Comparison of the expression and phosphorylation of the non-structural protein NS2 of three different orbiviruses: evidence for the involvement of an ubiquitous cellular kinase

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The non-structural protein NS2 of epizootic haemorrhagic disease (EHD), bluetongue (BT) and African horsesickness (AHS) viruses has each been expressed to high levels using a baculovirus vector gene expression system. It was found that the recombinant baculovirus-expressed EHDV NS2 protein was resolved as a doublet following PAGE. Peptide mapping of these protein bands indicated that they were identical. The difference in the sizes of the NS2 protein bands could not be attributed to the phosphorylation of NS2 or other post-translational modification such as N-glycosylation and remains obscure. The EHDV, BTV and AHSV baculovirus-expressed NS2 proteins were all phosphorylated in vitro without the addition of an exogenous kinase. An unphosphorylated form of EHDV NS2, obtained by expressing the NS2 gene as a fusion protein in Escherichia coli cells, could be phosphorylated in vitro by a protein kinase associated with the cytoplasm of insect cells. The phosphorylated version of this protein was found to be significantly less efficient in binding ssRNA, compared to the unphosphorylated version.

Introduction

Epizootic haemorrhagic disease, bluetongue and African horsesickness viruses (EHDV, BTV and AHSV) are three distinct serogroups of the Orbivirus genus in the Reoviridae family (Verwoerd et al., 1979; Gorman, 1992). These viruses are each characterized by a genome consisting of 10 dsRNA segments, each of which encodes at least one viral polypeptide (Bremer, 1976; Huismans, 1979; Huismans et al., 1979; Van Dijk & Huismans, 1988; Mechem & Dean, 1988). These include the seven structural proteins, VP1 to VP7 and three non-structural proteins, NS1, NS2 and NS3. During virus infection NS1 polymerizes to form tubular structures (Huismans & Els, 1979; Urakawa & Roy, 1988; Nel & Huismans, 1991) whereas NS2 forms virus inclusion bodies (VIBs) (Eaton et al., 1987; Thomas et al., 1990; Brookes et al., 1993). These characteristic structures are believed to be involved in viral morphogenesis. NS3 on the other hand is synthesized in much smaller amounts than NS1 and NS2 and probably plays a role in the release of virus progeny from infected cells (Hyatt et al., 1991, 1993).

The NS2 protein of BTV has been shown to bind ssRNA (Huismans et al., 1987; Thomas et al., 1990). This characteristic is presumed to be common to non-structural proteins of all members of the Reoviridae family and this has already been demonstrated to be the case for αNS of reovirus (Huismans & Joklik, 1976) and NS34 as well as NS35 of rotavirus (Boyle & Holmes, 1986; Kattoura et al., 1992). It is likely that the NS2 proteins of orbiviruses other than BTV will also have RNA-binding properties, although this has not been demonstrated as yet. Furthermore, Huismans et al. (1987) have shown that the NS2 protein is the only BTV phosphoprotein but the functional significance of NS2 phosphorylation is not known. It has also been found that purified BTV NS2 preparations could be phosphorylated in vitro without the addition of an exogenous protein kinase (Huismans et al., 1987) but to date the implication of this finding remains obscure. It is for instance unknown whether NS2 is capable of autophosphorylation, or whether another viral protein or perhaps a cellular protein kinase is involved in the phosphorylation of this protein. Thus far only the BTV and AHSV NS2 proteins have been reported to be phosphoproteins (Huismans et al., 1987; Devaney et al., 1988), and it is not known whether the EHDV NS2 protein is likewise phosphorylated.

It has been shown for a number of different viruses that the degree of phosphorylation may determine the ability of phosphoproteins to interact with nucleic acids. For instance, in the case of the simian virus 40 (SV40) large T antigen, it has been shown that phosphorylation down-modulates DNA-binding (Scheidtmann et al., 0001-2535 © 1994 SGM
control cells were infected with wild-type AcMNPV as described above. At 72 h post-infection the cells were harvested and cytoplasmic X-100). Three hundred gl (representing 3 x 106 cells) of the respective cytoplasmic fractions was loaded onto 5 ml linear 10 to 40 % sucrose gradients prepared from uninfected (10 mM-Tris-HCl, pH 7.4; 1 mM-EDTA; 150 mM-NaCl; 0.5% Triton TX-100). Three hundred gl (representing 3 x 106 cells) of the respective cytoplasmic fractions was loaded onto 5 ml linear 10 to 40 % sucrose gradients prepared in 0.15 M-STE-TX buffer and centrifuged for 16 h at 40000 r.p.m. at 4 °C in a Beckman SW50.1 rotor. Thirteen fractions (approx. 400 μl/fraction) were collected from the bottom of each gradient and the pellets were resuspended in 100 μl 001 M-STE-TX buffer. The gradient fractions were phosphorylated in vitro and analysed by SDS-PAGE and autoradiography.

In vitro phosphorylation of the recombinant baculovirus-expressed NS2 proteins. Proteins were phosphorylated in vitro as previously described (Huismans et al., 1987). Equal volumes of the respective gradient fractions and 2 x MTD buffer (0.02 M-Tris-HCl pH 8.0; 0.02 mM-MgCl2; 0.02 M-DTT) were mixed and incubated at 37 °C for 30 to 60 min in the presence of 0.5 to 1 μCi [γ-32P]ATP/reaction. The 32P-labelled proteins were subsequently analysed by SDS-PAGE and autoradiography.

Radiolabelling and peptide mapping of NS2 proteins. Monolayers of S. frugiperda cells in 35 mm tissue culture dishes (1 x 105 cells/well) were infected with the EHDV recombinant baculovirus and incubated at 28 °C for 24 h. The monolayers were rinsed with and incubated in methionine-free Eagle's medium for 1 h, after which the medium was replaced with 1 ml of the same medium but including [35S]methionine (30 μCi/ml; 3 h). After SDS-PAGE of radiolabelled proteins, the gels were dried and autoradiographed. NS2 protein bands were excised and subjected to peptide mapping according to the procedure of Cleveland et al. (1977) as described by Van Staden & Huismans (1991).

Phosphatase and tunicamycin treatment of recombinant baculovirus-infected cell extracts. A cytoplasmic extract prepared from S. frugiperda cells infected with the EHDV NS2 recombinant baculovirus was phosphorylated in vitro by mixing 200 μl thereof with 2 μCi of [γ-32P]ATP in 20 μl of a 10 x MTD buffer. The phosphorylated extract was treated with 30 μl calf intestinal alkaline phosphatase (CIP; Boehringer Mannheim) at 37 °C in CIP buffer (50 mM-Tris-HCl, pH 9.0; 1 mM-MgCl2; 0.1 mM-ZnCl2). Samples of the reaction mixture were removed after 30, 60 and 90 min of incubation and analysed by SDS-PAGE and autoradiography as well as by Western blot analysis. S. frugiperda cells infected with the EHDV NS2 recombinant baculovirus were treated with tunicamycin (Boehringer Mannheim) similar to the procedures described by Wu et al. (1992).

Cloning and expression of the EHDV NS2 gene in Escherichia coli. A full-length copy of the EHDV-2 NS2 gene was cloned into the BamHI site of the pGEX series of bacterial expression vectors (Pharmacia). Recombinant plasmids containing the NS2 gene-specific insert in the correct transcriptional orientation were transformed into competent E. coli JM109 cells and individual colonies of each were inoculated into 1 ml of LB-broth supplemented with ampicillin (50 μg/ml) and cultured overnight at 37 °C. Expression of the fusion protein was induced with IPTG (Boehringer Mannheim) as described by Smith & Johnson (1989). The cells were harvested and the bacterial lysates were analysed by SDS-PAGE and Western blot analysis using EHDV antisera.

Radiolabelling of the EHDV NS2 fusion protein synthesized in E. coli cells. Bacterial cells were grown in a low-phosphate glucose–peptone medium and labelled in vivo with either 30 μCi [35S]methionine (1 mCi/ml; 1000 Ci/mmole; Amersham) or 50 μCi 32P (10 Ci/ml; Amersham) per ml of culture as described by Barik & Banerjee (1991). Following harvest, cells were washed once with 1 x PBS and the proteins were analysed by SDS-PAGE and autoradiography.

In vitro phosphorylation of the NS2 fusion protein. The cells in IPTG-induced cultures of E. coli JM109 cells containing either the parental or recombinant pGEX-1 vector were harvested by centrifugation and lysed by resuspending the cells in ice-cold suspension buffer (25 mM-Tris–HCl, pH 8.0; 50 mM-glucose; 1 mM-EDTA; 5 mg/ml lysozyme) (Köchel et al., 1991). In the kinase assay, 30 μl of the different bacterial extracts were mixed with an equal volume of a cytoplasmic extract prepared from uninfected S. frugiperda cells or with 2 x MTD buffer.

Methods

Construction of recombinant baculoviruses and SDS-PAGE analysis of the recombinant proteins. The cloning of full-length cDNA copies of the NS2 genes of BTV-10, AHVS-9 and EHDV-2 has been described elsewhere (Nel & Huismans, 1990; Van Staden et al., 1991). The terminal homopolymeric sequences resulting from the cDNA cloning were removed by a PCR method as described by Nel & Huismans (1991). These amplified AHVS, BTV and EHDV NS2 genes were respectively cloned into the BamHI site of pAcDZ1 (Zuidema et al., 1990), pAcYM1 (Miller, 1988) and pAcC129-1 (Livingstone & Jones, 1991). Clones containing inserts in the correct transcriptional orientation were used to derive baculovirus recombinants following cotransfection of Spodoptera frugiperda cells with wild-type baculoviral DNA by lipofection (Feigner et al., 1987).

It was observed that the baculovirus-expressed EHDV and AHVS NS2 proteins separate into two distinct bands following SDS-PAGE. The composition and phosphorylation of these bands were further investigated in the case of EHDV NS2. In addition, an investigation into the factors responsible for the phosphorylation of the orbivirus NS2 proteins and its possible function was carried out. Evidence that phosphorylation affects sRNA-binding and involves an ubiquitous cellular kinase was obtained.
Orbivirus NS2 expression

3403

Fig. 1. Recombinant baculovirus expression of the NS2 proteins of AHSV, BTV and EHDV. Mock-infected or infected S. frugiperda cells were harvested and the cell lysates derived were analysed by SDS–PAGE. Lane 1, Mock-infected cells; lane 2, wild-type AcMNPV-infected cells; lanes 3 to 5, recombinant baculovirus-infected cells expressing the BTV-10 (lane 3), AHSV-9 (lane 4) or EHDV-2 (lane 5) NS2 proteins. Protein markers of indicated Mr are shown on the left of the figure and the position of the respective NS2 proteins and the AcMNPV polyhedrin protein is indicated by arrows.

The respective mixtures were then phosphorylated in vitro as described above and analysed by SDS–PAGE and autoradiography.

RNA-binding analysis. Harvested cells of induced cultures of E. coli JM109 containing either the recombinant or parental pGEX-1 vector were lysed (Bogerd et al., 1991), and the GST or NS2 fusion proteins purified by affinity chromatography (Smith & Johnson, 1989) with glutathione agarose (Sigma). The purified NS2 fusion protein (1.8 ml) was mixed with an equal volume of an S. frugiperda cytoplasmic extract (2 x 10^7 cells) and phosphorylated in the presence of 1 mM-ATP (final concentration). After 1 h at 37 °C, the reaction was terminated by the addition of EDTA (12.5 mM final concentration) and the reaction mix was subjected to glutathione affinity chromatography. Equal volumes of the purified fusion protein were divided into 10 different Eppendorf tubes containing approx. 10 mg poly(U)-Sepharose, pre-equilibrated in 0.01 M-STE-TX buffer. The ionic strength of the reaction mixes was varied by adjusting the molarities of STE-TX buffer, as indicated in Fig. 7. After gentle shaking on a rotating platform for 30 min, the poly(U)-Sepharose was pelleted at 2000 r.p.m. for 1 min and washed twice with 500 µl of the corresponding STE-TX buffer. The supernatants were pooled, precipitated with acetone and the unbound NS2 fusion protein was pelleted by centrifugation at 18 000 r.p.m. for 30 min at 4 °C in a Beckmann SW50.1 rotor. The pellets were resuspended in 20 µl of 0.01 M-STE-TX buffer. A control unphosphorylated NS2 preparation containing Grace's insect cell medium instead of a cell extract was set up and treated identically to that described above. The affinity of the GST protein alone for poly(U)-Sepharose was also assayed in 0.015 M-STE-TX buffer. The bound protein [poly(U)-

Fig. 2. Staphylococcus aureus V8 protease peptide mapping of the 46K and 48K species of the baculovirus-expressed NS2 protein of EHDV. The two 35S-labelled EHDV proteins were gel-purified and then partially digested during electrophoresis through a 15% polyacrylamide gel with 5 µg of S. aureus V8 protease. Lanes 1 and 2 show digested 48K and 46K NS2 bands, respectively. Undigested 46K and 48K NS2 species are indicated by arrows and protein markers of indicated Mr are shown to the left of the figure.

Seapharose pellets and unbound protein (precipitated supernatants) were analysed by SDS–PAGE. Quantification of RNA-binding was done by scanning of the stained protein bands representing the bound and unbound fractions. The peak areas were determined and expressed as a fraction of the total protein recovered, bound and unbound.

Results

Polyacrylamide gel analysis of NS2 proteins of three different orbiviruses

Recombinant baculoviruses, containing the entire coding region of the NS2 genes of BTV-10, AHSV-9 and EHDV-2, were constructed and SDS–PAGE analysis of recombinant baculovirus-infected S. frugiperda cell lysates indicated that all the different NS2 proteins were expressed in large amounts (Fig. 1). The NS2 of BTV-10 electrophoresed as a single band whereas the NS2 of AHSV-9 electrophoresed as a rather diffuse band that occasionally separated as a noticeable doublet. The NS2 expressed by the EHDV baculovirus recombinant, on the other hand, always separated into two distinct bands of 46K and 48K, respectively. Both unequivocally reacted
with EHDV antisera in a Western blot (not shown). Since it has been shown that EHDV NS2 does not contain any large overlapping open reading frames (Van Staden et al., 1991) and the two bands were found in all of several independently produced EHDV NS2 baculovirus recombinants, the relationship between the 46K and 48K NS2 bands was investigated by peptide map analysis. The peptide maps produced for these EHDV NS2 polypeptides were found to be identical, indicating that the 46K and 48K bands consist of the same amount of amino acids (Fig. 2).

**In vitro phosphorylation of NS2**

Considering their identical peptide maps, one factor likely to influence the electrophoretic mobility of EHDV NS2 proteins was thought to be differential phosphorylation. Although it is known that BTV NS2 can be phosphorylated *in vitro* without the addition of exogenous kinase (Huismans et al., 1987), the mechanism by which NS2 is phosphorylated *in vitro* is not known. To further investigate, the extent to which the different baculovirus-expressed NS2 proteins could be phospho-
Orbivirus NS2 expression

Fig. 3. Autoradiographs of in vitro phosphorylated fractions obtained after sucrose gradient fractionation of the different orbiviral NS2 proteins. S. frugiperda cells were infected with AcMNPV (a) or with the recombinant baculoviruses expressing the AHSV (b), BTV (c) and EHDV (d) NS2 proteins, respectively. Cytoplasmic extracts were centrifuged through linear 10 to 40% sucrose gradients and fractions were collected from the bottom (lanes 1) to the top (lanes 13) of the respective gradients. Each gradient fraction was subjected to an in vitro phosphorylation assay, resolved by SDS-PAGE and autoradiography. The position of the respective NS2 proteins as well as that of a strongly labelled 46K cellular protein is indicated by arrows. The pellet fractions are indicated by P in all cases.
Fig. 4. Analysis of dephosphorylated $^{32}$P-labelled EHDV NS2 by autoradiography and Western blotting. A cytoplasmic extract (200 µl), prepared from S. frugiperda cells infected with the EHDV NS2 baculovirus recombinant was phosphorylated in vitro as described in Methods. A sample (55 µl) was removed (lane 1) prior to treatment of the labelled extract with CIP. Samples (83 µl) were removed after 30 min (lane 3), 60 min (lane 4) and 90 min (lane 5) of incubation. A $^{32}$P-labelled sample of the cytoplasmic extract (50 µl) was incubated in CIP buffer without the CIP itself at 37 °C for 90 min (lane 2) and included as a control. (a) The samples were resolved by SDS-PAGE, followed by autoradiography. The sizes of the protein markers are indicated to the left of the figure. (b) An identically resolved gel was subjected to Western blot analysis using an EHDV antiserum. Lanes 1 to 5 were the same as in (a).

also found in uninfected cells (not shown) and has exactly the same size as NS2 of AHSV and BTV: this finding makes it more difficult to interpret the in vitro phosphorylation results obtained with these proteins (Fig. 3b and c). However, peptide mapping experiments were carried out with the phosphorylated cellular proteins sedimenting in fractions 9 and 10 of the gradients and the results were compared with the peptide maps obtained with the phosphorylated proteins of the same size in fractions 5 and 6 of both the AHSV and BTV gradients. The results, in combination with duplicate Western blots (not shown) indicated that the proteins were different and that the phosphorylated NS2-sized proteins in fractions 5 and 6 of the BTV and AHSV gradients (Fig. 3b and c) were indeed NS2. With the exception of the AHSV NS2, the extent to which NS2 was phosphorylated (Fig. 3b, c and d) did not correlate to the amount of protein.

In order to compare the in vitro phosphorylation of NS2 expressed in insect cells with that of in vivo phosphorylated wild-type NS2, peptide maps of the proteins from these different sources were prepared and compared to a digest of the in vitro phosphorylated NS2 obtained from recombinant baculovirus-infected cells. The peptide maps of the two proteins proved to be identical (not shown) and it was not possible to distinguish between the in vitro and the in vivo phosphorylated forms of the NS2 protein.

In vitro dephosphorylation of the NS2 protein

To determine whether dephosphorylation of NS2 influences its electrophoretic mobility, the 46K and 48K EHDV NS2 proteins were treated with CIP. In vitro phosphorylated cytoplasmic extracts prepared from the EHDV recombinant baculovirus-infected cells were incubated with CIP and samples taken at different time intervals were analysed by SDS-PAGE followed by autoradiography (Fig. 4a). Treatment with CIP resulted in the removal of the $^{32}$P label from both the 46K and 48K NS2 proteins and it appears as though an almost complete dephosphorylation could be achieved after only 30 min (Fig. 4a). Dephosphorylation of the 48K protein was more extensive than for the 46K protein, resulting in the apparent loss of all radioactivity. However, no concomitant increase in the amount of the 46K protein was observed and the loss of radioactivity was also not due to degradation of the NS2, since the proteins were present in similar amounts before and after
dephosphorylation. These results were confirmed by Western blot analysis of a duplicate gel to the autoradiographed gel (Fig. 4b), which indicated that the dephosphorylation did not alter the ratio or the electrophoretic migration of the 46K and 48K proteins. It could therefore be concluded that the observed difference in electrophoretic mobility is not due to NS2 phosphorylation. These results were confirmed in several independent experiments in which unlabelled extracts were dephosphorylated under the same conditions as those used for this study and/or with higher concentrations of CIP for longer periods of time. In all of these experiments no shift in the electrophoretic mobility of the 46K and 48K proteins was observed. Treatment of EHDV recombinant baculovirus-infected cells with tunicamycin, to detect N-linked carbohydrates, also had no effect on the electrophoretic migration of the NS2 proteins (results not shown).

Phosphorylation of EHDV NS2 synthesized in E. coli as a fusion protein

To further investigate the phosphorylation of NS2 proteins, EHDV NS2 was expressed in E. coli as described in Methods. It was assumed that, since bacterial kinases are generally specific for their natural substrates (Barik & Banerjee, 1991), the NS2 expressed in the bacteria would be unphosphorylated if a specific eukaryotic kinase is required for phosphorylation. IPTG-induced proteins were extracted from the E. coli cultures that were carrying EHDV NS2 recombinant or parental pGEX expression plasmids and analysed by SDS-PAGE. An over-produced 69K protein was present in the protein extract prepared from the pGEX-1 recombinant clone only. The identity of the fusion protein was confirmed by Western blot analysis with EHDV antiserum (not shown).

To determine whether the NS2–glutathione S-transferase (GST) fusion protein expressed in E. coli is phosphorylated, cultures of E. coli JM109 cells containing either the recombinant or the parental pGEX-1 vector were grown in a low-phosphate medium. 32P, was added to the cultures at the time of addition of IPTG and parallel cultures were labelled with [35S]methionine to confirm the synthesis of the NS2 fusion protein during the period of 32P-labelling. After SDS–PAGE of the bacterial lysates, autoradiography (Fig. 5) clearly indicated that the synthesis of the 69K NS2 fusion protein was induced by IPTG in the low-phosphate medium, as judged by a comparison of the [35S]methionine-labelled
proteins with that of the control non-induced cultures. However, no incorporation of the $^{32}$P-label by the abundant NS2 fusion protein was detected. A protein of approx. 34K served as a positive control in the phosphorylation studies in that it was labelled by $^{32}$P in both the IPTG-induced and the control non-induced cultures. It was therefore concluded that NS2 synthesized in *E. coli* is not phosphorylated *in vivo* by one of the bacterial kinases and that the NS2 fusion protein has no autophosphorylation activity.

**In vitro phosphorylation of the NS2 fusion protein by a cellular protein kinase**

In a subsequent investigation, it was determined whether the NS2 fusion protein could be phosphorylated *in vitro* by a component present in cell lysates from uninfected insect cells. Bacterial extracts were prepared from *E. coli* JM109 cells containing either the recombinant or parental pGEX-1 vector. Each of these extracts was incubated either alone or with an equal volume of a cytoplasmic extract prepared from uninfected *S. frugiperda* cells, in the presence of $[^{32}P]ATP$. The resulting SDS–PAGE autoradiograph (Fig. 6a) revealed that neither the NS2 fusion protein nor the GST protein was phosphorylated when the respective extracts were incubated with $[^{32}P]ATP$ alone (Fig. 6, lanes 3 and 4). However, when the respective extracts were mixed with the *S. frugiperda* cytoplasmic extract and incubated in the presence of $[^{32}P]ATP$, the NS2 fusion protein was strongly labelled (Fig. 6, lane 1). From these results it could be concluded that the fusion protein and by extension the NS2 synthesized in recombinant baculovirus-infected insect cells is phosphorylated by a protein kinase(s) present in the *S. frugiperda* cellular extract and that the kinase(s) is specific for NS2. The results shown in Fig. 6(a) also revealed the presence of an additional radiolabelled protein (67K). However, Western blot
phosphorylated protein did not bind to the poly(U)-Sepharose at low salt concentrations investigated (0.015 M-STE-TX), as much as 25% of the phosphorylated and unphosphorylated proteins were found to bind to poly(U)-Sepharose at a given molarity of STE-TX buffer, is shown as a fraction of the total (bound plus unbound) protein. Both the phosphorylated and unphosphorylated proteins were found to bind to the poly(U)-Sepharose at low salt concentrations. However, even at the very lowest salt concentrations investigated (0.015 M), as much as 25% of the phosphorylated protein did not bind to the poly(U)-Sepharose. An increase in salt concentration to 0.03 M-STE-TX even further reduced this to 42% unbound, whereas in comparison more than 95% of the unphosphorylated NS2 was bound at salt concentrations of 0.03 M-STE-TX or less. Both proteins do not bind at the very highest salt concentrations investigated, but there is again a difference in that 23% of the unphosphorylated NS2 still bound at 0.2 M-STE-TX, whereas none of the phosphorylated protein was found to bind under the same conditions.

**Discussion**

We have expressed high levels of the NS2 proteins of EHDV-2, BTV-10 and AHSV-9 in *S. frugiperda* cells by means of infection with different baculovirus recombinants. The EHDV NS2 and occasionally the AHSV NS2 were each resolved into two bands in SDS-polyacrylamide gels. It has also previously been observed that BTV NS2 may be presented as two closely spaced bands upon electrophoresis in SDS-PAGE gels (Huismans & Basson, 1983; Huismans et al., 1987). One of the aims of the present investigation was to determine why the NS2 proteins of these viruses can be represented by doublet bands after SDS-PAGE. For this purpose the EHDV NS2, consistently and clearly represented by two different bands, was used as a model. Since the NS2 sequence of EHDV (Van Staden et al., 1991) does not contain any large overlapping open reading frames, the possibility that the different EHDV NS2 species originated as an artifact of *in vivo* translation by separate translation events could be excluded. This was confirmed in an independent experiment by *in vitro* transcription and translation of a full-length DNA copy of the EHDV-2 NS2 gene (J. Theron, J. M. Uitenweerde, H. Huismans & L. H. Nel, unpublished). Furthermore, peptide mapping of the 46K and 48K EHDV NS2 protein species indicated that they were identical and it was therefore postulated that post-translational modifications such as phosphorylation or glycosylation could account for the presence of the two NS2 protein species in infected cells.

We have established that the EHDV NS2 protein does not contain any N-linked carbohydrates and this result is in agreement with that reported for AHSV NS2 (Grubman & Lewis, 1992). It is not known whether O-linked carbohydrates may be added during NS2 processing and the possibility that such a modification plays a role in altering the electrophoretic properties of the protein cannot be excluded. We have subsequently shown that baculovirus-expressed EHDV and AHSV NS2 proteins could be phosphorylated *in vitro*. If phosphorylation of the proteins was indeed responsible for differentially altering their electrophoretic characteristics, it follows that complete dephosphorylation should lead to reversion into a single electrophoretic form.
However, it was found that each of the two EHDV NS2 protein bands were phosphorylated and that dephosphorylation of these protein species with CIP (specific for phosphoserines; Klausing et al., 1988) did not change their electrophoretic migration at all. If EHDV NS2 is phosphorylated at threonine as well as serine residues, it is possible that dephosphorylation (phosphorylation) of phosphoserines has no detectable effect on the electrophoretic migration of the NS2 species, whereas dephosphorylation (phosphorylation) of phosphothreonine residues may have a pronounced effect. This has been found to be the case for the large T antigen of SV40 (Gräßer & König, 1992). However, BTV as well as AHSV have been reported to be phosphorylated only at serine residues (Devaney et al., 1988; Thomas et al., 1990). Based on the similarity of these proteins with EHDV NS2, as well as the apparently total dephosphorylation by CIP, it appears likely that the EHDV NS2 may also be phosphorylated at serine residues only. Devaney et al. (1988) have shown that BTV NS2 could be separated into two different forms in two-dimensional gels (isoelectric focussing followed by SDS–PAGE) and the authors concluded that an unidentified charge altering modification, rather than variation in phosphorylation, was responsible for the observed NS2 isoforms.

The results presented in this paper indicated that different orbivirus NS2 proteins could be phosphorylated in vitro, and essentially ruled out the phosphorylation of the proteins by another orbivirus protein. To investigate the mechanism by which the phosphorylation occurs, we have expressed the EHDV NS2 protein in E. coli and determined its phosphorylation status. The NS2 synthesized as a fusion to GST, was found to be not phosphorylated, which suggested that there was an absence of autophosphorylation activity. Thus, NS2 may be phosphorylated by a kinase present in the cytoplasm of virus-infected cells. To investigate this, a kinase assay experiment was performed in which the unphosphorylated bacterial-expressed NS2 was mixed with a cytoplasmic extract prepared from uninfected insect cells and it was found that the bacterial-expressed NS2 fusion protein could indeed be phosphorylated in this way. In addition, it was found that the kinase associated with the cytoplasm of S. frugiperda cells exhibited a specificity for NS2, since the GST affinity tail was found to be unphosphorylated in control experiments. Although these results constitute the first direct evidence that NS2 is phosphorylated by a kinase of cellular origin, the identity of the kinase remains unknown. It appears likely that a single protein kinase is involved since it has been demonstrated that sucrose gradient purification or even poly(U)–Sepharose column purification of BTV NS2 did not change its ability to be phosphorylated by the simple addition of [γ-32P]ATP (Huismans et al., 1987), and it therefore follows that the cellular kinase was co-purified with the NS2 protein.

A source of completely unphosphorylated NS2, such as described here for the NS2 produced in prokaryotic cells, provided an opportunity to investigate likely functions of NS2 phosphorylation. Subsequently, a clear correlation between phosphorylation of NS2 and a corresponding decrease in ssRNA-binding, has been demonstrated in vitro. Throughout a wide range of different ionic strengths, the phosphorylated protein preparations were found to be 20 to 30% less effective in binding ssRNA, compared to the unphosphorylated control preparations. In addition, phosphorylated NS2 appeared to be more sensitive to increased ionic strength as the RNA-binding capability of phosphorylated NS2 was completely abolished at 0·2 M-STE-TX, whereas more than 20% of the unphosphorylated NS2 could still bind RNA. We have no indication of the percentage of sites or molecules which was phosphorylated in our in vitro experiments and as yet no information on the factors which control phosphorylation of NS2 is available. It is also likely that our assays do not reflect in vivo occurrences precisely, whereas the bacterial-expressed fusion NS2 will also differ somewhat from the native viral protein. Nevertheless, we have now obtained evidence that the EHDV NS2 protein is specifically phosphorylated by a cellular kinase and that this alteration down-modulates ssRNA-binding by the protein. It is therefore suggested that the control of NS2 phosphorylation plays an important regulatory role in the replication of orbiviruses and that this aspect may constitute a unique characteristic amongst members of the Reoviridae.

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References


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