and 67 are mutated to alanine) were described previously (Eichwald et al., 2004b).

Cells (2.5 × 10⁵) were transfected with 2 µg siRNA in 1 ml serum-free medium containing 5 µl Transfectam reagent (Promega). Six hours after transfection, cells were washed twice with serum-free medium and incubated for an additional 34–48 h in medium supplemented with 10% FCS.

Recombinant vaccinia virus vTF7.3 was used to obtain efficient transient expression of genes under the control of the T7 promoter. Cells were transfected with siRNAs as described above and, 32 h after transfection, were infected with recombinant vaccinia virus at 20 p.f.u. per cell in serum-free medium. One hour after vaccinia virus infection, the cells were washed, transfected with 1 µg each plasmid and 2.5 µl Lipofectamine 2000 (Invitrogen) and incubated for an additional 16 h.

Antibodies. NSP5, NSP2 and VP1 antisera were produced in guinea pigs and rabbits with glutathione S-transferase or His fusion proteins as described previously (Gonzalez & Burrowe, 1991). A mAb against tubulin and a polyclonal antibody against actin were obtained from Sigma. CK1α goat polyclonal antibody was obtained from Santa Cruz.

Cellular lysis and immunoprecipitations. Lysates were prepared in 100 µl TNN lysis buffer (100 mM Tris/HCl pH 8.0, 250 mM NaCl, 0.5% NP-40) in the presence of 1 µl protease inhibitor cocktail (Sigma) for 10 min at 4 °C and subsequently centrifuged at 10000 g...
Cells were fixed in 3.7 % formaldehyde for 5 min. Usually 1–10 μl of the supernatant extract was analysed by SDS-PAGE and Western immunoblotting, and one half of the total extract was used for immunoprecipitation.

Cellular extracts were immunoprecipitated by adding 1.5 μl guinea pig anti-NSP5 serum, 1 μl 100 mM PMSF, 50 μl 50 % protein A-Sepharose CL-4B beads (Pharmacia) in TNN buffer and 60 μl TNN buffer, for 2 h or overnight at 4 °C. Beads were then washed three times with TNN buffer and samples analysed by SDS-PAGE.

When indicated, α-protein phosphatase (α-Ppase) treatment of cellular extracts was performed with 2 μl (400 U μl⁻¹) α-Ppase (New England Biolabs) and 2 μl sample. The reaction was incubated for 2 h at 30 °C and stopped with 5 μl loading buffer.

**In vivo** [³²S]methionine labelling and DSP cross-linking. MA104 cells were infected with rotavirus 48 h after transfection. Four hours after infection, cells were starved in DMEM lacking methionine for 30 min, then 300 μCi (11.1 MBq) [³²S]methionine (Promix [³²S] cell labelling mix, specific activity: >1000 Ci (37 000 Gbq) mmol⁻¹, 10 μCi (370 MBq) ml⁻¹, GE Healthcare) were added for 30 min. The cells were washed in PBS, incubated for 10 min in 600 μM DSP [dithiobis(succinimidylpropionate)] (Pierce) in PBS at 4 °C, then washed three times in 2.5 ml TBS (50 mM Tris/HCl pH 7.5, 150 mM NaCl). Cells were subsequently lysed in 100 μl TNN lysis buffer supplemented with 1 μl protease inhibitor cocktail (Sigma) for 10 min at 4 °C and centrifuged at 10 000 g for 5 min. The supernatants were immunoprecipitated as described above. Protein A beads were washed four times in TNN buffer and once in PBS; samples were resuspended in 20 μl loading buffer, boiled and analysed by SDS-PAGE. Visualization of [³²S]-labelled proteins was by autoradiography at −70 °C using X-ray film (Hyperfilm; Amersham Biosciences) enhanced by fluorography using Amplify (Amersham Biosciences).

**Western blotting.** Cellular extracts were resolved by SDS-PAGE. After electrophoresis, samples were transferred to a PVDF membrane (Immobilon-P; Millipore) for 2 h at 200 mA or overnight at 50 mA. The membrane was blocked in PBS/5 % skimmed milk for 30 min and incubated for 1 h in PBS/5 % skimmed milk containing the primary antibody. The dilutions of primary antibody were: 1:800 for anti-NSP5 guinea pig serum; 1:1000 for anti-NSP2 guinea pig serum; 1:1000 for anti-VP7 rabbit serum; 1:2000 for anti-VP2 guinea pig serum; 1:2000 for anti-tubulin mAb (Sigma); 1:500 for anti-NSP5 guinea pig serum; 1:1000 for anti-NSP1 guinea pig serum; 1:1000 for anti-VP7 rabbit serum; 1:2000 for anti-VP2 guinea pig serum; 1:2000 for anti-tubulin mAb (Sigma). The membrane was washed with TNN buffer for 10 min at 4 °C, then washed three times in 2.5 ml TBS (50 mM Tris/HCl pH 7.5, 150 mM NaCl). Beads were then washed three times in TNN buffer and once in PBS; samples were resuspended in 20 μl loading buffer, boiled and analysed by SDS-PAGE. Visualization of [³²S]-labelled proteins was by autoradiography at −70 °C using X-ray film (Hyperfilm; Amersham Biosciences) enhanced by fluorography using Amplify (Amersham Biosciences).

**Immunofluorescence microscopy.** Cells were fixed in 3.7 % paraformaldehyde in PBS for 10 min at room temperature. Coverslips were washed in PBS, blocked with PBS containing 1 % BSA for 30 min and incubated with primary antibody diluted 1:100 in PBS containing 1 % BSA in a moist chamber at room temperature for 1 h. After three washes with PBS, slides were incubated either with another primary antibody for double staining or directly for 45 min with rhodamine isothiocyanate- or fluorescein isothiocyanate-conjugated secondary antibodies (at a dilution of 1:100; Jackson Immunolabs). After three washes, nuclei were stained with Hoechst 33342 dye (2 μg ml⁻¹) for 10 min, washed and mounted with ProLong mounting medium (Molecular Probes). Samples were analysed by confocal microscopy (LSM 510; Carl Zeiss) or with the confocal SNAPS system using a fluorescence microscope (DMLB; Leica). Around 500 cells were counted per measurement point and experiments were repeated at least three times.

**Viral genomic dsRNA.** Total genomic dsRNA was prepared as described previously (Gonzalez et al., 1989). Briefly, cells were lysed in TNN buffer for 10 min at 4 °C and incubated with 50 μg proteinase K ml⁻¹ (Sigma) for 1 h at 50 °C. After two phenol extractions at 60 °C, sodium acetate was added to 0.3 M final concentration and samples were precipitated with ethanol. Samples were finally resuspended in water and electrophoresed in a 10 % polyacrylamide gel, 0.75 mm thick, for 5 h at 30 mA. Viral RNA segments were stained with ethidium bromide and subjected to densitometry evaluation using Kodak 1D 3.6 image analysis software.

**RESULTS**

**RNA interference with CK1α impairs NSP5 hyperphosphorylation.**

We have previously reported that, in vitro, the phosphorylation of Ser 67 in the NSP5 deletion mutant Δ3 was catalysed by CK1α and this caused the activation of Δ3 to induce the phosphorylation of the substrate, the NSP5 deletion mutant Δ2 (Eichwald et al., 2004a). In order to evaluate whether CK1α also phosphorylates NSP5 in vivo and if it follows the same pattern of activation, we treated cells co-transfected with gene constructs encoding mutants Δ2 and Δ3 with an siRNA that specifically targets CK1α (si/CK1α) without blocking other CK1 isoforms (Hino et al., 2003; Liu et al., 2002). This siRNA treatment decreased the quantity of CK1α to levels undetectable by Western blotting (Figs 1a, b, c and 3). In order to unequivocally identify Δ2 in the PAGE mobility shift assay, the mutant was tagged at the amino terminus with the 11 aa SV5 tag (SV5–Δ2). As shown in Fig. 1(a), SV5–Δ2 expressed alone gave rise to a unique non-phosphorylated band in the presence and absence of CK1α (lanes 1 and 2). However, when SV5–Δ2 was co-expressed with Δ3, strong hyperphosphorylation was observed, with the appearance of three slower-migrating bands, which were largely diminished in CK1α-silenced cells (lanes 3 and 4), suggesting that, in vivo, phosphorylation of Δ3 by CK1α was necessary for this mutant to promote Δ2 phosphorylation.

As NSP2 has been shown to promote NSP5 hyperphosphorylation, most probably through the initial phosphorylation of Ser 67, we investigated the effect of si/CK1α in cells co-expressing the two proteins. As shown in Fig. 1(c), the NSP2-mediated hyperphosphorylation of NSP5 was also inhibited significantly in cells transfected with si/CK1α (reflected in the increased intensity of the 26 kDa band and the concomitant decrease of the slower-migrating forms), demonstrating a key role for this enzyme in NSP5 hyperphosphorylation. In agreement with the involvement of CK1α in the phosphorylation of serine 67 of NSP5, mutants in which this serine was changed to alanine (NSP5/S67A) or where all the serines present in the domain that contains serine 67 were changed to alanine (NSP5a; Eichwald et al., 2004a) were both insensitive to NSP2-promoted hyperphosphorylation in the presence of CK1α (Fig. 1d).
NSP5 hyperphosphorylation appears to be a hierarchical process, with the first step of Ser 67 phosphorylation and a number of subsequent phosphorylation steps. In order to evaluate whether CK1α activity was also acting downstream of Ser 67 phosphorylation, we analysed the phosphorylation of mutant NSP5/S67D, which mimics phosphorylation of Ser 67. As reported previously (Eichwald et al., 2004a), NSP5/S67D showed the appearance of slower-migrating forms compared with the wild-type NSP5 (Fig. 1b, lanes 1 and 2). However, silencing of CK1α had only a marginal effect on the formation of the slower-migrating forms, suggesting that, in vivo, kinases other than CK1α are responsible for the phosphorylation events downstream of Ser 67 phosphorylation.

**Effect of CK1α on NSP5 derived from virus-infected cells**

In SA11-infected cells, NSP5 appears as a band of 26 kDa, a more abundant one of 28 kDa and a series of slower-migrating hyperphosphorylated bands with apparent molecular masses of up to 34 kDa. This characteristic pattern can be observed in control cells or cells treated with an irrelevant siRNA (si/irr) (Fig. 2a, lanes 1–2). However, silencing of CK1α had only a marginal effect on the formation of the slower-migrating forms, suggesting that, in vivo, kinases other than CK1α are responsible for the phosphorylation events downstream of Ser 67 phosphorylation.
excluding the possibility that the hyperphosphorylated forms of NSP5 could be present in the insoluble fraction (data not shown), as has been reported for tagged forms of NSP5 (Campagna & Burrone, 2006; Sen et al., 2006).

Despite the different phosphorylation pattern obtained in CK1α-silenced cells, the amount of NSP5 accumulated in virus-infected cells did not change when compared with cells treated with si/irr (Fig. 2a, b). In order to better understand this, cellular extracts were treated with λ-phosphatase before PAGE. Previous results had shown that λ-phosphatase converts all slower-migrating bands of NSP5 into the unique 26 kDa band (Afrikanova et al., 1996). As shown in Fig. 2(c), the intensity of the bands for samples that were si/CK1α- or si/irr-transfected and treated with λ-phosphatase was comparable, indicating that the impaired phosphorylation of NSP5 did not affect its stability. Moreover, when we examined the levels of production of the other viral proteins in cells labelled with [35S]methionine, we could not observe any change in cells treated with si/CK1α (Fig. 3a). Similarly, the use of specific antibodies in Western blots did not show any decrease in protein levels compared with si/irr, in contrast to treatment with an siRNA against NSP5 mRNA (si/SAP11), which, as reported previously (Campagna et al., 2005), dramatically decreased the level of viral protein production (Fig. 3b).

**Phosphorylation of NSP5 does not affect its interaction with VP1 and NSP2**

In virus-infected cells, NSP5 interacts with NSP2 and with the viral polymerase VP1 (Afrikanova et al., 1998; Arnoldi et al., 2007). In order to evaluate whether phosphorylation of NSP5 had an effect on its interaction with these viral proteins, cells transfected with an irrelevant siRNA or si/CK1α and then infected with SA11 virus in the presence of [35S]methionine were cross-linked with the bivalent crosslinker DSP. Cellular extracts were then immunoprecipitated with an anti-NSP5 antibody and resolved on a reducing SDS-PAGE gel to visualize the two co-precipitating proteins. As shown in Fig. 4, despite significantly impaired NSP5 phosphorylation in si/CK1α-treated cells, no difference in the ability of NSP5 to interact with either VP1 or NSP2 was observed compared with si/irr-treated cells, suggesting that the phosphorylation status of NSP5 is not essential for its capacity to interact with these two viroplasm-associated proteins.

**NSP5 hyperphosphorylation and virus replication**

In order to investigate the effect of NSP5 phosphorylation on virus replication, cells treated with si/CK1α were infected at an m.o.i. of 10 and analysed for their ability
to produce dsRNA and infectious virions in comparison with si/irr-treated cells. Whereas at 10 h post-infection (p.i.), there was no decrease in dsRNA production, at later times (15–36 h p.i.), a decrease in dsRNA production was observed. Concordantly, at later time points, viral infectivity was also decreased in CK1α-silenced cells compared with cells treated with an irrelevant siRNA (Fig. 5a). An aliquot of the extract used to isolate dsRNA was assayed by Western blot in order to control the status of phosphorylation of NSP5 and the quantity of proteins loaded (Fig. 5b). The decrease of both dsRNA production and infectivity was also observed reproducibly when a lower m.o.i. (0.1) was used (data not shown).

Altered morphology of viroplasms in CK1α-silenced cells

As NSP5 was shown to be essential for the formation of cytoplasmic viroplasms in virus-infected cells, we investigated the role of NSP5 phosphorylation in this process. Viroplasm formation was assessed by immunofluorescence with an antibody against NSP2 at different times p.i. with rotavirus strains OSU and SA11. Whilst the number of infected cells was comparable in control and si/CK1α-treated cells, the morphology of viroplasms was strikingly different in CK1α-silenced cells compared with control cells (Fig. 6a). Upon infection, viroplasms of regular spherical shape are formed as soon as 2 h p.i. and, from around 6 h p.i., the total number of viroplasms per cell decreases, with a concomitant increase in their size without any substantial change in their regular shape (Eichwald et al., 2004b). As can be observed in Fig. 6(a) (first and third columns), a pattern of morphogenesis with a decreasing number and an increasing size was obtained in SA11- and OSU-infected cells treated with si/irr, even though larger numbers of viroplasms remained in OSU-infected cells and they seemed to have a lower tendency to fuse. In contrast, formation of viroplasms in cells treated with si/CK1α was clearly different, particularly at around 10–15 h p.i. The shape of viroplasms was altered significantly; they did not appear as the classical spherical forms, but rather in irregular shapes. In many cells, there were structures resembling aggregations of smaller viroplasms. More strikingly, viroplasms produced in si/CK1α-treated cells infected with OSU assumed sickle-like forms, with the size of these structures increasing over time (Fig. 6a, fourth column). In both cases, the shape of viroplasms was strikingly different between cells treated with si/irr and cells treated with si/CK1α. More detailed images of the structures formed compared with viroplasms found in cells treated with si/irr are shown in Fig. 6(b).

On the other hand, despite the altered morphology of viroplasms, NSP2, VP2 and NSP5 were still able to co-localize in such irregular structures (Fig. 7a). In addition, CK1α, which, in non-infected cells, is normally distributed in the cytoplasm and at lower concentrations in the nuclei (Burzio et al., 2002), was partially localized to the viroplasms of virus-infected cells (Fig. 7b).
Taken together, these results indicate that lack of hyperphosphorylation of NSP5 is associated significantly with abnormal morphogenesis of viroplasms.

**DISCUSSION**

The phosphorylation of NSP5 is a complex process that requires, in the initial steps, the interaction of at least three different partners, namely the substrate NSP5, NSP2, as a possible inducer of conformational change in NSP5 to make Ser 67 accessible for phosphorylation, and the cellular kinase responsible for such activity (Eichwald et al., 2004a). In a series of in vitro experiments, we have shown previously that CK1α was possibly the enzyme involved in Ser 67 phosphorylation (Eichwald et al., 2004a). We have also proposed that Ser 67 phosphorylation is essential for initiating the cascade of NSP5 hyperphosphorylation. The aim of the work reported here was to explore whether CK1α also played a role in NSP5 hyperphosphorylation in vivo and to investigate the possible consequences for the virus replication cycle. We used RNA interference with the mRNA of CK1α to deplete this enzyme activity specifically in cells. The results obtained show clearly that NSP5 phosphorylation is largely impaired in cells in which CK1α is depleted, indicating that CK1α is required for NSP5 phosphorylation in cultured cells. The least-phosphorylated isoform of NSP5 (26 kDa for the wild-type protein) became the most prevalent one in the three different cases tested: (i) phosphorylation of the substrate NSP5-A2 with the activator NSP5-Δ3; (ii)
Fig. 6. CK1α-silenced cells show altered viroplasm morphology. (a) Time course of viroplasm formation in siRNA-treated cells. Immunofluorescence of MA104 cells transfected with siCK1α or si/irr and infected with the indicated virus strain. Bar, 10 μm.
(b) Magnification of viroplasms in si/irr- or siCK1α-transfected cells infected with SA11 or OSU strains for 15 h.
phosphorylation of wild-type NSP5 induced by NSP2; and (iii) phosphorylation of NSP5 during virus infection. In addition, we found CK1α localized in viroplasms in infected cells. Previous reports have presented evidence for co-localization of members of the CK1 family with their substrates (Takano et al., 2004). Phosphorylation of mutant S67D, which mimics a phosphorylated Ser 67, was insensitive to interference with CK1α. All these results are consistent with the proposed model of CK1α initiating the cascade through phosphorylation of Ser 67, and indicate that kinases other than CK1α are involved in vivo in the downstream process, contrary to what has been suggested previously based on in vitro experiments (Eichwald et al., 2004a). In this regard, it is interesting to note that the only kinases that are known to be primed by hierarchical phosphorylation in the recognition of substrates are CK1, CK2, GSK-3 and the Golgi kinase (Marin et al., 2003).

A similar role for CK1α has recently been demonstrated for the phosphorylation of the HCV NS5A protein, a non-structural protein that, like NSP5, interacts with the viral polymerase and is localized in cytoplasmic structures where viral replication occurs (Quintavalle et al., 2006).

As previous results have shown that depletion of NSP5 impaired production of viral proteins strongly (Campagna...
et al., 2005; Lopez et al., 2005), we investigated whether a lack of NSP5 hyperphosphorylation had an effect on this process as well. We found that the amount of viral proteins produced was unaltered in CK1α-depleted cells, even a long time after infection, despite the reduction in viral dsRNA. This result is in agreement with those obtained by silencing NSP4, which suggested that production of viral proteins depends mainly on the mRNAs produced by the infecting virion(s) rather than by newly formed double-layered particles in viroplasms (Silvestri et al., 2005).

We also found that non-phosphorylated NSP5 was still capable of interaction with NSP2 and VP1, a result consistent with previous studies using NSP5 deletion mutants, which showed that the status of phosphorylation did not affect interaction with NSP2 (Eichwald et al., 2002) and, more recently, with VP1 (Arnoldi et al., 2007).

Furthermore, localization to viroplasms of NSP2, as well as of VP2, was not affected by the depletion of CK1α. NSP5 phosphorylation status has been reported to have no effect on the interaction with VP2 (Berois et al., 2003). Despite this, we have recently observed strongly enhanced hyperphosphorylation of NSP5 when it is co-expressed with VP2 (Eichwald et al., 2002) and, more recently, with VP1 (Arnoldi et al., 2007).

The most striking consequence of the inhibition of NSP5 phosphorylation was the strong alteration in the morphology of viroplasms. Previous studies had tried to correlate NSP5 phosphorylation and its localization to viroplasms, with contradictory results (Eichwald et al., 2002, 2004b; Poncet et al., 1997). The use of RNA interference and its high efficiency in blocking NSP5 phosphorylation allowed us to analyse the wild-type form of the protein, as well as the formation of viroplasms, directly in virus-infected cells. Our results showed that the pattern of NSP5 phosphorylation has no effect on its localization to viroplasms, in full agreement with previous data showing that, in infected cells, NSP5 mutants that were unable to be hyperphosphorylated localized to viroplasms (Eichwald et al., 2002, 2004b). Despite this ability to localize, impaired phosphorylation of NSP5 clearly affected the morphology of viroplasms. We noticed practically no effect at early times p.i. (although a more diffuse, punctate pattern was noticed), whereas at later times, a clear alteration of viroplasm architecture was apparent. This suggests that NSP5 phosphorylation, rather than participating in the initiation of viroplasm formation, plays a significant role in the correct morphogenesis and development of viroplasms.

Depletion of CK1δ probably impairs the phosphorylation of cellular proteins in addition to NSP5. However, the fact that NSP5 localizes in viroplasms, mainly on their outer face (Eichwald et al., 2004b), and is necessary for their formation (Campagna et al., 2005; Lopez et al., 2005), together with the direct role of CK1α in NSP5 phosphorylation strongly supports the interpretation that the lack of NSP5 phosphorylation was directly responsible for the observed change in viroplasm morphology. In addition, viroplasm morphogenesis was altered differently for the two virus strains tested, mainly in shape, thus suggesting that the effect was not due to impaired phosphorylation of a cellular protein, but rather to differences in NSP5 primary sequence (Gonzalez & Burrone, 1989; Welch et al., 1989). In the first case, one would have expected the same phenotype in the two strains.

Non-structural protein NS2 of bluetongue virus (BTV) of the family Reoviridae is also phosphorylated by a member of the casein kinase family (CK2; Modrof et al., 2005). NS2 is one of the main components of viral inclusion bodies (VIBs). Interestingly, as in the case of NSP5, the lack of phosphorylation of NS2 does not affect its ability to interact with the structural viral inner core proteins, although NS2 is able to form VIBs on its own, whereas NSP5 is not. Furthermore, unlike NSP5, NS2 phosphorylation has a strong effect on the formation of VIBs (Modrof et al., 2005). Despite the differences, the phosphorylation status of both proteins appears to be linked to the architecture of viral inclusion bodies.

Lack of phosphorylation of NSP5 appears to moderately affect the capacity of infected cells to sustain production of viral genomic dsRNA, suggesting a function of NSP5 phosphorylation in virus replication. It is interesting to note that the decrease in production of dsRNA and infectious virus was correlated in time with the appearance of the disorganized structure of viroplasms.

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