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Coronavirus JHM: a Virion-associated Protein Kinase

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SUMMARY

Coronavirus JHM contains six major proteins, one of which, the 60000 mol. wt. nucleocapsid protein pp60, is phosphorylated. In JHM-infected cells ip 60K, the intracellular precursor to pp60 is also phosphorylated. Associated with purified JHM virions is a protein kinase which will phosphorylate pp60 and a variety of exogenous substrates *in vitro*. The enzyme has the characteristics of a cyclic nucleotide-independent protein kinase. Both the *in vivo* reaction and the enzyme activity *in vitro* transferred the γ-phosphate of ATP to serine residues on the nucleocapsid protein.

INTRODUCTION

The coronaviruses are associated with a variety of diseases including respiratory and enteric disorders in humans, bronchitis in birds, transmissible gastroenteritis and encephalitis in pigs and demyelinating encephalitis and hepatitis in rodents (McIntosh, 1974; Tyrrell *et al.*, 1978; Robb & Bond, 1980). In particular, coronavirus JHM induces a variety of central nervous system disorders in mice and rats and can be used as a model for virus-induced demyelination (Nagashima *et al.*, 1978, 1979).

JHM virions are pleomorphic and bear characteristic club-shaped projections. The virions mature by budding from internal cellular membranes and not from the plasma membrane (McIntosh, 1974). The genome of JHMV is a non-segmented single-stranded infectious RNA of mol. wt. $5{\cdot}4 \times 10^6$, and virion RNA is polyadenylated (Lai & Stohlman, 1978; Wege *et al.*, 1978). The virion contains six major proteins, four of which are glycoproteins (Wege *et al.*, 1979; Siddell *et al.*, 1980).

Protein kinases are associated with a variety of animal viruses, both enveloped and non-enveloped (Blair & Russell, 1978; Tan, 1975). The function of these virus-associated kinases remains obscure, but the phosphorylation of virion proteins is often an indication of such a virus-associated kinase. As the nucleocapsid protein of JHMV is known to be phosphorylated (Stohlman & Lai, 1979), we decided to investigate whether there was a protein kinase associated with JHMV. We detected in JHM virions a protein kinase activity which, in the absence of exogenous protein substrates, specifically phosphorylates the virion nucleocapsid protein *in vitro*. The same protein is the only virion protein that is significantly phosphorylated in infected cells.

METHODS

Virus and cells. Coronavirus JHM was obtained, plaque-purified and stocks prepared in monolayers of Sac(−) cells as previously described (Siddell *et al.*, 1980). To prepare purified virus, 5×10^8 cells growing in suspension culture were infected as described previously (Siddell *et al.*, 1980) and the virus was purified as described by Wege *et al.* (1979), except that two cycles of equilibrium gradient centrifugation were used. In outline, the purification

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procedure involves medium clarification, high salt treatment, PEG precipitation and velocity and equilibrium centrifugation in sucrose gradients. The purified virus, after pelleting, was resuspended and stored at −20 °C in 10 mM-tris-HCl pH 7·5, 10 mM-2-mercaptoethanol, 5 mM-$MgCl_2$ and 50 mM-KCl. ^{35}S-methionine-labelled virus was prepared as described previously (Siddell *et al.*, 1980). ^{32}P-labelled virus was purified by the same procedures using clarified medium taken from a suspension of 2×10^8 cells which had been infected at a multiplicity of 0·3 $TCID_{50}$/cell, maintained after infection in medium minus phosphate (MEM without phosphate containing 50 mM-Hepes pH 7·4 and 2% dialysed foetal calf serum) and pulse-labelled with 20 μCi/ml ^{32}P-orthophosphate from 9 h after infection to harvesting. The purified virus was pelleted, resuspended in 20 mM-tris-HCl pH 6·8 containing 0·05% Nonidet P40 (NP40) and 100 μg/ml pancreatic ribonuclease. The sample was extracted with phenol/chloroform/isoamyl alcohol (50:50:1) and the proteins were precipitated from the phenol phase with 5 vol. ethanol. The precipitated proteins were redissolved in electrophoresis sample buffer and heated to 100 °C for 2 min.

Pulse-labelling of infected cells. Cells were infected and pulse-labelled with ^{35}S-methionine as described (Siddell *et al.*, submitted for publication), using 100 μCi ^{35}S-methionine/ml labelling medium. Similarly, 9 h after infection cells were pulse-labelled for 1 h in medium minus phosphate containing 75 μCi ^{32}P-orthophosphate/ml. After labelling, cell or cytoplasmic lysates for one- or two-dimensional gel electrophoresis respectively were prepared (Siddell *et al.*, submitted for publication).

Protein kinase assay. Protein kinase activity was assayed by mixing aliquots of virus (normally 10 μg) with 0·05% NP40 at room temperature followed by 1 μCi γ-^{32}P-ATP (1 μCi/nmol, 002X, New England Nuclear Chemical, Dreieich, F.R.G.) in a buffer containing 10 mM-tris-HCl pH 7·5, 10 mM-2-mercaptoethanol, 5 mM-$MgCl_2$ and 50 mM-KCl. The total vol. was 15 μl and incubation was at 30 °C, normally for 30 min. The reaction was terminated by adding 10 μl of electrophoresis sample buffer and heating at 100 °C for 2 min. The incorporation of γ-^{32}P from ATP in the *in vitro* reaction was determined by excising the phosphoprotein-containing area of the dried polyacrylamide gel after electrophoresis, solubilization with Soluene 100 and scintillation counting in Dimilume (Packard Instrument International SA, Zürich, Switzerland). As the efficiency of this method was not determined the value obtained is a minimum one.

Polyacrylamide gel electrophoresis. The procedures for the preparation of cell or cytoplasmic lysates for one- or two-dimensional gel electrophoresis, the use of 15% discontinuous SDS–polyacrylamide gels described by Laemmli (1970) and the NEPHGE two-dimensional gel electrophoresis described by O'Farrell *et al.* (1977) have all been described in detail (Siddell *et al.*, submitted for publication). The procedures for the staining, drying and autoradiography of gels have been described by Smith *et al.* (1976).

Analysis of phosphoamino acids. In experiments where the *in vitro* product was analysed the γ-^{32}P-ATP was increased to 4 μCi per reaction. After incubation, ten 15 μl reaction mixtures were pooled and dialysed against 4 l of distilled water overnight. The dialysed sample was lyophilized and sealed under vacuum with 2 M-HCl. Hydrolysis was performed at 108 °C for 5·5 h. The products of *in vivo* phosphorylation were electrophoresed on one-dimensional gels and the major product was excised. The gel slice was directly treated with 2 M-HCl for 5·5 h at 108 °C in a sealed ampoule.

After hydrolysis the samples were clarified, lyophilized and resuspended in 130 μl 0·05 M-HCl containing 38 mM each of standard phosphoserine and phosphothreonine. The samples were fractionated on 0·6 × 10 cm columns of Dowex 50 equilibrated with 0·05 M-HCl. Phosphoserine and phosphothreonine were located in the column fractions by ninhydrin staining and radioactivity by scintillation counting. In all cases more than 95% of the applied radioactivity was recovered.

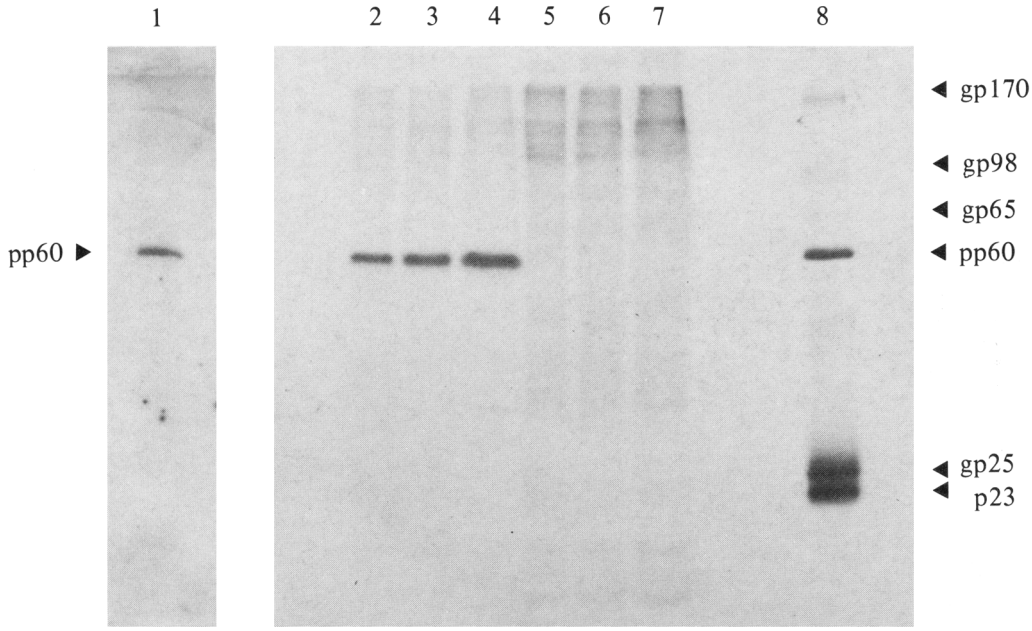

Fig. 1. Intracellular and virion phosphoproteins of JHMV analysed by SDS–polyacrylamide gel electrophoresis. ^{35}S-methionine labelling and ^{32}P-orthophosphate labelling of virus and the pulse-labelling of polypeptides with ^{32}P-orthophosphate in infected or mock-infected cells were as described in the text. Track 1, ^{32}P-orthophosphate-labelled virus. Tracks 2, 5 μl; 3, 10 μl; 4, 15 μl of cell lysate from infected cells. Tracks 5, 5 μl; 6, 10 μl; 7, 15 μl of cell lysate from mock-infected cells. Track 8, ^{35}S-methionine-labelled virus. The virion proteins are designated by their mol. wt. ($\times 10^{-3}$) and a prefix, gp or pp, which indicates glycosylation or phosphorylation of the protein.

Virus concentration. Protein determinations were performed with the Biorad protein assay (Biorad Laboratories, München, F.R.G.). The mol. wt. markers, purchased from Amersham/Buchler, Braunschweig, F.R.G. (CF 262) are ^{14}C-labelled myosin (200K), phosphorylase B (92·5K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K) and lysozyme (14K).

RESULTS

Virion proteins

Coronavirus JHM is comprised of six major ^{35}S-methionine-containing proteins which are designated on the basis of their mol. wt. and whether or not they are glycosylated (Fig. 1, track 8). One-dimensional gel electrophoresis of purified virions labelled *in vivo* with ^{32}P-orthophosphate shows that only the major virion protein p60 is significantly phosphorylated (Fig. 1, track 1). Although we have not been able to incorporate sufficient radioactivity to confirm the phosphoamino acid linkage in pp60 (hereafter, we will use the term phosphoprotein 60 or pp60) it is unlikely that the radioactivity associated with pp60 is due to the non-specific absorption of RNA or phospholipid because the virus proteins were treated with RNase, phenol-extracted and ethanol-precipitated before electrophoresis.

Intracellular proteins

One-dimensional gel electrophoresis of infected or uninfected cell lysates pulse-labelled with ^{32}P-orthophosphate reveals that specifically in the infected cell there is a significant proportion

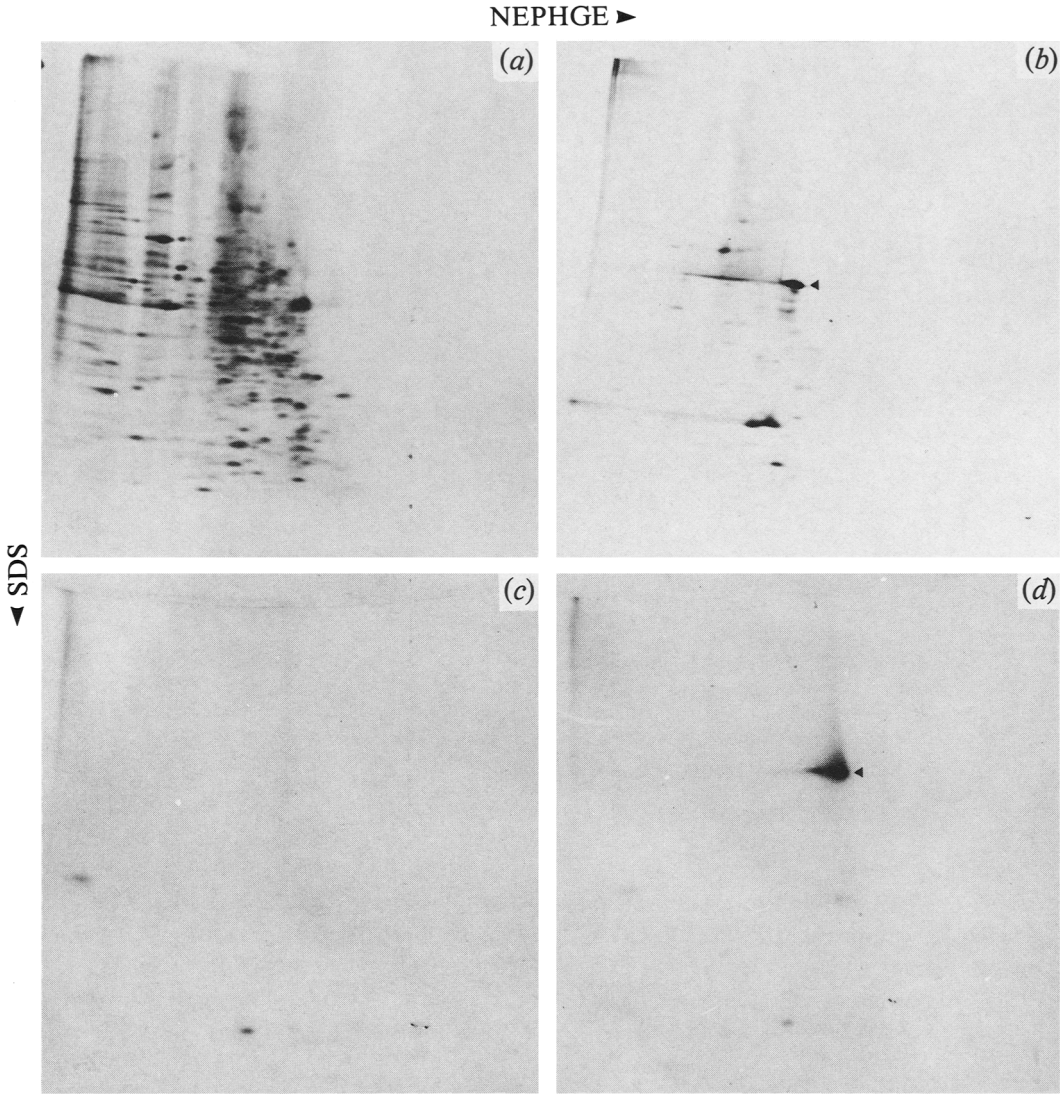

Fig. 2. Two-dimensional NEPHGE gel electrophoresis of JHMV-infected lysates. Sac(−) cells were pulse-labelled with ^{35}S-methionine or ^{32}P-orthophosphate 9 h after infection and cytoplasmic lysates were prepared and electrophoresed as described in the text. (*a*) ^{35}S-methionine, mock-infected lysate; (*b*) ^{35}S-methionine, infected lysate; (*c*) ^{32}P-orthophosphate, mock-infected lysate; (*d*) ^{32}P-orthophosphate, infected lysate. The ip 60K is arrowed in the gels of infected cell lysates.

of the radioactivity associated with a protein of 60000 mol. wt. (Fig. 1, tracks 2 to 7). (Hereafter, K will be used to denote mol. wt. × 10^3.) The major protein synthesized in JHM-infected cells is ip 60K which, by tryptic peptide fingerprinting, is known to be the precursor to pp60 (Siddell *et al.*, 1980). It seems likely, therefore, that the intracellular phosphoprotein now detected is ip 60K and this suggestion is strongly supported by two-dimensional NEPHGE electrophoresis of infected cell lysates pulse-labelled with ^{35}S-methionine or ^{32}P-orthophosphate. Fig. 2(*b*, *d*) shows that both ip 60K and the intracellular phosphoprotein are very basic proteins with similar electrophoretic mobility in NEPHGE gels. We conclude, therefore, that the 60K phosphoprotein is ip 60K and that the virion nucleocapsid protein is phosphorylated intracellularly. We did not detect any evidence

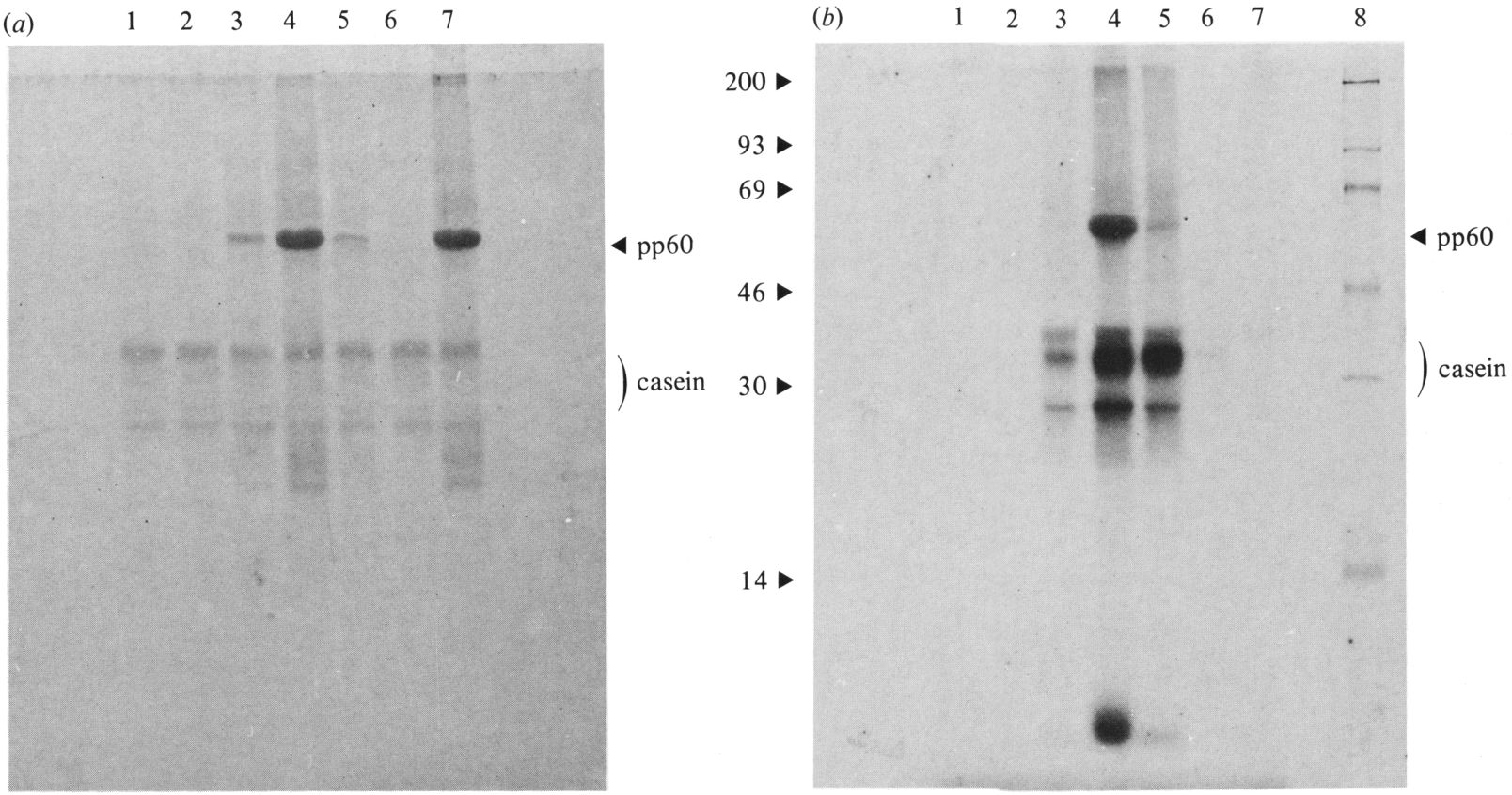

Fig. 3. Co-purification of protein kinase activity with JHMV. After purification JHMV was centrifuged to equilibrium on a second sucrose density gradient. Six fractions were collected across the gradient, diluted with buffer and centrifuged to pellet virus. The pellets were resuspended in buffer and aliquots were assayed for kinase activity as described in the text, using 10 μg casein as exogenous substrate. The reactions were terminated, and the gradient fractions and *in vitro* products analysed by SDS-polyacrylamide gel electrophoresis. (*a*) Coomassie blue staining of the gel. Tracks 1 to 6 represent gradient fractions 1 to 6; track 7 is equivalent to track 4 except that the virus aliquot was boiled for 1 min before assay. (*b*) Autoradiograph showing the products of *in vitro* phosphorylation of aliquots of fractions 1 to 6 of the gradient (tracks 1 to 6) and an aliquot of fraction 4 which had been boiled (track 7). Mol. wt. markers are shown in track 8. Sedimentation was from right to left.

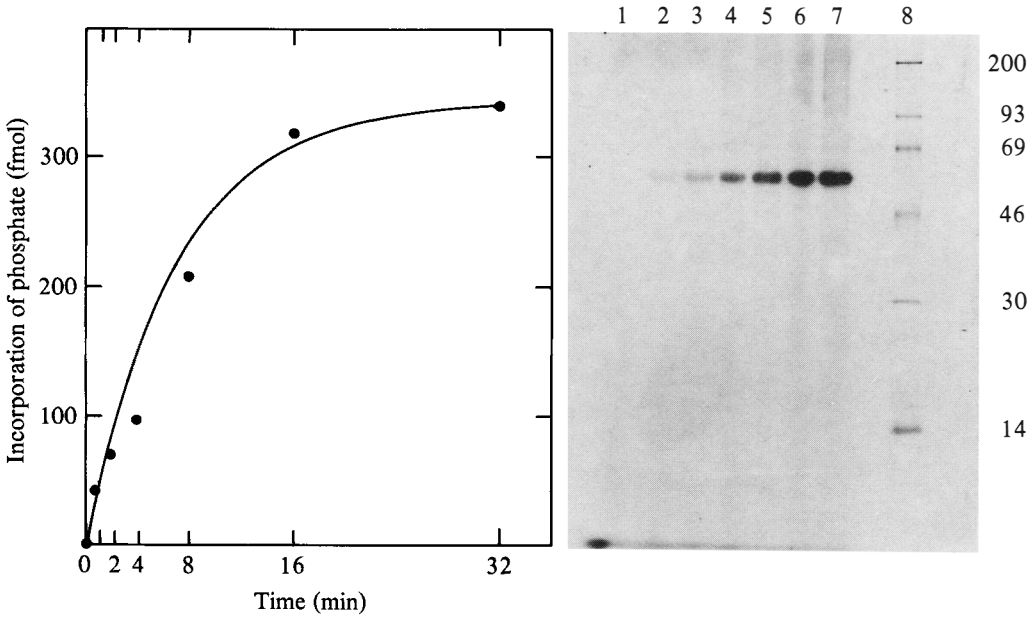

Fig. 4. The time course of the *in vitro* protein kinase reaction. 10 μg amounts of purified NP40-distributed virus were assayed by protein kinase activity without exogenous substrate with incubation periods of 0, 1, 2, 4, 8, 16 and 32 min and analysed by SDS–polyacrylamide gel electrophoresis (tracks 1 to 7). Mol. wt. markers are shown in track 8. Incorporation of phosphate into pp60 was determined as described in Methods.

for multiple forms of the intracellular phosphoprotein representing different degrees of phosphorylation.

Virion-associated kinase

The specificity and activity of the kinase reaction which phosphorylates ip 60K in infected cells suggested that the enzymic activity responsible was a consequence of virus infection and by analogy to many other viruses may be associated with released virion particles. We therefore assayed purified virus for *in vitro* kinase activity and Fig. 3 demonstrates that there is indeed a virion-associated kinase. JHMV sediments to equilibrium in sucrose gradients at a density of 1·18 g/ml (Wege *et al.*, 1979) and when analysed by polyacrylamide gel electrophoresis virus proteins can be identified in fractions of this density (Fig. 3 *a*, tracks 3, 4 and 5). When the same gradient fractions are assayed using casein as a substrate only those which contain virus show kinase activity (Fig. 3 *b*, tracks 3, 4 and 5). It is clear that the virion protein pp60 also acts as an efficient phosphate acceptor in this reaction. The same result has been obtained using histones as exogenous substrate. Boiling a sample of the gradient fractions containing purified virus for 1 min destroyed the ability of the associated kinase to phosphorylate both exogenous and endogenous substrates (Fig. 3 *b*, track 7).

The time course of the virion-associated kinase reaction is shown in Fig. 4. The reaction is essentially linear for 15 min at 30 °C in pH 7·5 buffer and we calculate that the rate of incorporation is at least 225 pmol/mg virus/h. Virus stored at −70 °C for 6 months did not exhibit any appreciable decrease in kinase activity. Fig. 4 also shows that in the absence of exogenous substrates the *in vitro* kinase reaction phosphorylates only pp60.

Table 1 shows some characteristics of the kinase reaction measured using the endogenous reaction. Disruption of JHMV with the non-ionic detergent NP40 resulted in an approx.

Table 1. *Characteristics of the* in vitro *protein kinase reaction*

Virus (μg)	Treatment	Incorporation (%)
10	—	58
10	+NP40	100*
1	+NP40	12
20	+NP40	175
20	+NP40, boiled	3
10	+NP40, 5 mM-EDTA†	5
10	+NP40, ribonuclease‡	93
10	+NP40, Pronase‡	5
10	+NP40, 10^{-3} M-cAMP§	79

* The standard reaction, which contained 10 μg purified NP40- disrupted virus, incubated without exogenous substrate at 30 °C for 30 min is taken as 100%. Phosphate incorporation into pp60, was determined as described in the text.

† The incubation buffer contained 5 mM-EDTA.

‡ After incubation the mixtures were treated for 15 min at 37 °C with 250 μg/ml and 500 μg/ml (final concentrations) of pancreatic ribonuclease or Pronase P.

§ The incubation buffer contained 10^{-3} M-cyclic AMP.

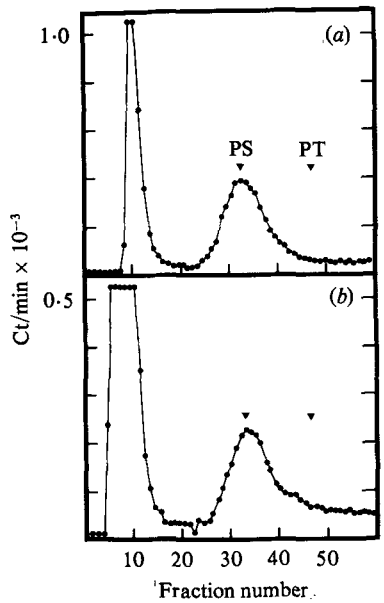

Fig. 5. Phosphoamino acid analysis of *in vivo* and *in vitro* phosphorylated nucleocapsid protein. The phosphoamino acid linkages to the (*a*) *in vivo* or (*b*) *in vitro* phosphorylated nucleocapsid protein were analysed by acid hydrolysis and chromatography of phosphoamino acids on Dowex 50 as described in the text. PS, Phosphoserine; PT, phosphothreonine.

twofold increase in the kinase activity. There was a total inhibition of activity when 5 mM- or 10 mM-EDTA was added to the reaction mixture, demonstrating the divalent cation requirement of the enzyme. Treatment with RNase had no effect on the radioactivity incorporated, whilst Pronase treatment reduced the phosphate incorporated to background levels. Cyclic AMP, tested over the range of 10^{-3} to 10^{-5} M did not stimulate *in vitro* phosphorylation. We have found no alteration in the specificity of the phosphorylation reaction under any ionic or other changes in conditions that we have tested.

The phosphoprotein linkage to pp60

The nature of the phosphoprotein bond produced *in vivo* and produced by the *in vitro* reaction using endogenous substrates was further investigated by acid hydrolysis and chromatography on Dowex 50 columns. Evidence that both phosphorylation reactions result in a phosphoprotein linkage is shown in Fig. 5 (*a*, *b*). The data also suggest that the majority of these linkages are phosphoserine. Nearly all of the radioactivity applied to the columns was recovered and under the conditions of hydrolysis used phosphoserine is more labile than phosphothreonine. Whilst our analysis does not exclude phosphothreonine as a product of the kinase reaction we consider this unlikely as the nucleocapsid protein of the closely related MHV-A59 has been shown to be specifically phosphorylated at serine residues (Stohlman & Lai, 1979).

DISCUSSION

We have shown that the nucleocapsid protein of JHMV is phosphorylated, confirming the report of Stohlman & Lai (1979). In the infected cell the major phosphoprotein is ip 60K, the precursor to pp60, and one phosphoamino acid linkage to this protein is phosphoserine.

Associated with JHMV is a protein kinase which is independent of cyclic AMP stimulation, has a requirement for divalent cations, phosphorylates serine residues and has a broad substrate specificity *in vitro*, phosphorylating both histones and casein, as well as the major virion structural protein pp60. Thus, this enzyme has many features in common with those identified in a variety of other enveloped viruses (Tan, 1975).

The broad specificity of the virion-associated enzyme *in vitro* is, however, not reflected by the pattern of polypeptide phosphorylation in infected cells, where only the nucleocapsid precursor ip 60K is significantly phosphorylated. Whether the same enzyme is responsible for the kinase activity both *in vivo* and *in vitro* has not yet been shown. Also, we do not know whether ip 60K is only partially phosphorylated *in vivo* and is therefore able to provide a substrate for the *in vitro* reaction, whether host cell phosphatases are able to dephosphorylate sites on ip 60K which subsequently provide a substrate for the *in vitro* reaction, or whether independent sites are phosphorylated *in vivo* and *in vitro*.

The kinase activity detected remains associated with virions during treatment with 0·5 M-NaCl, PEG precipitation and several cycles of velocity and equilibrium centrifugation. Therefore, if the enzyme is a contaminating host cell protein it must be tightly integrated into the structure of the virion. Alternatively, the enzyme may be a virion protein and if so, it might be expected to be coded for in virus RNA. In principle, one way to show this would be to synthesize the enzymic activity *in vitro* using virus mRNA. Another approach would be the use of mutants of JHMV.

Finally, the functional role, if any, of the kinase we have described in the infection process remains to be investigated. It has been suggested that the phosphorylation of virion proteins could play such diverse roles as regulating the uncoating of virus in the host cell or regulating the recognition of polypeptides during the assembly of virus (Lamb, 1975). Sturman *et al.* (1980) have shown in the closely related MHV-A59 that there is a complex temperature-dependent interaction between the virion nucleocapsid and the low mol. wt. transmembrane protein E1. The possible role played by the phosphorylation of pp60 in this reaction needs to be considered.

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REFERENCES

BLAIR, G. E. & RUSSELL, W. C. (1978). Identification of a protein kinase activity associated with human adenoviruses. *Virology* **86**,157–166.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LAI, M. M. C. & STOHLMAN, S. A. (1978). RNA of mouse hepatitis virus. *Journal of Virology* **26**, 236–248.

LAMB, R. A. (1975). The phosphorylation of Sendai virus proteins by a virus particle-associated protein kinase. *Journal of General Virology* **26**, 249–263.

MCINTOSH, K. (1974). Coronaviruses: a comparative review. *Current Topics in Microbiology and Immunology* **63**, 85–129.

NAGASHIMA, K., WEGE, H., MEYERMANN, R. & TER MEULEN, V. (1978). Coronavirus induced subacute demyelinating encephalitis in rats: a morphological analysis. *Acta Neuropathologica* **44**, 63–70.

NAGASHIMA, K., WEGE, H., MEYERMANN, R. & TER MEULEN, V. (1979). Demyelinating encephalomyelitis induced by a long term coronavirus infection in rats. *Acta Neuropathologica* **45**, 205–213.

O'FARRELL, P. Z., GOODMAN, H. M. & O'FARRELL, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133–1142.

ROBB, R. A. & BOND, C. W. (1980). Coronaviridae. In *Comprehensive Viro.* , vol. 14, pp. 195–247. Edited by H. Fraenkel-Conrat and R. R. Wagner. New York: Plenum Press.

SIDDELL, S. G., WEGE, H., BARTHEL, A. & TER MEULEN, V. (1980). Coronavirus JHM: cell-free synthesis of structural protein p60. *Journal of Virology* **33**, 10–17.

SMITH, A. E., WHEELER, T., GLANVILLE, N. & KÄÄRIÄINEN, L. (1976). Translation of Semliki Forest virus 42S RNA in a mouse cell-free system to give virus coat protein. *European Journal of Biochemistry* **49**, 101–110.

STOHLMAN, S. A. & LAI, M. M. C. (1979). Phosphoproteins of murine hepatitis viruses. *Journal of Virology* **32**, 672–675.

STURMAN, L. S., HOLMES, K. V. & BEHNKE, J. (1980). Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *Journal of Virology* **33**, 449–462.

TAN, K. B. (1975). Comparative study of the protein kinase associated with animal viruses. *Virology* **64**, 566–570.

TYRRELL, D. A. J., ALEXANDER, D. J., ALMEIDA, J. D., CUNNINGHAM, C. H., EASTERDAY, B. C., GARWES, D. J., HIERHOLZER, J. C., KAPIKIAN, A., MACNAUGHTON, M. R. & MCINTOSH, K. (1978). Coronaviridae: second report. *Intervirology* **10**, 321–328.

WEGE, H., MÜLLER, A. & TER MEULEN, V. (1978). Genomic RNA of the murine coronavirus JHM. *Journal of General Virology* **41**, 217–227.

WEGE, H., WEGE, H., NAGASHIMA, K. & TER MEULEN, V. (1979). Structural polypeptides of the murine coronavirus JHM. *Journal of General Virology* **42**, 37–47.

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