Identification of a protein kinase involved in the phosphorylation of the C-terminal region of human respiratory syncytial virus P protein

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P protein, the structural phosphoprotein of the Long strain of respiratory syncytial (RS) virus, is phosphorylated at serine residues. Some of these residues are candidates for modification by casein kinase II, as they are contained in consensus sequences. A cellular protein kinase, able to phosphorylate the P protein in vitro and apparently associated with purified RS virions, has been partially purified from HEp-2 cells. It shows several characteristics similar to those of casein kinase II. The P protein is modified in vitro by this activity mainly at serine residues located near the C terminus, which are also modified during virus infection. Thus, the P protein is phosphorylated in vivo in two regions, a central region as previously described, and another located in the C-terminal part of the molecule. The protein kinase involved in the phosphorylation of the C-terminal domain is similar to a cellular casein kinase II.

Introduction

The transcription and replication complexes of respiratory syncytial (RS) virus are likely to be the viral nucleocapsids, as established for other non-segmented negative-strand RNA viruses. Thus, it is thought that RS virus nucleocapsids are composed of the genome RNA of 15222 nucleotides (Mink et al., 1991) associated with the nucleocapsid protein (NP), which form the template for RNA synthesis, involving the L protein which displays the RNA polymerase activity and the P protein, a phosphoprotein (Kolakofsky & Roux, 1987).

Analyses of mutant phenotypes and the results of in vitro reconstitution experiments indicate that the L proteins of vesicular stomatitis virus (VSV), a rhabdovirus (Banerjee, 1987), and Newcastle disease virus (Hamaguchi et al., 1983) are responsible for multiple enzymatic functions such as initiation, elongation and termination of the products of transcription and replication. The precise role of the P phosphoproteins in RNA synthesis remains undefined, but the fact that activities of L are detected only in the presence of the P protein suggests that the latter is an essential transcription factor (Banerjee, 1987; Emerson & Yu, 1975).

The current knowledge of the VSV P protein has been summarized recently (Banerjee & Barik, 1992). Three distinct functional domains have been defined. The N-terminal domain I is highly acidic and contains the constitutively phosphorylated sites, located between positions 35 and 78 (Bell & Prevec, 1985; Hsu & Kingsbury, 1985). Domains II and III are basic and apparently involved in the binding of the P protein to the L protein and the NP–RNA template, respectively. An L-associated kinase activity (Chattopadhyay & Banerjee, 1987) has also been described, which modifies in vitro two serine residues (236 and 242) in domain II. This specific phosphorylation regulates binding of P to L and to the NP–RNA template, which is essential for RNA synthesis in vitro (Gill et al., 1986; Chattopadhyay & Banerjee, 1987). It has also been demonstrated that unphosphorylated P protein is transcriptionally active upon reconstitution with viral L protein and NP–RNA template preparations (Barik & Banerjee, 1991).

Recently, a model for activation of the P protein by a phosphorylation cascade pathway has been proposed (Barik & Banerjee, 1992a). The unphosphorylated P protein (P₀) is converted to a phosphorylated P₁ species by a cellular kinase, present in BHK cell extracts, which phosphorylates residues in domain I. This protein kinase has been purified and identified as casein kinase II (Barik & Banerjee, 1992b). The precise serine residues (those at positions 59 and 61) modified by casein kinase II, both in vitro and in vivo, have been identified using mutated versions of the P protein (Takacs et al., 1992). An intrinsic protein kinase activity of the L protein has also been suggested (Hammond et al., 1992). In Sendai virus, the purified L protein also displays a kinase activity.
which acts on the NP and P proteins (Einberger et al., 1990).

To understand the role of P protein phosphorylation in RS virus development, the phosphorylated residues of this protein were analysed and partially located (Navarro et al., 1991). It was also found that, in contrast to the situation for the VSV P phosphoprotein, the phosphorylation level of the P protein in vivo does not affect nucleocapsid function in RNA synthesis (Villanueva et al., 1991).

Here we attempt to determine whether or not the kinase activity(ies) responsible for phosphorylation of the P protein is virus-encoded.

The results indicate that P protein phosphorylation in vivo occurs at two regions, the central domain previously described (Navarro et al., 1991) and an additional C-terminal domain. Phosphorylation in the second is mediated by a cellular casein kinase II-like protein. It also appears that this protein kinase activity may be present in association with purified virions.

**Methods**

**Cells and viruses.** The cell lines HEp-2, HeLa, Vero and CVI were obtained from the ATCC; culture conditions have been described previously (Villanueva et al., 1991). Long strain of human RS virus used throughout this study was plaque-purified and passaged at low multiplicity in HEp-2 cells (Villanueva et al., 1991).

**Infection, cell labelling and immunopurification of the P protein.** The conditions previously described by Villanueva et al. (1991) and Navarro et al. (1991) have been followed.

**Purification of RS virions.** The conditions for RS virus infection were as previously described (Villanueva et al., 1991). Forty-eight hours post-infection, cells were scraped off the tissue culture plates with a rubber policeman and pelleted by low-speed centrifugation. The supernatant was precipitated twice with polyethylene glycol 6000 (Merck) at a final concentration of 6%. The precipitated material was collected and precipitated with 70% (NH₄)₂SO₄. The precipitated material was washed twice with 0.5 ml/dish of PBS. The last pellet was resuspended in kinase buffer and the cellular debris pelleted by low-speed centrifugation. The supernatant, considered as the cellular extract, was collected and precipitated with 70% (NH₄)₂SO₄. The precipitated material was recovered by centrifugation at 10000 g for 30 min at 4 °C. The material sedimented at 45% sucrose was collected and pelleted through a 33% sucrose cushion in TNE buffer at 240000 g for 2 h at 4 °C.

**Disruption of RS virions.** Purified virions were diluted in TNE buffer containing 1% NP40 to protein concentrations ranging from 0.5 to 1 mg/ml. After 30 min at room temperature with occasional shaking, the protein preparation was centrifuged at 16000 g for 30 min at 4 °C or subjected to immunoaffinity chromatography as described (Navarro et al., 1991).

**Chemical and enzymatic treatments of the ³²P-labelled P protein.** The unlabelled or ³²P-labelled P protein produced in RS virus infections was purified by immunoaffinity chromatography with monoclonal antibody 1P, specific for the P protein (Garcia et al., 1993), following previously described procedures (Navarro et al., 1991). The unlabelled P protein was used as the substrate for in vitro phosphorylation; the enzyme was obtained from HEp-2 cell extracts. In vitro phosphorylation was also achieved using purified virions as both the substrate and enzyme. Phosphoamino acid analyses were performed following previously described conditions (Navarro et al., 1991).

Approximately 40 µg of protein ³²P-labelled in vivo or in vitro, with a partially purified casein kinase II-like activity, was digested with 1 µg of trypsin for 2 h at 37 °C. The reaction was stopped by addition of 5 mM-PMSF. The peptides obtained were separated by HPLC using a C18 column. The N-terminus of the peptides were determined as previously described (Navarro et al., 1991). In some cases, after SDS-PAGE (Studier, 1972), the ³²P-labelled P protein band was sliced out and treated with either 80% formic acid, 1 µg trypsin or with 1 µg chymotrypsin overnight at 37 °C. The material eluted from the gel was vacuum-dried, resuspended in Student’s sample buffer and analysed in a 20% acrylamide gel.

**Kinase assay.** Protein kinase activity was determined in 50 µl of kinase buffer (60 mM-Tris-HCl pH 8.0, 5 mM-MgCl₂, 10 mM-DTT, 0.1% NP40) containing 5 µM-ATP or 1 or 5 µM-GTP and 25 µCi [γ-³²P]ATP or 25 µCi [γ-³²P]GTP. The mixture was incubated for 30 min at 37 °C. The reaction was terminated by the addition of Student’s sample buffer and the phosphorylated material was analysed by SDS-PAGE (Studier, 1972) and autoradiography. As the protein kinase substrate (when the P protein was not present in the protein kinase preparation), 1.8 µg of a protein preparation containing NP and P proteins, purified by immunoaffinity chromatography from cytoplasmic extracts of RS virus-infected HEp-2 cells (Navarro et al., 1991) was used. The peptide RRREEETEEE (ETE peptide) (10 mM) was also used as a substrate. Its phosphorylation was assayed using 50 µM-ATP (Casnellie, 1991).

**Partial purification of a protein kinase activity from uninfected HEp-2 cells.** Confluent HEp-2 cells, growing in eight 55 cm² plastic Petri dishes, were scraped off, collected by low-speed centrifugation and washed twice with 0.5 ml/dish of PBS. The last pellet was resuspended in kinase buffer and the cellular debris pelleted by low-speed centrifugation. The supernatant, considered as the cellular extract, was collected and precipitated with 70% (NH₄)₂SO₄. The precipitated material was recovered by centrifugation at 10000 g for 30 min at 4 °C, resuspended in kinase buffer and chromatographed on Sepharose 4B containing covalently bound NP and P proteins, previously purified by immunoaffinity chromatography (Navarro et al., 1991). The sample was chromatographed twice at a flow rate of 1.7 ml/h and the column was washed with five column volumes of kinase buffer at the same flow rate. Successive elutions were performed with kinase buffer containing 0.1 and 0.25 M-NaCl. The bulk of kinase activity was eluted at 0.1 M-NaCl and further chromatographed through a heparin-acrylamide column (Sigma). The flow rate was 3 ml/h and the sample was chromatographed twice. The column was then washed with five column volumes of sample buffer and eluted with kinase buffer containing 0.5 or 1 M-NaCl. Protein concentrations were measured by the Bradford method (Bradford, 1976) and kinase activity was determined as described above. Cell extracts from RS virus-infected HEp-2 cells and CVI cells were also used as a source of protein kinases. Rabbit anti-casein kinase II serum was obtained from Upstate Biotechnology.

**M determination of protein kinase catalytic subunit(s).** The method described by Kameshita & Fujisawa (1989) was followed. Briefly, the proteins contained in the different fractions from purification steps were separated in SDS-polyacrylamide gels containing 1 mg/ml casein. After electrophoresis, the proteins fractionated in the gel were renatured and assayed for protein kinase activities using the casein present in the gel as the substrate under the conditions previously described. After removal of the non-incorporated [γ-³²P]ATP by several washes with 5% TCA and 1% pyrophosphate, the putative protein kinases were identified by autoradiography.
Results

Phosphorylation of the P protein by a virion-associated protein kinase activity

To determine whether a protein kinase activity is associated with RS virus, purified virions were assayed for this activity. Fig. 1 shows that a protein kinase that phosphorylates the P protein is associated with virus particles. In addition to the P protein, other polypeptides were phosphorylated, although none comigrated in SDS-PAGE with the viral proteins, suggesting that they may be of cellular origin. The presence of kinase activity(ies) is dependent on the addition of detergent in the kinase assay and it uses either GTP (lanes 1, 2, 4 and 5) or ATP (lanes 3 and 6) as the phosphate donor. The similarity in the phosphorylation patterns obtained using ATP or GTP is compatible with the presence, in purified RS virus particles, of one or more protein kinases, able to use either phosphate donor on the P protein. This contrasts with the situation described for VSV (Beckes & Perrault, 1991).

To localize the protein kinase activity present in the virus particle, purified virions were disrupted by detergent treatment and envelope and nucleocapsid proteins were fractionated by centrifugation or affinity chromatography, as indicated in Methods. The protein composition and the protein kinase activity of the fractions are shown in Fig. 2.

The differences in the phosphorylation level observed for the virion preparations shown in Fig. 1 (lanes 3 and 6) and in Fig. 2 (a, lanes 1 and 4; b, lanes 1 and 5) are probably due to differences in the detergent treatment, i.e. addition of 0.1% NP40 to the kinase mixture in Fig. 1, or treatment with 1% NP40 for 30 min before the kinase assay in Fig. 2. In addition, as different virus particle preparations were used in each case, slight differences in inhibitor or phosphatase levels may also account for the experimental results observed.

The high-speed supernatant fraction (Fig. 2a, lane 2) contains the bulk of F1, small amounts of NP and P proteins and 50% of membrane proteins M1 and M2. The pellet (a, lane 3) contains the bulk of the NP and P proteins and the remainder of the M1 and M2 membrane proteins. The protein kinase activity was found to be associated with both the envelope proteins and the particulate material (a, lanes 5 and 6, respectively). Virus proteins were also fractionated by affinity chromatography on a column containing an antibody against the P protein coupled to Sepharose 4B. The fractions not retained on the column (b, lane 3) are composed of the bulk of the F1 and M1 proteins, NP protein and small amounts of P and M2 proteins. The fractions eluted from the column (b, lane 4) contain the bulk of the M2, NP and P proteins, and a small amount of M1. A protein of approximate Mr 200K, which may be the RS virus L protein, copurifies with putative nucleocapsids after immunoaffinity chromatography, as determined by using longer length SDS-polyacrylamide gels (data not shown).

Protein kinase activity was shown to be associated mainly with the fractions not retained on the column and extracted from it with sample buffer (b, lane 7). Kinase activity is also found in the column fractions obtained during sample application (b, lane 2), and probably corresponds to that of undisrupted virions. However, labelling of the P protein was enhanced when the fractions separated by chromatography (b, lanes 7 and 8) were mixed (b, lane 9), suggesting that the P protein does not possess kinase activity. In other words, the P protein phosphorylation observed is not due to autophosphorylation. It also seems that the protein kinase activity copurifies with viral envelope components when the virion components have been fractionated by chromatography.

To eliminate the possibility that a proportion of the kinase activity is inactivated by the chromatography
elution conditions, a pre-chromatography kinase sample was tested under these conditions; no changes in kinase activity were detected. Thus, these data indicate that purified RS virions contain at least one kinase, not associated with the nucleocapsid, which is able to use either ATP or GTP to phosphorylate the P protein.

**Determination of phosphoamino acids and phosphopeptides in the P protein**

To determine whether the P protein phosphorylation produced by *in vitro* modification using purified virions is similar to that found in RS virus-infected cells, we have identified the amino acids modified under both conditions and have located them within the P protein molecule.

HEp-2 cells were infected with RS virus and labelled with $^{32}$P, and the $^{32}$P-labelled P protein was then purified by immunoaffinity chromatography (Navarro *et al.*, 1991) (Fig. 3, lanes 1, 3 and 5). Purified virions were also used as a source of both P protein and kinase activity for *in vitro* assays (Fig. 3, lanes 2, 4 and 6). The $^{32}$P-labelled P protein was extracted after SDS-PAGE and subjected to either phosphoamino acid analysis (Fig. 3a) or chemical and enzymatic treatments to cleave it (Fig. 3b). As shown for P protein produced during RS virus infection (Navarro *et al.*, 1991) (Fig. 3a, lane 1), the *in vitro* phosphorylated P protein (Fig. 3a, lane 2) also contains phosphoserine as the main phosphorylated amino acid.

The phosphopeptide cleavage maps (Fig. 3b) obtained when the $^{32}$P-labelled P proteins from both sources were treated with formic acid (lanes 1 and 2), trypsin (lanes 3 and 4) or chymotrypsin (lanes 5 and 6) were found to be similar. These results suggest that P protein, modified *in vitro* by virion-associated kinase(s), is phosphorylated at serine residues located in same region of the P protein molecule as those phosphorylated during RS virus infection.

**P protein exogenously added to uninfected or RS virus-infected cell extracts is phosphorylated in vitro**

Cell extracts from uninfected (Fig. 4, lanes 3 and 8, and 5 and 10) or RS virus-infected HEp-2 cells (Fig. 4, lanes 2 and 7, and 4 and 9) were assayed for protein kinase activities displayed on endogenous proteins (Fig. 4, lanes 2 and 7, and 3 and 8) or on added P protein (Fig. 4, lanes 4 and 8, and 5 and 10). The phosphorylated proteins were visualized, after separation by SDS-PAGE, by Coomassie blue staining (lanes 1 to 5) or autoradiography (lanes 6 to 10). In both cases, protein kinase activities were found on both of the substrates assayed.

The labelled P protein was sliced out and treated with formic acid, trypsin and chymotrypsin. After SDS-PAGE, the phosphopeptides produced in all cases have
the same electrophoretic mobilities as those shown in Fig. 3 (data not shown). Similar results were obtained when uninfected or RS virus-infected extracts from

- HEp-2 cells growing in tissue culture
- Remove medium and wash twice with PBS
- Scrape off
- Low-speed centrifugation
  - Supernatant
  - Pellet
- Resuspend in kinase buffer
- Low-speed centrifugation
  - Cell extract = supernatant
  - Pellet
- Precipitation with 70% (NH₄)₂SO₄
- Chromatography through Sepharose 4B column with NP-P covalently bound
  - Flowthrough
  - Retained material eluted with kinase buffer, 0-1 M-NaCl
- Chromatography through heparin-acrylamide
  - Flowthrough
  - Retained material eluted with kinase buffer, 0-5–1 M-NaCl

Fig. 5. Summary of the purification of protein kinase activity from HEp-2 cells.

Fig. 4. Phosphorylation of the P protein by uninfected or RS virus-infected HEp-2 cell extracts. Cell extracts from uninfected (30 µg, lanes 3 and 8) or RS virus-infected (30 µg, lanes 2 and 7) cells were assayed for kinase activity in the absence (lanes 2 and 7, and 3 and 8) or presence (lanes 4 and 9, and 5 and 10) of exogenous P protein. Lane 1 indicates the preparation of the P protein used in lanes 4 and 9, and lanes 5 and 10. After SDS-PAGE, the proteins were stained with Coomassie blue (lanes 1 to 5) and the phosphopeptides were identified by autoradiography (lanes 6 to 10).

Fig. 3. Determination of the residues phosphorylated in vivo and in vitro. ³²P-labelled P protein obtained from RS virus-infected cells (a, lane 1; b, lanes 1, 3 and 5) or by in vitro phosphorylation (a, lane 2; b, lanes 2, 4 and 6) using purified virions as the source of both enzyme and substrate were hydrolysed to characterize the labelled phosphoamino acids (a). S, T and Y correspond to the positions of phosphoserine, phosphothreonine and phosphotyrosine markers, developed after electrophoresis with ninhydrin. Both labelled P proteins were cleaved (b) by incubation with either formic acid (lanes 1 and 2), trypsin (lanes 3 and 4) or chymotrypsin (lanes 5 and 6) and the resulting polypeptides were characterized by gel electrophoresis.
Fig. 6. Kinase activity of chromatography fractions obtained on heparin-acrylamide. Purified NP and P proteins (1.8 μg) were used as substrates in the kinase assay of the fractions indicated. The proteins were then separated by SDS-PAGE and stained with Coomassie blue (lanes 1 to 8). Phosphorylated proteins were visualized after autoradiography (lanes 9 to 16). Lanes 1 and 9 correspond to 0.72 μg of the material eluted with 0.1 M-NaCl-containing kinase buffer from the P protein-Sepharose 4B column; lanes 2 and 10 contain 1 μg of the material not retained by the heparin-acrylamide column; lanes 3 and 11, and 4 and 12 represent 1 μg of the material contained in the fractions corresponding to the washing of the column; lanes 5 and 13, 6 and 14, 7 and 15, and 8 and 16 correspond to material contained in 5 μl of the fractions eluted from the column with 1 M-NaCl kinase buffer. Lane V, purified virus; lanes M, Mr markers.

HeLa, Vero or CV1 cells were tested (data not shown). This suggests that the population of P protein molecules produced during RS virus infection is not completely phosphorylated or dephosphorylated during the isolation procedures. P protein is therefore a suitable substrate for in vitro phosphorylation mediated by cellular protein kinase(s) present in different cell lines.

Partial purification of the protein kinase activity that modifies the P protein

A protein kinase was isolated by fractionation of a HEp-2 cell extract as described in Methods (Fig. 5), using immunopurified P protein as a substrate.

The bulk of proteins present in cell extracts after precipitation with 70% (w/v) (NH4)2SO4 was chromatographed through a Sepharose 4B column containing covalently bound NP and P proteins. Most kinase activity was found in the material retained and eluted with 0.1 M-NaCl. The kinase activity, present in purified virions, was not retained in the immunoaffinity column (Fig. 2b) when the sample was loaded onto the column in 0.1 M-NaCl.

Considering that three of the nine putative phosphorylated serines of the P protein (those at positions 86, 119 and 161) (Navarro et al., 1991) have an acidic residue in position +3 and that one (at position 116) may have phosphoserine (if serine 119 is modified), it can be postulated that all of these serines could be substrates for a casein kinase II-like protein kinase(s). Thus, the next purification step involved chromatography of the protein fraction containing the kinase activity through heparin-acrylamide. The fraction containing the greater protein kinase activity was eluted with kinase buffer containing 1 M-NaCl.

The kinase activity of the fractions eluted from the heparin-acrylamide column is shown in Fig. 6; in every case, the major substrate for the kinase was the P protein.

Table 1. Summary of protein kinase purification

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity*</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>70% (NH4)2SO4 precipitate</td>
<td>0.68</td>
<td>4</td>
</tr>
<tr>
<td>Eluted material from NP-P proteins covalently bound to a Sepharose 4B column</td>
<td>2.29</td>
<td>13.5</td>
</tr>
<tr>
<td>Eluted material from heparin-acrylamide column</td>
<td>34-212</td>
<td>200-1247</td>
</tr>
</tbody>
</table>

* Specific activity is defined as pmol 32P incorporated into 18 μg of NP-P per μg of protein assayed for protein kinase activity.
Table 2. Properties of purified kinase activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Addition</th>
<th>Kinase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>10 mM-spermidine</td>
<td>130</td>
</tr>
<tr>
<td>3</td>
<td>0.1 M-NaCl</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>48 mM-heparin</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>10 mM-CaCl₂</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>Rabbit serum</td>
<td>110</td>
</tr>
<tr>
<td>7</td>
<td>Anti-CKII* rabbit serum</td>
<td>55</td>
</tr>
</tbody>
</table>

(ii) Phosphate donor

<table>
<thead>
<tr>
<th>[³²P]ATP (µM)</th>
<th>GTP (µM)</th>
<th>³²P incorporation in P protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>19</td>
</tr>
</tbody>
</table>

* CKII: Casein kinase II.

Characterization of purified kinase activity

Considering that serine residues at positions 86, 116, 119 and 161 in the P protein molecule are the candidates for phosphorylation both in vivo (Navarro et al., 1991) and in vitro (as described here), and that all of these serines are surrounded by residues matching putative sequence signals for casein kinase II, we have determined whether a protein kinase preparation from HEp-2 cells had the properties described for casein kinase II. The results are summarized in Table 2. All properties analysed are displayed by this partially purified kinase preparation, including the ability to phosphorylate the ETE peptide (Casnellie, 1991) and 50% inhibition by a specific anti-casein kinase II 3'0 subunits.

The partially purified kinase is able to phosphorylate the P protein using either ATP or GTP as the phosphate donor, as suggested by the results presented in Table 2(ii). The calculated $K_m$ values were between 2 and 9 µM and 10 and 22 µM, respectively, in agreement with the values previously described (10 µM and 30 µM respectively) by Marshak & Carroll (1991).

![Fig. 7. Determination of the apparent $M_r$ of protein kinase catalytic subunits. (a) The proteins contained in purified RS virions (40 µg) (lanes 3 and 6) and mock- (30 µg) (lanes 2 and 5) or RS virus- (30 µg) (lanes 1 and 4) infected HEp-2 cell extracts were separated after electrophoresis on a 12% polyacrylamide gel containing casein (1 mg/ml). Following the conditions described in Methods (Kameshita & Fujisawa, 1989), polypeptides with kinase activity were developed using [³²P]ATP (lanes 1 to 3) or [³²P]GTP (lanes 4 to 6) as phosphate donors. The autoradiography of this gel is shown and positions of prestained $M_r$ markers are indicated in the left margin of the figure. (b) A similar experiment using ATP as the phosphate donor and the proteins present in the following samples: lane 1, 40 µg of purified virions; lanes 2 and 3, 30 µg of mock- and RS virus-infected HEp-2 cells; lane 4, 40 µg of CV1 cells. The other lanes correspond to 20 µl of the pooled fractions with kinase activity eluted from Sepharose 4B with covalently bound NP-P (lanes 5 and 7) and heparin-acrylamide columns (lanes 6 and 8) when the purification process was performed with extracts of mock- (lanes 5 and 6) or RS virus-infected HEp-2 cells (lanes 7 and 8). The apparent $M_r$ values of the catalytic subunits of the protein kinase(s) present in purified RS virions and mock- or RS virus-infected extracts or in partially purified protein fractions from them were determined.
using the method of Kameshita & Fujisawa (1989). The results (Fig. 7a) indicate that at least two proteins of $M_r$ 40K to 42K are able to phosphorylate casein in this assay. These proteins are present in both mock- and RS virus-infected HEp-2 cells and in purified virions. These proteins have an electrophoretic mobility similar to those of the catalytic subunits $z/\alpha'$ described for casein kinase II (37K to 44K) of different origins (Marshak & Carroll, 1991). There are different proportions of these subunits in purified virions and cytoplasmic extracts. According to the previous identification, these catalytic subunits are able to use GTP as a phosphate donor (a, lanes 4 to 6). This casein kinase-like activity is the partially purified activity able to phosphorylate the P protein in vitro (Fig. 7b).

**Location on the P protein of in vitro phosphorylated residues by the partially purified protein with casein kinase II-like activity**

The results previously presented indicated that the residues modified in vitro are located in the same part of the P protein molecule as those modified during virus infection; however, there is no evidence that they are the same residues.

To determine that the casein kinase II-like activity modifies the same residues in vitro as those during virus infection, in vivo and in vitro phosphate-labelled P protein was trypsin-treated. The resulting peptides were separated by HPLC and the sequences of the N-terminal residues of the phosphorylated peptides were determined. The HPLC profiles are presented in Fig. 8, corresponding to in vivo and in vitro labelled P protein (a and b, respectively). In both cases, two phosphorylated peptides, T1 and T2, are present. The first phosphopeptide (T1), eluted in fraction 20, has a higher level of radioactivity when labelled in vivo than when labelled in vitro. Its partial N-terminal sequence indicates that it starts at residue number 53 of the P protein and has an electrophoretic mobility corresponding to 20K. This peptide should contain the in vivo phosphorylated domain previously reported, with one, some or all serine residues phosphorylated at positions 86, 94, 99, 116, 117, 119, 143, 156 and 161 (Navarro et al., 1991). The second phosphopeptide (T2), eluted in fraction 40, has a higher level of radioactivity than the first in the case of both in vivo and in vitro labelled P protein. The N terminus occurs at residue number 209 of the P protein molecule and T2 would include serine residues 211, 215, 220, 232 and 237. These results indicate that the P protein molecule is phosphorylated in vivo, and perhaps in vitro, at serine residues located in two different domains (Fig. 8c), one located in the central part of the protein molecule which contains the previously described serine residues (Navarro et al., 1991), and a second located in the C-terminal part of the molecule. The latter is phosphorylated mainly in vitro.
Discussion

The objective of this study was to determine whether the protein kinase that modifies the RS virus P protein is encoded by the viral genome or is of cellular origin, and to characterize this protein kinase.

In the VSV system, phosphorylation of the P protein during in vitro transcription requires protein kinase activities present in the other components (NP template and L preparations) (Barik & Banerjee, 1992a). One of the kinases is of cellular origin (casein kinase II) (Barik & Banerjee, 1992a, b) and the other may be virus-encoded (probably displayed by the L protein) (Barik & Banerjee, 1991); both activities are present in purified VSV virions (Barik & Banerjee, 1991). One of the virion-associated protein kinases displays properties of cellular casein kinase II (communications by Barik and Perrault in The Eighth International Conference on Negative Strand Viruses, 1991).

The presence of protein kinase(s) in viral particles has been described for several other enveloped (Beckes et al., 1989; Gerlich et al., 1982; Howard & Buchmeier, 1983; Imblum & Wagner, 1974; Lemaster & Roizman, 1980) and non-enveloped animal viruses (Grubman et al., 1984; Ratka et al., 1989; Tsuzuki & Luftig, 1985). The P protein (Fig. 1) is the only viral protein that is phosphorylated by the virion-associated protein kinase(s). The kinase activity(ies) is able to use ATP or GTP as the phosphate donor. When virions are disrupted by detergent treatment, the bulk of the kinase activity remains membrane-associated. However, nucleocapsid preparations display kinase activity to different extents, depending on the fractionation method used (Fig. 2), as described also for the VSV system. Thus, no clear association of protein kinase activity with the viral envelope or nucleocapsid was observed. It is of interest to point out that this protein kinase activity, regardless of its origin, phosphorylates the P protein at serine residues located in the same part of the molecule as the in vivo modified P protein (Navarro et al., 1991). This implies that not all P protein molecules present in purified virions are completely phosphorylated or dephosphorylated during the virus purification process, and that the virion-associated protein kinase(s) may be identical to that responsible, at least in part, for modification of the P protein in infected cells.

To determine whether the kinase(s) is of cellular origin, mock- or RS virus-infected HEp-2 cells were used as a source of kinase to modify P protein in vitro purified from RS virus-infected cells. Similar results were obtained when either preparation was used. Modification of the P protein was also found when extracts from uninfected or RS virus-infected Vero, HeLa or CV1 cells were used. Moreover, no differences were found in the phosphopeptide maps of the P protein when uninfected or infected cell extracts were used (data not shown).

These results suggest that the cellular protein kinase activity(ies) is a ubiquitous kinase present in the different cell lines assayed. This activity has been partially purified from mock- or RS virus-infected HEp-2 and CV1 cells and has been characterized as being casein kinase II-like. The reasons for this are as follows. (i) It uses GTP and ATP as a phosphate donor (Fig. 1 and 8) with \( K_a \) values similar to those described for casein kinase II (Marshak & Carroll, 1991). (ii) Its activity can be inhibited by incubation with anti-casein kinase II serum (Table 2). (iii) The apparent \( M_r \), determined for its catalytic subunits (about 40K to 42K) corresponds to that described for casein kinase II subunits isolated from different sources (Marshak & Carroll, 1991). However, the results in Fig. 7 suggest that the casein kinase II-like activity contained in purified virions has a higher proportion of the catalytic subunit showing a faster electrophoretic mobility than that found in uninfected cell extracts. (iv) It is also able to phosphorylate the ETE peptide, described as a suitable substrate for casein kinase II (Casnellie, 1991). (v) It can be isolated by its affinity for negatively charged molecules such as heparin, which inhibit the activity of the protein kinase preparation (Table 2). The association of the kinase with heparin in vitro accounts for its association with the virion particle, probably through negatively charged domains of the viral proteins.

When the tryptic peptides of in vivo or in vitro \(^{32}P\)-labelled P protein were separated by HPLC, the same two phosphopeptides were produced but with different levels of phosphorylation.

The phosphopeptide phosphorylated at the higher level in both cases starts at amino residue 209. This was unexpected for the P protein modified in vivo, as this residue is outside the previously reported phosphorylated domain, which contains serine residues at positions 86, 94, 99, 116, 117, 119, 143, 156 and 161, some of which are modified by phosphorylation (Navarro et al., 1991). In the previous analyses, an unphosphorylated peptide which starts at position 192, with an apparent \( M_r \) of 10K was obtained by chemical cleavage with formic acid. Apart from its apparent size, this peptide does not contain all amino acids present from positions 192 to 241 (the C terminus). It has been described that amino acid changes in this part of the P protein molecule may affect its electrophoretic mobility (Caravokyri & Pringle, 1992). The proximity between the start of this formic acid peptide with that of the tryptic phosphopeptide (containing phosphoserine) suggests that the in vitro modified residues are close to the C terminus.

The other phosphopeptide with the lower specific radioactivity starts at amino acid residue 53 and has an apparent \( M_r \) of 20K; this peptide also contains serine
residues at positions 54, 58 and 60. The possibility that some of these could be modified in vivo seems unlikely as all previously identified P protein phosphopeptides start after residue number 89 (Navarro et al., 1991). This region did not appear to be a good substrate for casein kinase II-like activity in vitro when an NP-P complex was tested as the substrate. There are two possible explanations for this result; either this region contains a domain buried in the NP-P complex or it is phosphorylated mainly by a virus-encoded protein kinase. The first implies such a P protein domain would be close to the interaction site of the P protein with the NP protein. As for the second possibility, it has been reported that the RS virus L protein contains an ATP-binding motif similar to that present in protein kinases (Stiee et al., 1988). In any case, the action of a putative virus-encoded kinase on the P protein during RS virus infection remains an open question.

Casein kinase II phosphorylates serine and threonine residues when an acidic amino acid is present in the +3 position (Pearson & Kemp, 1991). In the central domain, the serine residues of the P protein at positions 116, 117, 119, 143, 156 and 161 were shown to be the major residues phosphorylated, although phosphorylation of serines at positions 86, 94 and 99 could not be ruled out (Navarro et al., 1991). Of these, the residues at positions 86, 116, 119 and 161 are included in the consensus sequence and are, therefore, possible candidates for casein kinase II phosphorylation. In the C-terminal domain, serine residues at positions 232 and 237 are candidates for modification.

These data thus suggest that P protein phosphorylation takes place in two domains and that modification in one at least is due to a casein kinase II-like protein kinase. A similar activity, perhaps with a different catalytic subunit composition, was found to be associated with the virions.

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