Vesicular Stomatitis Virus Growth in Drosophila melanogaster Cells. II. Modifications of Viral Protein Phosphorylation

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SUMMARY

The phosphoproteins of vesicular stomatitis virus released from infected Drosophila melanogaster cells were examined. The membrane (M) protein was more phosphorylated than after multiplication in chicken embryo cells, even in Drosophila cell cytoplasm before its association with cellular membranes. Analysis of phosphopeptides generated after partial proteolysis and of phosphoamino acids obtained after complete acid hydrolysis showed that M phosphorylation was quantitatively and qualitatively changed, while NS protein phosphorylation was only slightly modified.

Phosphorylation and dephosphorylation are common post-translational processes controlling cellular protein functions. Most viruses contain phosphoproteins associated with their genomes and these proteins are often involved in viral RNA synthesis and replication (Lesnaw et al., 1979; Sen et al., 1977; Wilcox et al., 1980). The interactions of proteins with nucleic acids can be modified by the degree of protein phosphorylation and this may play a regulatory role in virus multiplication.

Vesicular stomatitis virus (VSV), a negative-strand RNA-containing enveloped virus, possesses two phosphoproteins, NS and M (Clinton et al., 1978a), which are involved in viral RNA synthesis (Carroll & Wagner, 1979; Clinton et al., 1978b; Martinet et al., 1979; Testa et al., 1980; Kingsford & Emerson, 1980). As phosphorylation depends on the host cell (Imblum & Wagner, 1974; Moyer & Summers, 1974) and could alter virus multiplication, it might be related to the control mechanism which strongly modifies VSV growth in insect cells where VSV (and also alphaviruses) is not cytopathogenic (Mudd et al., 1973; Richardson et al., 1980). Cellular synthesates are not inhibited after infection while virus multiplication and intracellular viral protein synthesis are controlled (Richardson et al., 1980; Wyers et al., 1980). This leads to a persistent infection.

In the present study, we compared the phosphorylated viral proteins in VSV grown in chicken embryo (CE) cells and in Drosophila melanogaster cells (line 77-OM3). These two types of cells were infected with VSV at high multiplicity (10 p.f.u./cell) and grown in the presence of Shields' medium supplemented with 2~ dialysed foetal calf serum, 20 mM-HEPES pH 7 (Wyers et al., 1980) and modified according to the radioactive precursor used to label the viral proteins. For 14C-labelled VSV, the medium contained 1 mM-leucine, and 1-25 µCi/ml [14C]leucine; for 32P-labelled VSV, the medium was free of phosphate ions and supplemented with 80 µCi/ml [32P]Pi; for 14C- and 32P-double-labelled VSV, a leucine- and phosphate-free Shields' medium was supplemented with 1 mM-leucine, 2-5 µCi/ml [14C]leucine and 80 µCi/ml [32P]Pi. Labelled viruses harvested after 24 h at 26 °C or 37 °C for Drosophila and CE cells respectively were purified as previously described (Wyers et al., 1980) and proteins analysed by electrophoresis on an SDS-polyacrylamide slab gel.

As shown in Fig. 1(a), in VSV released from CE cells 32P was found to be associated mainly with NS and to a lesser extent with M protein. Identical results have been obtained for VSV
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Fig. 1. Identification of phosphoproteins in VSV grown in Drosophila cells (lanes 1 and 1'), CE cells (2 and 2') and actinomycin D-treated Drosophila cells (3 and 3') in the presence of $^{32}$P, (80 μCi/ml) or $[^{14}$C]leucine (1.25 μCi/ml) as previously described (Wyers et al., 1980). Viruses were harvested after 24 h, purified, disrupted with SDS and analysed on SDS–polyacrylamide slab gel containing: (a) 10% acrylamide and 0.13% bisacrylamide (Laemmli, 1970) or (b) 10% acrylamide, 0.4% bisacrylamide and 7 M-urea (Kingsford & Emerson, 1980). $[^{14}$C]-labelled VSV grown in Drosophila cells (lane 4) was used as a marker to identify the viral $^{32}$P-labelled proteins.

released from BHK cells (Clinton et al., 1978a). After multiplication in Drosophila cells, NS and M were also phosphorylated. However, $^{32}$P-labelling was clearly higher in the M protein, while it remained similar for NS. Artefactual M labelling was ruled out since treatments with RNase A, DNase I or a lipid-solvent as well as hot trichloroacetic acid had no effect on phosphate–protein association whatever the phosphoprotein or the origin of the virus (data not shown). The variation observed was not due to a modification in NS and M protein ratios, which were practically identical in both viruses as seen after analysis of $^{14}$C-labelled protein (Fig. 1 a). In fact, as it has already been shown (Wyers et al., 1980), only the amounts of L and G are reduced in VSV released from Drosophila cells while other viral protein ratios are not significantly changed.

Viral protein phosphorylation, depending on cellular kinases, could be modified by the host mechanism which controls VSV multiplication in insect cells. This process has been shown to be counteracted by actinomycin D; in the presence of the drug, viral protein synthesis is no longer inhibited and VSV multiplies freely in Drosophila cells (Wyers et al., 1980). To test the effect of the drug on phosphorylation, Drosophila cells were treated with 1 μg/ml actinomycin D prior to infection and during VSV multiplication. In that case, $^{32}$P incorporation into NS and M proteins was similar to that found in the absence of actinomycin D; in particular, M protein was as phosphorylated as in untreated Drosophila cells (Fig. 1 a).

The degrees of phosphorylation of NS and M proteins were compared in $^{14}$C- and $^{32}$P-double-labelled samples that had been subjected to protein electrophoresis, gel slicing and radioactivity
counting. Compared to M, NS carried 30 times more phosphate per molecule in VSV released from CE cells and 7 times more than this after multiplication in Drosophila cells. On the other hand, the degree of phosphorylation of M was 3.5-fold higher in VSV grown in Drosophila cells, either treated or untreated with actinomycin D, than in VSV grown in CE cells. Several $^{14}$C- and $^{32}$P-double-labelling experiments revealed also small reproducible differences in NS protein: whereas the NS protein ratio compared to N (given by $^{14}$C-labelling) was identical in all viruses, NS contained 20% less phosphate per molecule in VSV grown in Drosophila cells than in VSV grown in CE cells (data not shown). NS protein has been resolved by electrophoresis in an SDS gel containing 7 M-urea into two major species, NS1 and NS2, which differ in their phosphate contents (Kingsford & Emerson, 1980). They are present in amounts too low to allow a reliable assessment of their ratios in the virion after $^{14}$C-labelling. As shown in Fig. 1(b), each virus, whatever its origin, contained NS forms which migrated in an identical way, but less phosphate was found in NS$_2$ compared to NS$_1$ when VSV was grown in Drosophila cells, either untreated or treated with actinomycin D (determined by scanning gel autoradiography and also by gel cutting and counting). These results suggested that the virus grown in Drosophila cells contained either less NS$_2$ or a form of it which is less phosphorylated than in virus grown in CE cells. These differences could explain the slightly decreased phosphorylation in NS after multiplication in Drosophila cells.

The differences observed for NS and M proteins could be due to phosphorylation either on identical sites with variable efficiency or on different sites depending on the host cell, but also to a different distribution of phosphoamino acids. Therefore, $^{32}$P-labelled M and NS proteins were purified from VSV released from each cell type. Partial protease digestion (Cleveland et al., 1977) and analysis of the peptides generated, as well as complete acid hydrolysis and separation of phosphoamino acids, were used to study these possibilities.

For the different NS proteins, identical $^{32}$P-labelled phosphopeptides were produced at different steps of proteolysis whatever the origin of the virus (Fig. 2); similar results were obtained using $\alpha$-chymotrypsin or papain. These data indicate that the same peptides were phosphorylated in both cell systems and probably that NS phosphorylation differences were not due to a selection of new sites in Drosophila cells. Results of phosphoamino acid analysis are presented in Table 1; they were close to those described by Clinton & Huang (1981) in BHK cells. While NS phosphorylation sites remained unchanged, as well as the nature and ratio of the phosphoamino acids, the equilibrium between the two states of phosphorylation was different in the mature virions grown in Drosophila cells; if this occurred also in the cytoplasm of these cells, it could affect the regulation of viral RNA synthesis, via a mechanism of phosphorylation and dephosphorylation (Witt & Summers, 1980; Kingsford & Emerson, 1980; Testa et al., 1980).

The results from M protein digestion were more complex. After $\alpha$-chymotrypsin treatment (Fig. 2), one $^{32}$P-labelled high molecular weight peptide was unexpectedly missing in M proteolysis products when VSV was propagated in Drosophila cells. On the other hand, all the other $^{32}$P-labelled peptides were identical to those found when VSV was grown in CE cells, but their degrees of phosphorylation were higher in Drosophila cells (in fact the peptide analysis shown in Fig. 2 needed half the amount of M protein required after multiplication in CE cells); this explains the differences observed previously in M protein phosphorylation. When Drosophila cells were treated with actinomycin D, M proteolysis generated $^{32}$P-labelled peptides (Fig. 2) which were identical, but much more phosphorylated than after multiplication of VSV in CE cells. In particular, the high molecular weight peptide which was not labelled in VSV grown in untreated Drosophila cells, was highly phosphorylated in the presence of the drug. Identical results were obtained after limited papain hydrolysis (data not shown). In the case of phosphoamino acid analysis, other differences could be related to the host cell and were even more pronounced when Drosophila cells were used as host: phosphotheonine and phosphothreonine were undetectable, whereas they were both present after multiplication in CE and BHK cells (Table 1). Moreover, they were found in M protein when actinomycin D was added to Drosophila cells during VSV multiplication, but each represented less than 2% of the total phosphoamino acids.

These findings indicated that, in Drosophila cells, M phosphorylation was (i) absent from a
Fig. 2. NS and M proteins purified by electroelution from an SDS–polyacrylamide gel, digested with α-chymotrypsin (6, 60 or 300 μg) under the conditions described by Cleveland et al. (1977). The ensuing degradation products were separated by electrophoresis on an SDS–polyacrylamide gel (15% acrylamide, 0.2% bisacrylamide). Phosphopeptides were detected by autoradiography. VSV was propagated and labelled as described in Fig. 1 in CE cells (a), or in Drosophila cells either untreated (b) or treated with actinomycin D (c). Samples equivalent to 15 μg virus were analysed, except for M protein from virus grown in CE cells where 30 μg was used. The arrow indicates the phosphopeptide missing in M protein from VSV grown in Drosophila cells.

Table 1. Relative amounts of phosphoserine, phosphothreonine and phosphotyrosine in M protein from mature virions

<table>
<thead>
<tr>
<th>Viral protein</th>
<th>Host cells</th>
<th>32P-Ser</th>
<th>32P-Thr</th>
<th>32P-Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>BHK†</td>
<td>60</td>
<td>40</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>CE</td>
<td>72</td>
<td>28</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Drosophila</td>
<td>71</td>
<td>28</td>
<td>&lt;1</td>
</tr>
<tr>
<td>M</td>
<td>BHK†</td>
<td>75</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CE</td>
<td>94</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Drosophila</td>
<td>99</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Drosophila + actinomycin D</td>
<td>97</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*The spots corresponding to each phosphoamino acid were scraped off and counted after two-dimensional electrophoresis on a pre-coated thin-layer cellulose plate. The first dimension electrophoresis was carried out for 2 h at 1000 V with 7.8% glacial acetic acid and 2.5% formic acid, the second for 1 h at 750 V with 4.6% glacial acetic acid and 1% pyridine. Results are expressed as a percentage of the radioactivity in each amino acid divided by the total radioactivity in all three amino acids. These totals were 1185 ct/min and 575 ct/min respectively for NS and M proteins from VSV grown in CE cells, 975 ct/min and 1860 ct/min respectively when VSV was released from Drosophila cells and 1300 ct/min for M protein contained in VSV grown in actinomycin-treated Drosophila cells.

† Determined by Clinton & Huang (1981). In their experiments about 3000 ct/min of NS, and NS2 proteins and 500 ct/min of M protein were applied to the paper before high voltage two-dimensional electrophoresis.

particular peptide, (ii) impaired at threonine and tyrosine residues and (iii) increased only on serine residues. The first two effects could be related, but it was not possible to determine the phosphoamino acids present in this peptide because it was not sufficiently labelled for such an analysis. On the other hand, the high degree of phosphorylation which remained unchanged in
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Fig. 3. The state of phosphorylation of soluble M protein in infected cell cytoplasm. VSV-infected CE cells (a) and Drosophila cells either untreated (b) or treated with actinomycin D (c) were labelled for 1 h with $^{32}$P (100 μCi/ml) and $[^3H]$leucine (5 μCi/ml), 4 h after infection. Cells were harvested, washed and broken in low-salt buffer (5 mM-sodium phosphate pH 7.4, 1.5 mM-MgCl$_2$, 10 mM-NaCl, 1 mM-phenylmethylsulphonyl fluoride, 10 mM-Na$_2$P$_2$O$_7$, 1 mM-(NH$_4$)$_6$Mo$_7$O$_{24}$, 1 mM-ATP). The soluble cytoplasmic M protein was purified as described in the text and analysed by gel electrophoresis. The $^{32}$P (○) and $[^3H]$ (●) radioactivities were counted after gel slicing. $[^3H]$-labelled virions were analysed in the same way to indicate the positions of the NS, N and M viral proteins.

the presence of actinomycin D appeared to be a special property of Drosophila cells and, perhaps, of insect cells.

As M protein plays a pivotal role in virus assembly, its unusual state of phosphorylation due to host enzyme(s) in Drosophila cells could drastically change VSV multiplication, especially if free M protein was already phosphorylated in the cell cytoplasm. So far it is not known, even in vertebrate cells, whether M protein is converted into the form found in the mature virion in either the cytoplasm or the membrane.

To study this point, free M protein was purified from VSV-infected CE and Drosophila cells labelled simultaneously with $[^3H]$leucine and $^{32}$P, from 4 to 5 h post-infection. Protease and phosphatase inhibitors and kinase substrates were added during all purification steps to protect phosphoproteins against proteolysis, phosphorylation and dephosphorylation artefacts (Stott & Williamson, 1978). The cytoplasmic extracts were prepared and fractionated by centrifugation in a sucrose gradient (David, 1977). In the soluble cytoplasmic fraction, G protein was always absent, indicating that there was no contamination with membrane during centrifugation; thus, the free M protein was separated from the membrane-bound M protein discarded with the pellet. Two additional steps were necessary to purify M protein: phosphocellulose chromatography (Carroll & Wagner, 1979) followed by specific viral protein immunoprecipitation. Immune complexes were dissociated and $[^3H]$- and $^{32}$P-labelled viral proteins analysed by electrophoresis. The curves obtained after gel cutting and counting are shown in Fig. 3. Although high molecular weight $^{32}$P-labelled contaminants were still present in Drosophila cells (Fig. 3b, c), no labelled cellular product co-migrated with M, as revealed by analysis of purified uninfected cell cytoplasmic extract labelled with $^{32}$P (data not shown). Free M protein was phosphorylated in the cell cytoplasm and fivefold higher in Drosophila cells either untreated or treated with actinomycin D than in CE cells. Therefore, M protein phosphorylation occurred, in vivo, either during or immediately after synthesis, and before membrane association, and, in addition, was dependent on host cells.

M is known to be essential for the budding of virions by interacting with G protein and the plasma membrane of the infected cells (Reidler et al., 1981; Zakowski et al., 1981), and with nucleocapsids also (Newcomb & Brown, 1981; Wilson & Lenard, 1981). A phosphorylation step could be implicated in these processes; in particular, virion disruption has been observed after in vitro phosphorylation of VSV proteins (Witt et al., 1981). Modification of G–M interaction could explain the G protein deficiency observed in the mature viruses released from Drosophila cells...
(Wyers et al., 1980). Decreased affinity between M and N proteins could impede the condensation of the nucleocapsids into the compact form found in native virions and could be a limiting factor controlling VSV production in insect cells.

M protein is also implicated in the regulation of VSV RNA synthesis (Carroll & Wagner, 1979; Clinton et al., 1978b; Martinet et al., 1979) perhaps via its affinity for the nucleocapsid. Studies on M–nucleocapsid interactions and RNA synthesis in vitro, are in progress using the more phosphorylated M protein synthesized in Drosophila cells. These studies may reveal correlations between the state of M phosphorylation and function and bring further insight into the problem of control of VSV multiplication in insect cells.

REFERENCES


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