Phosphorylation of the N and $M_1$ Proteins of Rabies Virus

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SUMMARY

Phosphorylation of rabies virus proteins was followed in vivo and in vitro. The N and $M_1$ proteins were both found to be phosphorylated. The $M_1$ protein was present in the virion in two phosphorylated states, but only the hypophosphorylated form of $M_1$ was found in infected cells. The hypothesis that some of the $M_1$ molecules become hyperphosphorylated during the maturation process by a membrane-bound kinase was examined. The phosphorylation of the viral proteins by the kinase present in purified rabies virions was studied using an in vitro transcriptase assay: under the conditions of the assay, additional phosphate groups were rapidly attached to the N protein. The $M_1$ protein was similarly hyperphosphorylated although more slowly. Whether the hyperphosphorylation of the N protein is responsible for the poor efficiency of the in vitro transcriptase reaction is not clear. No detectable change in the phosphorylation of cellular proteins was observed in the course of rabies virus infection.

For several enveloped viruses including vesicular stomatitis virus (VSV) it is well documented that the degree of phosphorylation of some structural proteins controls important steps of the infection process, but very little is known concerning the phosphorylation of rabies virus proteins. Sokol & Clark (1973) reported that the nucleocapsid protein was phosphorylated, and subsequently Dietzschold et al. (1979) demonstrated that the $M_1$ protein is present in the virion as two forms, $M_{11}$ and $M_{111}$, which differ in their phosphate content and are found in virions in a 1:2 ratio. $M_{111}$, which was less phosphorylated than $M_{11}$, migrated faster in polyacrylamide gel electrophoresis. In addition, Sokol & Clark (1975) have found that purified virions contain a bound protein kinase which can catalyse the transfer of the gamma phosphate group of ATP or dATP to N and $M_1$ proteins.

By labelling rabies virus strain CVS-infected BHK-21 cells with $[^{35}S]$methionine under conditions of osmotic shock in order to lower cellular syntheses (Madore & England, 1977), we have observed that cytoplasmic $M_1$ migrates as a single band which corresponds to $M_{111}$. The synthesis of the viral glycoprotein was inhibited by the high salt treatment. The absence of any detectable $M_{11}$ in the cytoplasm suggested that $M_{11}$ is derived from $M_{111}$ after additional phosphorylation either during the maturation process or even later in the virion. This last possibility has been tested by incubating freshly prepared $^{32}P$-labelled CVS virions at 4°C for 1, 2, 3 or 4 days. No variation of the proportion of $M_{11}$ to $M_{111}$ was observed (data not shown).

In this study, the synthesis and phosphorylation of N and $M_1$ proteins were followed in infected cells by labelling with $[^{35}S]$methionine and $^{32}P_i$. We also wanted to see if the modifications of cellular metabolism induced by rabies virus are accompanied by a change in the phosphorylation of any cellular proteins.

In order to simplify the protein pattern, cells were fractionated according to Penman's procedure (Burr et al., 1980). Briefly, cell monolayers in Petri dishes were treated with a non-ionic detergent which resulted in the solubilization of 70 to 85% of the proteins. A detergent-insoluble fraction mainly composed of cytoskeletal structures, some of the internal membranes and the nuclei remained attached to the surface of the dish. When $[^{35}S]$methionine-labelled
Fig. 1. Comparison of rabies virus proteins present in virions and in infected cells. BHK-21 cells (3 × 10⁶) infected 22 h before with the CVS strain of rabies virus (m.o.i. 3) were subjected to an osmotic shock and labelled with 10 μCi [³⁵S]methionine (Madore & England, 1977) in 0.1% bovine serum albumin in MEM containing one-fifth the normal quantity of amino acids. After 4 h at 37 °C, cells were washed and resuspended in 200 μl Laemmli buffer; 0.5 μl of cell extracts was mixed with 10 μl [³⁵S]methionine-labelled SDS-disrupted virions and the mixture was analysed in a 10% SDS polyacrylamide gel. The gel was sliced and the 1 mm-thick slices were incubated overnight at 60 °C in 100 μl of H₂O₂. The radioactivity (³⁵S, —; ³⁴S, —) was determined in a LKB liquid scintillation counter. [³⁵S]Methionine (400 mCi/mmol) and [³⁴H]leucine (40 Ci/mmol) were purchased from the Commissariat à l’Energie Atomique, Saclay, France.

Fig. 2. Solubilization of VSV and rabies virus proteins after detergent treatment of infected BSR cells. BSR cells (10⁶) either (a) uninfected or (b) infected 4 h before with VSV (m.o.i. 10) or (c) 22 h before with rabies virus (m.o.i. 3) were labelled with [³⁵S]methionine as in Fig. 1. The cells were then washed and either disrupted in SDS-Laemmli buffer or treated with NP40 in order to remove detergent-soluble proteins, according to Burr et al. (1980) but modified as follows. Cells were washed twice with isotonic saline buffer, then twice with CSK buffer [10 mM-PIPES pH 6.8, 100 mM-KCl, 300 mM-sucrose, 2.5 mM-MgCl₂, 1 mM-PMSF and aprotinin (100 U/ml)]. The detergent-soluble fraction was removed after incubation for 3 min with 1 ml CSK buffer plus 1% NP40. Two washes with CSK buffer were then carried out. The detergent-soluble fraction was precipitated at −20 °C by addition of 4 vol. ethanol and the resulting pellet was disrupted in 200 μl SDS-Laemmli buffer. The detergent-insoluble fraction was directly solubilized in 200 μl SDS-Laemmli buffer and heated at 95 °C for 3 min. One-tenth of the material was analysed on a 10% SDS-polyacrylamide slab gel. The gel was dried and autoradiographed with an RP X-Omat film. Lanes 1, total cell extract; lanes 2, detergent-insoluble fraction.

Soluble and insoluble fractions were analysed in polyacrylamide-SDS gels it was found that viral proteins L, N, M₁ and M₂ mainly remained associated with the cytoskeleton fraction. The viral G protein was not clearly visible in cell extracts (Fig. 1, Fig. 2). A similar experiment with VSV indicated that the glycoprotein was solubilized, while L, N, NS and M remained in the detergent-insoluble fraction (Fig. 2).

When the same experiment was performed after ³²P labelling of the cells, the N protein could be clearly detected 18 and 24 h after infection (Fig. 3b,c). Phosphorylated N protein was mainly associated with the cytoskeletal fraction. As expected, very few, if any, phosphate label was associated with the M₁ protein in the insoluble fraction. No quantification was possible since a cellular protein was present in the same region of the gel. A light ³²P-labelled band migrating at the position of M₁ protein was noticeable in the soluble fraction of cells infected for 18 or 24 h.

Apart from the appearance of phosphorylated N and M₁, no progression in the phosphorylation of cellular proteins could be observed during the course of viral infection (Fig. 3). This suggested that the effect of the virus on cellular metabolism was not via a modification
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Fig. 3. In vivo and in vitro phosphorylation of proteins from rabies virus-infected BSR cells. (b, c) In vivo labelling was performed for 3 h at 37 °C with MEM without phosphate containing 20 μCi 32P, on 10⁶ BSR cells infected 0, 6, 18 or 24 h before with the CVS strain (m.o.i. 3). After two washes with cold phosphate-buffered saline (PBS), cells were fractionated with detergent as in Fig. 2. (a) In the in vitro experiment, cells were fractionated with NP40 and the detergent-insoluble fraction was labelled with 150 μl CSK buffer containing 5 mM-MgCl₂ and 10 μCi [γ-32P]ATP (5000 Ci/mmol, Amersham) for 5 min at 4 °C. The reaction was stopped with 200 μl of SDS-Laemmli buffer and heated at 95 °C for 3 min. The proteins of the (a, b) insoluble and (c) soluble fractions were analysed on a 10% SDS-polyacrylamide slab gel (Banerjee et al., 1974). Autoradiography of the dried gel was done with an RP X-Omat film.

in the phosphorylation of cellular proteins. Similarly, we have found no difference in the phosphorylation of cellular proteins in three BHK-21 cell lines persistently infected by rabies virus (Tuffereau et al., 1985).

In order to determine when additional phosphate groups become attached to the M₁₁ protein, the detergent-insoluble fraction of infected BSR cells was incubated with [γ-32P]ATP. Neither N nor M₁ could be phosphorylated by this method, although they were present in the insoluble fraction (Fig. 3a). The observations that M₁₁ is not hyperphosphorylated by cytoskeleton-bound kinases and that M₁₁ was not detectable in infected cells suggests that some of the M₁₁ molecules could become hyperphosphorylated during the maturation process, probably in the external membranes, and could be rapidly externalized in the virions.

Several cellular proteins were phosphorylated by cytoskeleton-bound kinases. A cellular protein of high mol. wt. (approx. 150K) was heavily labelled, with no change during the infection process (Fig. 3). This protein was not phosphorylated in vivo. With this exception, a notable proportion of the proteins were phosphorylated in vivo as well as in vitro. No difference was found in the phosphorylation pattern of cellular proteins before or after infection with rabies virus.

It has been postulated that in the case of VSV the degree of phosphorylation of the NS protein regulates the rate of transcription and also possibly the equilibrium between transcription and replication (Clinton et al., 1978; Hsu et al., 1982; Kingsford & Emerson, 1980; Tan & Clark, 1977; Watanabe et al., 1974; Witt & Summers, 1980). In an attempt to understand why transcription of rabies virus has such a low efficiency in vitro, we assayed the in vitro
Comparison of the kinase activity of (a) VSV and (b) rabies purified virions in \textit{in vitro} transcriptase assays. Rabies virus kinase activity was assayed according to Flamand \textit{et al.} (1978). In addition to 20 \(\mu\)g of purified virions the reaction mixture contained 0.1 mmol each of UTP, CTP and GTP, 0.01 mmol of unlabelled ATP and 10 \(\mu\)Ci \(^{32}\)P-ATP, 8 mmol Bicine pH 8.9, 1 mmol MgCl\(_2\), 4 mmol NaCl, 0.6 mmol dithiothreitol and 0.02 \(\mu\)l of Triton-N 101 in 125 \(\mu\)l total. Incubation was at 30 °C. The reaction was stopped after 0, 15, 30, 60 or 90 min by adding 20 \(\mu\)l of a saturated solution of sodium pyrophosphate. It was mixed with 1 vol. double-strength Laemmli buffer. Labelled proteins were separated by 10% SDS-PAGE and visualized by autoradiography on RP X-Omat film. VSV kinase activity was assayed on 10 \(\mu\)g of purified virions as described by Aaslestad \textit{et al.} (1971) under conditions which differed from the above in the concentration of NaCl (10 mmol), dithiothreitol (0.2 mmol) and Triton-N 101 (0.2 \(\mu\)l). Conditions of labelling and autoradiography were as for rabies virus and Fig. 3. V, viral markers from rabies virus- or VSV-infected BSR cells.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4}
\caption{Fig. 4. Comparison of the kinase activity of (a) VSV and (b) rabies purified virions in \textit{in vitro} transcriptase assays. Rabies virus kinase activity was assayed according to Flamand \textit{et al.} (1978). In addition to 20 \(\mu\)g of purified virions the reaction mixture contained 0.1 mmol each of UTP, CTP and GTP, 0.01 mmol of unlabelled ATP and 10 \(\mu\)Ci \(^{32}\)P-ATP, 8 mmol Bicine pH 8.9, 1 mmol MgCl\(_2\), 4 mmol NaCl, 0.6 mmol dithiothreitol and 0.02 \(\mu\)l of Triton-N 101 in 125 \(\mu\)l total. Incubation was at 30 °C. The reaction was stopped after 0, 15, 30, 60 or 90 min by adding 20 \(\mu\)l of a saturated solution of sodium pyrophosphate. It was mixed with 1 vol. double-strength Laemmli buffer. Labelled proteins were separated by 10% SDS-PAGE and visualized by autoradiography on RP X-Omat film. VSV kinase activity was assayed on 10 \(\mu\)g of purified virions as described by Aaslestad \textit{et al.} (1971) under conditions which differed from the above in the concentration of NaCl (10 mmol), dithiothreitol (0.2 mmol) and Triton-N 101 (0.2 \(\mu\)l). Conditions of labelling and autoradiography were as for rabies virus and Fig. 3. V, viral markers from rabies virus- or VSV-infected BSR cells.}
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The kinetics of phosphorylation of rabies virus and VSV proteins in parallel with \textit{in vitro} transcriptase assays. As expected (Sokol & Clark, 1975; Clinton & Huang, 1981; for review, see Hsu & Kingsbury, 1980) we found a kinase activity associated with purified virions of VSV and rabies virus. As reported by Witt & Summers (1980), at high salt concentration (0.08 M-NaCl) the VSV-associated enzyme phosphorylated almost exclusively the NS protein (Fig. 4). The kinetics of phosphorylation of this protein were similar to those of the M\(_1\) protein of rabies virus. Some phosphorylation of the M protein was also detectable.

The rabies virus-associated enzyme phosphorylated both the N and M\(_1\) proteins (Fig. 4). The phosphorylation of N was near-maximal within less than 15 min. In contrast, additional

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5}
\caption{Fig. 5. Kinetics of phosphorylation of (a) VSV and (b) rabies virus proteins \textit{in vitro}. The autoradiograms shown in Fig. 4 were scanned and the relative absorbance of labelled proteins was plotted as a function of time. (a) ■, NS; ○, M. (b) ■, M\(_1\); ○, N.}
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phosphate groups were attached to the M₁ protein over at least 90 min (Fig. 5). The presence of an additional band migrating slightly slower than M₁ suggested the presence of an hyperphosphorylated form of M₁, M₁ H. In this in vitro reaction, the M₁ protein accepted twice as many labelled phosphate groups as the N protein although it is much less abundant (1751 molecules of N per 919 molecules of M₁, according to Dietzschold et al., 1979).

It is possible that hyperphosphorylation of the N protein could account for the low activity of the rabies transcriptase in vitro. Whether the phosphorylated status of the N protein in the cytoplasm is also responsible for the lower rate of transcription and replication of rabies virus is unclear. Be that as it may, the fact that the N protein of rabies virus is phosphorylated whereas the N protein of VSV is not, is an important difference between the two viruses.

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