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Review

Regulation of positive-strand RNA virus replication: The emerging role of phosphorylation

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Abstract

Protein phosphorylation is a reversible post-translational modification that plays a fundamental role in the regulation of many cellular processes. Phosphorylation can modulate protein properties such as enzymatic activity, stability, subcellular localization or interaction with binding partners. The importance of phosphorylation of the replication proteins of negative-strand RNA viruses has previously been documented but recent evidence suggests that replication of positive-strand RNA viruses – the largest class of viruses, including significant human, animal and plant pathogens – may also be regulated by phosphorylation events. The objective of this review is to summarize current knowledge regarding the various regulatory roles played by phosphorylation of nonstructural viral proteins in the replication of positive-strand RNA viruses.

Keywords: Positive-strand RNA virus; Replication; Phosphorylation; Regulation

Contents

1. Introduction

Positive-strand RNA viruses account for over one-third of all virus genera (van Regenmortel et al., 2000), and include significant human, animal and plant pathogens, e.g. the severe acute respiratory syndrome coronavirus SARS, hepatitis C virus (HCV), and tobacco mosaic virus. Upon infection of a cell, the genomic RNA is first translated to produce viral proteins, including the nonstructural proteins involved in replication of the viral genome. Viral replication requires the assembly of replication complexes – intricate "factories" featuring the close association of both viral and host components in virus-induced intracellular membrane compartments (reviewed in Buck, 1996; Ahlquist et al., 2003; Salonen et al., 2005; Sanfaçon, 2005; Nagy and Pogany, 2006). Successful assembly of such replication complexes is likely to depend on many critical interactions between viral RNA, proteins and lipids. Within these complexes, viral RNA-dependent RNA polymerase (RdRp) plays a pivotal role, catalyzing synthesis of new viral RNA genomes from the original infecting RNA in a two-stage process: the incoming positive-strand RNA is first used as a template to generate a

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complementary (negative-strand) RNA, which in turn directs the synthesis of progeny positive-strand RNAs. As a general rule, a large excess of positive- over negative-strands is produced, but the molecular mechanisms regulating this observed asymmetry and its temporal control remain largely unknown (Buck, 1996; Ahlquist et al., 2003). The fact that positive-strand RNA virus genomes can serve as templates for both translation and replication also suggests a need to coordinate their use during the viral multiplication cycle. While significant progress has been made in deciphering the molecular mechanisms of the replication of positive-strand RNA viruses, crucial details of many important regulatory steps remain to be elucidated.

In recent years, evidence has accumulated suggesting that a number of viral nonstructural proteins are phosphoproteins. Phosphorylation is well known as a major reversible post-translational modification of proteins, playing a fundamental role in the regulation of many cellular processes in eukaryotic cells.

Activation of protein kinases and phosphatases provides a powerful means of controlling the phosphorylation state of a protein and consequently its biological function as phosphorylation can modulate properties such as enzymatic activity, protein stability, subcellular localization or interaction with binding partners (reviewed in Cohen, 2000). Increased knowledge about the phosphorylation of viral nonstructural replication proteins may broaden our understanding of the molecular processes in which they are involved, and help to unravel important regulatory pathways.

The importance of phosphorylation of nonstructural proteins has previously been documented in the case of negative-strand RNA viruses (reviewed in Lenard, 1999) but recent evidence suggests that replication of positive-strand RNA viruses may also be regulated by phosphorylation events. The objective of this review is to present an overview of current knowledge of the various regulatory roles that phosphorylation of nonstructural viral proteins can play in the replication of positive-strand RNA viruses (Table 1).

2. Phosphorylation can regulate interactions between viral replication proteins

The formation of multi-protein complexes requires specific interactions between the individual components. The first evidence suggesting that phosphorylation might regulate interactions between viral proteins within the viral replication complex came from studies performed on Dengue virus type 2 (DEN-2), a member of the family *Flaviviridae* (Kapoor et al., 1995). The NS5 protein, which harbors motifs indicative of RdRp activity, was shown to be phosphorylated on serine residues in virus-infected cells. Subcellular fractionation and phosphatase treatments revealed the existence of multiple forms of NS5 due to different extents of phosphorylation, with the hyperphosphorylated form being located predominantly in the nucleus. Interestingly, coimmunoprecipitation experiments revealed that the replication protein NS3 – which contains proteinase and RNA helicase domains – interacted preferentially with the hypophosphorylated cytoplasmic form of NS5, suggesting that differential phosphorylation might regulate the interaction between NS3 and NS5, and thus their participation as components of viral RNA replication complexes. Subsequently, yeast two-hybrid experiments revealed that the same region of NS5 interacted both with NS3 and with the cellular nuclear import receptor importin-beta, suggesting that NS3 and importin might compete for interaction with NS5 (Johansson et al., 2001). Kapoor et al. (1995) suggested that phosphorylation of NS5 by cellular kinase(s) could cause the disruption of the NS3–NS5 complexes involved in viral replication in order to promote transport of NS5 to the nucleus to fulfill an as yet uncharacterized function, but this remains to be demonstrated.

Evidence for a link between phosphorylation and inhibition of an interaction between replication proteins has been established in the case of cucumber mosaic virus (CMV), a plant virus belonging to the family *Bromoviridae* (Kim et al., 2002). The 2a protein encompassing the RdRp domain was reported to be phosphorylated in infected cells at a late stage of the replication cycle and was also shown to be phosphorylated *in vitro* by membrane-associated host kinase(s). *In vitro* phosphorylation assays carried out with truncated versions of the 2a protein revealed the presence of at least three potential phosphorylation sites: one in a central region comprising the conserved RdRp catalytic core, and the other two in the N-terminal part of the protein. Coimmunoprecipitation experiments and yeast two-hybrid assays revealed that the N-terminal region is necessary and sufficient for interaction with the replication protein 1a, which contains motifs indicative of RNA-capping and helicase functions (Kim et al., 2002; Suzuki et al., 2003). Interestingly, *in vitro* phosphorylation of CMV 2a protein (or its N-terminal region) led to inhibition of the interaction with the 1a protein, raising the possibility that the phosphorylation status of the 2a polymerase might regulate interactions between viral proteins within the replication complex. Phosphorylation of the 2a polymerase at a late stage of infection would thus prevent the formation of new 1a–2a protein complexes, or induce disassembly of existing complexes. It is also possible, as suggested by Kim et al. (2002), that the pool of phosphorylated 2a may have another function besides replication, such as interactions with host factors leading to virus movement or host defense responses.

3. Phosphorylation can regulate interactions between viral and host proteins within the replication complex

As viral replication complexes contain both viral and host components (Buck, 1996; Ahlquist et al., 2003), it is anticipated that phosphorylation events could also regulate interactions between viral and cellular proteins. This idea is supported by recent studies on the NS5A replication protein of HCV, a member of the family *Flaviviridae*. NS5A is a multifunctional subunit of the replication complex, playing key roles in both viral replication and modulation of host cell physiology (reviewed in Macdonald and Harris, 2004). Two phosphorylated forms of NS5A, termed p56 and p58, can be distinguished by their electrophoretic mobility. The 56 kDa form contains unphosphorylated NS5A and basal phosphorylated forms arising from phosphorylation at residues in the centre and near the

Table 1
Replication phosphoproteins of positive-stand RNA viruses and proposed phosphorylation-dependent functions

Virus	Replication phosphoprotein	Proposed phosphorylation-dependent regulatory functions	References
Bromoviridae CMV	2a[a]	Inhibition of interaction between viral replication proteins 2a and 1a	Kim et al. (2002)
	1a	?	Kim et al. (2006)
Flaviviridae BVDV	NS5A	?	Reed et al. (1998)
Flaviviridae DEN-2	NS5[a]	Inhibition of interaction between viral replication proteins NS5 and NS3	Kapoor et al. (1995)
Flaviviridae HCV	NS5B[a]	Enhancement of viral RNA synthesis	Hwang et al. (1997), Kim et al. (2004)
	NS5A	Inhibition of interaction between NS5A and a host protein hVAP-A	Kaneko et al. (1994), Tanji et al. (1995), Evans et al. (2004)
		Regulation of protein stability	Pietschmann et al. (2001)
Flaviviridae TBEV	NS5[a]	?	Morozova et al. (1997)
Flaviviridae YFV	NS5[a]	?	Reed et al. (1998)
Picornaviridae PV	3D[a]	?	Ransone and Dasgupta (1989)
Potyviridae PVA	VPg	Regulation of viral RNA synthesis	Puustinen et al. (2002)
		Induction of pathogenesis in host organism	K. Mäkinen, personal communication
Togaviridae SFV	nsP3	Enhancement of viral RNA synthesis	Peranen et al. (1988)
		Induction of pathogenesis in host organism	Vihinen and Saarinen (2000), Vihinen et al. (2001)
Togaviridae SIN	nsP3	Fine-tuning of viral RNA synthesis	Li et al. (1990), LaStarza et al. (1994), De et al. (2003)
Tombusviridae CNV	p33	Inhibition of RNA-binding	Shapka et al. (2005)
		Fine-tuning of viral RNA synthesis	Stork et al. (2005)
		Induction of pathogenesis in host organism	
Tombusviridae TCV	p28	?	Shapka et al. (2005)
Tymoviridae TYMV	66K[a]	Regulation of protein stability	Héricourt et al. (2000)
		Fine-tuning of viral RNA synthesis	Jakubiec et al. (2006)

Virus abbreviations: CMV, cucumber mosaic virus; BVDV, bovine viral diarrhea virus; DEN-2, Dengue virus type 2; HCV, hepatitis C virus; TBEV, tick-borne encephalitis virus; YFV, yellow fever virus; PV, poliovirus; PVA, potato virus A; SFV, Semliki Forest virus; SIN, Sindbis virus; CNV, cucumber necrosis virus; TCV, turnip crinkle virus; TYMV, turnip yellow mosaic virus.

[a] Viral proteins with the RdRp signature, corresponding to the catalytic subunit of the viral replication complex, ?: unknown function.

C-terminus, while the 58 kDa protein – referred to as the hyperphosphorylated form – is phosphorylated within a serine-rich central region of the protein (Kaneko et al., 1994; Tanji et al., 1995; Huang et al., 2004). Hyperphosphorylation appears to be a highly regulated process that depends on the expression of other nonstructural HCV proteins (Kaneko et al., 1994).

Several lines of evidence suggest that the phosphorylation state of NS5A can regulate HCV replication in cell culture: analyses of the spontaneous occurrence of adaptative mutations leading to substitutions of phosphorylated serine residues (Blight et al., 2000), engineered substitutions of putative phosphoacceptor sites (Appel et al., 2005), and treatment with kinase inhibitors (Neddermann et al., 2004) revealed that, in most cases, a reduction in NS5A hyperphosphorylation correlated with enhanced replication of an HCV-derived subgenomic replicon in cell culture. Such results imply that, in this context, extensive phosphorylation of NS5A is not required for its function in viral replication, a finding consistent with the fact that a derivative of NS5A whose phosphorylation was undetectable *in vivo* was still able to support replication of the HCV replicon (Huang et al., 2005).

Interestingly, a possible molecular basis for the deleterious effects of NS5A hyperphosphorylation on viral replication was proposed by Evans et al. (2004), who observed an inverse correlation between the phosphorylation state of several NS5A variants and their capacity to interact in the yeast two-hybrid system with a host protein termed hVAP-A (for human vesicle-associated membrane protein-associated protein A). hVAP-A – an integral membrane protein involved in intracellular vesicle trafficking – has been proposed as a membrane docking site for assembly of HCV replication complexes (Tu et al., 1999; Gao et al., 2004). Strikingly, in the replicon system, NS5A mutations that impair the interaction with hVAP-A strongly reduce the efficiency of viral replication (Evans et al., 2004). Collectively, these findings support a model in which the deleterious effect of NS5A hyperphosphorylation on viral replication is due to disruption of the interaction with hVAP-A, which impairs the assembly of viral replication complexes on target membranes. This hypothesis is consistent with the observation that hyperphosphorylated NS5A is found neither in affinity purified HCV replication complexes (Waris et al., 2004) nor in the detergent-resistant membrane fractions proposed to be the site of viral RNA replication (Shi et al., 2003; Gao et al., 2004).

As the exact function of p58 in viral replication remains unknown, one might envisage that this form of NS5A plays a distinct role in the virus life cycle, perhaps, as suggested by Appel et

al. (2005), during particle assembly. As NS5A is also thought to interfere with numerous cellular signaling pathways, including the interferon response, induction of apoptosis and regulation of cell growth (reviewed in Macdonald and Harris, 2004), it is also conceivable that the hyperphosphorylated form of NS5A might be required for one or more of these functions. Phosphorylation of NS5A is a conserved feature among hepacivirus and pestivirus within the family *Flaviviridae* (Table 1), supporting the argument that phosphorylation plays an important role in the infection cycle of these viruses.

4. Phosphorylation and stability of viral replication proteins

Modulation of the function of viral replication complexes can also be achieved through differential cleavage of the proteins involved during the viral multiplication cycle (Lemm et al., 1994), or changes in their stoichiometry (Schwartz et al., 2004), presumably via modifications in the interaction network within the replication complex. In this context, protein stability is an important parameter and much evidence in recent years, has suggested that the replication proteins of positive-strand RNA viruses, e.g. the nsp4 polymerase proteins of Sindbis virus (SIN) and Semliki Forest virus (SFV) (family *Togaviridae*) (de Groot et al., 1991; Merits et al., 2001) and the 3D polymerase of hepatitis A virus (family *Picornaviridae*) (Losick et al., 2003) are subject to specific degradation pathways. Indeed, the ubiquitination and degradation of HCV NS5B polymerase, has been reported to have an inhibitory effect on viral HCV replicon replication (Gao et al., 2003).

The idea that stability of a viral replication protein could be influenced by its phosphorylation status was first put forward in the case of HCV NS5A, as the p58 form of NS5A was less stable than p56 in HCV replicon-infected cells (Pietschmann et al., 2001). Another recent example is the case of turnip yellow mosaic virus (TYMV), a member of the family *Tymoviridae*. TYMV 66K protein, which encompasses the RdRp domain, was shown to be phosphorylated both during viral infection and when expressed in isolation, and several phosphorylated residues have been identified (Héricourt et al., 2000; Jakubiec et al., 2006). Two phosphorylation sites are located in the N-terminal region of the protein, within a PEST sequence – a conditional signal for protein degradation (Rechsteiner and Rogers, 1996) – that is invariably identified in this region of tymovirus RdRps (Héricourt et al., 2000). Infectivity assays revealed that mimicking phosphorylation of those residues had a detrimental effect both on the accumulation of 66K protein and on viral replication, thus it was proposed that phosphorylation of these sites may serve to regulate the accumulation level of 66K via activation of the latent PEST signal controlling the metabolic stability of the protein (Jakubiec et al., 2006). Mimicking phosphorylation would lead to increased turnover of RdRp, which would thus become a limiting component in the replication machinery, resulting in the inhibition of viral replication. A recent finding that mutation of these phosphorylation sites affects 66K stability *in vivo*, whereas expression of the TYMV 140K replication protein – reported to inhibit 66K phosphorylation – led to its stabilization, supports this hypothesis (Jakubiec et al., 2006, our unpublished data). These results suggest that controlling the stability of a viral replication protein may have a profound influence on the efficiency of viral replication, and highlight the role that other viral proteins may play in that regulatory process.

5. Phosphorylation can regulate protein/RNA interactions

It has long been known that phosphorylation of proteins can regulate their nucleic acid binding properties (Boyle et al., 1991; Mayrand et al., 1993), but evidence that phosphorylation can regulate interaction between viral RNA and replication proteins of positive-strand RNA viruses was reported only recently. The p33 protein of cucumber necrosis virus (CNV), a plant virus belonging to the family *Tombusviridae*, is a nonstructural replication protein involved in RNA template selection and recruitment into replication complexes. p33 was recently demonstrated to be phosphorylated within CNV replication complexes (Shapka et al., 2005). Based on *in vitro* assays in which synthetic peptides are phosphorylated by membrane-associated host kinase(s) and/or protein kinase C (PKC), two phosphorylation sites were mapped to residues adjacent to the RNA-binding domain (Shapka et al., 2005). Interestingly, *in vitro* phosphorylation by PKC of recombinant p33, or amino acid substitutions mimicking phosphorylation, both reduced the ability of p33 to bind the viral RNA *in vitro*, while a kinase treatment promoted release of the viral RNA from a prebound p33:RNA complex (Stork et al., 2005). These results suggest that phosphorylation of p33 can inhibit its binding to viral RNA, presumably by neutralizing a positively charged motif in the adjacent RNA-binding site. Further insight into the potential function of p33 phosphorylation during CNV replication was provided by the characterization of viral mutants bearing phosphorylation-mimicking or phosphorylation-deficient substitutions. This analysis revealed that mimicking phosphorylation strongly impaired the activity of purified replication complexes isolated from yeast – a heterologous host supporting tombusvirus replication – and also drastically inhibited the accumulation of viral products in infected yeast or plant cells (Shapka et al., 2005; Stork et al., 2005). These findings are consistent with a model in which the phosphorylation state of p33 could serve as a switch during the replication cycle, allowing the binding or release of viral RNA. As suggested by Stork et al. (2005), in addition to fine-tuning of the replication process (see below), it is possible that phosphorylation of p33 at a late stage of infection could prevent the assembly of new replication complexes, and/or promote the release of viral RNA from the existing complexes, rendering it available for other processes such as translation, encapsidation or virus movement.

Conservation of putative phosphoacceptor residues within homologous proteins of other members of the family *Tombusviridae* suggests that regulation of RNA-binding by phosphorylation might be a common feature of viruses belonging to this family. Consistent with this suggestion, the homologous turnip crinkle virus-encoded p28 has been shown to be phosphorylated *in vivo* (Shapka et al., 2005).

6. Phosphorylation can be involved in fine-tuning of replicase catalytic function

During infection, viral RdRps act in combination with other viral and host factors to catalyze synthesis of new viral RNA genomes. Phosphorylation might modulate various steps of the RNA synthesis process (i.e. template or nucleotide selection, initiation of complementary strand synthesis, transition from initiation to elongation, fidelity, processivity of the enzyme or product release), and thus influence the efficiency, the ratio, or the timing of production of the different viral RNA species. There are now several examples in which phosphorylation of viral replication proteins or viral RdRp has been reported to finely tune this RNA synthesis process.

The alphavirus nsP3 proteins are nonstructural replication proteins involved in regulation of RNA synthesis (reviewed in Kaariainen and Ahola, 2002), and the nsP3s of SIN and SFV were among the first positive-strand RNA virus replication proteins reported to be phosphoproteins (Peranen et al., 1988; Li et al., 1990). Both are phosphorylated on serine and threonine residues that map to a C-terminal hypervariable domain (LaStarza et al., 1994; Vihinen and Saarinen, 2000; Vihinen et al., 2001), and phosphorylated nsP3 was detected in cellular membrane fractions enriched for viral replication complexes (Peranen et al., 1988; Barton et al., 1991) suggesting an active role for phosphorylated nsP3 in alphavirus RNA replication. Consistent with this suggestion, SFV nsP3 deletion derivatives with reduced or undetectable phosphorylation exhibited a decreased rate of RNA synthesis at early stages of viral infection in cell culture (Vihinen et al., 2001), while reduced phosphorylation of SIN nsP3 caused by thermosensitive mutations correlated with decreased negative-strand RNA synthesis (De et al., 2003), opening the possibility that phosphorylation of nsP3 might have a regulatory function at this stage of the replication cycle. Recently, SFV nsP3 was reported to carry neurovirulence determinants (Tuittila et al., 2000), and a phosphorylation-deficient mutant displayed severely reduced pathogenicity in mice (Vihinen et al., 2001) suggesting that, in addition to a role in fine-tuning of the replication process, phosphorylation of nsP3 might also be involved in regulation of viral pathogenicity.

Strikingly, in the case of CNV, a phylogenetically distant plant virus, analysis of p33 phosphorylation mutants revealed that substitutions affecting the phosphorylation sites adjacent to the RNA-binding domain also altered the synthesis and relative accumulation of the different viral RNA species produced during infection (Stork et al., 2005; Shapka et al., 2005). In particular, phosphorylation-deficient mutants displayed a change in the ratio of sgRNAs synthesized and showed a reduced ratio in positive- versus negative-strand synthesis. This finding opens the possibility that p33 phosphorylation might be involved in fine-tuning of the viral replication process during early stages of the viral cycle. Interestingly, pathogenicity in plants was also affected, as a phosphorylation-deficient mutant exhibited delayed accumulation and milder symptoms (Shapka et al., 2005). Similarly, substitutions affecting phosphorylated residues within the genome-linked VPg protein appeared to affect both replication and pathogenicity of potato virus A, a plant virus belonging to the family *Potyviridae* (Puustinen et al., 2002; K. Mäkinen, personal communication).

Phosphorylation-dependent regulatory mechanisms of the RNA synthesis process may also depend on the phosphorylation status of the polymerase itself, as recently reported in the case of TYMV (Jakubiec et al., 2006). Remarkably, one of the residues identified as being phosphorylated during viral infection was mapped within the conserved palm subdomain of RdRp. This subdomain harbors the polymerase catalytic site and contains the conserved sequence motifs (A–E) found in all polymerases (Poch et al., 1989; Ferrer-Orta et al., 2006). The phosphorylated residue is located in the so-called motif A – predicted to be involved in nucleotide recognition and binding – at a position that is strictly or highly conserved in two out of three subgroups of RdRps (Koonin, 1991). Infectivity assays with viral mutants bearing substitutions of the phosphoacceptor residue revealed that mimicking phosphorylation abolished viral replication, while a phosphorylation-deficient mutant was infectious but displayed a drastically reduced ratio of positive- versus negative-strand synthesis (Jakubiec et al., 2006). These results are consistent with the idea that phosphorylation of this residue within the palm subdomain is a dynamic process that may serve as a switch to control strand asymmetry during the replication cycle. Interestingly, the evolutionary conservation of this target residue from alphavirus to picornavirus RdRps (Koonin, 1991) suggests that it may also constitute a phosphorylation target site in other viruses. Consistently, the existence of phosphorylated residue(s) within the palm subdomain of CMV polymerase 2a and within the palm and finger subdomains of HCV polymerase NS5B have been reported (Kim et al., 2002, 2004), although the precise target sites remain to be identified. In the latter case, the isolation of PRK2 (protein kinase C-related kinase 2) as an interacting partner of HCV NS5B (Kim et al., 2004) supports the idea that NS5B phosphorylation can regulate HCV RNA replication. Indeed, downregulation of PRK2 expression by RNA interference was shown to inhibit NS5B phosphorylation *in vivo* and correlated with decreased accumulation of HCV replicon RNA, whereas overexpression of PRK2 had the opposite effect.

These observations raise the possibility that regulation of viral RNA synthesis through phosphorylation of the polymerase protein might be a common feature of positive-strand RNA viruses, which is of particular interest given the essential function of RdRp in viral replication, and the common architecture and mechanism of polymerase catalysis.

7. Concluding remarks

Although the identification of phosphorylated proteins and residues remains challenging due to the low abundance of nonstructural proteins produced during viral infection and the substoichiometric and often labile nature of phosphorylation, it is likely that the recent progress in phosphoproteomics (Reinders and Sickmann, 2005; Hjerrild and Gammeltoft, 2006) will contribute to increasing the list of phosphoproteins identified among positive-strand RNA virus replication proteins.

As summarized in this review, the available evidence points to phosphorylation as a powerful controlling factor in the life cycle of positive-strand RNA viruses. The biological role of phosphorylation during viral replication still awaits characterization for a number of viral nonstructural phosphoproteins (Table 1). Elucidation of the underlying molecular mechanisms will not only contribute to our basic understanding of the viral replication process, but may also prove critical for developing methods to inhibit or contain infection.

A parallel challenge for the near future will be to move towards identification of the kinase(s) and phosphatase(s) involved, as these may constitute attractive targets for the development of anti-viral drugs (Cohen, 2002; Sawyer et al., 2005).

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