Phosphorylation of Influenza Virus Nucleoprotein in vivo

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SUMMARY

Two-dimensional analysis of polypeptides from A/FPV/Rostock/34 (FP/R)-infected chick embryo fibroblast cells using non-equilibrium pH gradient gel electrophoresis followed by polyacrylamide gel electrophoresis, showed that nucleoprotein (NP) was the only detectable virus phosphoprotein and was present in both the nucleus and cytoplasm. The kinetics of accumulation of phosphorylated NP in the nucleus and cytoplasm were similar, suggesting that the presence or absence of phosphate groups did not control the entry of NP into the nucleus. In the course of this study, two-dimensional analysis of [3SS]methionine-labelled FP/R-infected cells revealed some major differences from previously published work which are discussed.

The nucleoprotein (NP) and non-structural protein 1 (NS1) of influenza type A strains are reported to be phosphorylated in vivo (Privalsky & Penhoet, 1977, 1978), while a virus particle kinase phosphorylates the NP (Sugiyama et al., 1976; Kamata & Watanabe, 1977) or the M protein (Rylatt, 1980) in vitro. The function of these virus phosphoproteins is not understood although phosphorylation affects the efficiency of the transcriptase activity which resides in the virion nucleoprotein complex (Kamata & Watanabe, 1977). However, in general, phosphorylation and dephosphorylation are commonly found mechanisms controlling the functions of cellular proteins (Uy & Wold, 1977; Greengard, 1978). The influenza virus-infected cell is a well-characterized system which lends itself to the study of the function of virus phosphoproteins, particularly since the phosphoprotein NP is involved in a number of activities. For instance, it forms replicative and structural nucleoprotein complexes (Caliguiri & Gerstein, 1978), interacts with both virion and complementary RNA (Pons, 1975), and is transported into the nucleus (Breitenfeld & Schäfer, 1957; Taylor et al., 1969, 1970; Lazarowitz et al., 1971; Krug & Etkind, 1973; Hay & Skehel, 1975) and subsequently back into the cytoplasm (Flawith & Dimmock, 1979). We have investigated the phosphorylation of influenza proteins in vivo to determine whether phosphorylation might affect the movement of NP between the nucleus and cytoplasm.

A/FPV/Rostock/34 (FP/R) (H7N1; Assad et al., 1979) was propagated by inoculating 10^4 p.f.u. into the allantoic cavity of 10-day-old embryonated hens' eggs. Primary chick embryo fibroblast (CEF) cells (Morser et al., 1973) in 5 cm plastic dishes were inoculated with 20 to 30 p.f.u. in allantoic fluid at 37 °C for 1 h and subsequently incubated in medium 199 + 2 % calf serum. For radiolabelling with phosphate, cells were rinsed in phosphate-free Eagle's minimum essential medium (MEM) and incubated with 250 μCi [32P]orthophosphate (The Radiochemical Centre, Amersham) in 0.5 ml of the same medium. Nuclear and cytoplasmic fractions were prepared by the nuclear monolayer method (Hudson & Dimmock, 1977): monolayers were rinsed with ice-cold phosphate-buffered saline (PBS) and the cytoplasm solubilized with two extractions of 0.5 ml 10 mM-tris--HCl pH 7.4, 0.25 M-sucrose, 1 mM-MgCl2 and 2 % (v/v) Nonidet P40 (NP40) (BDH). Nuclei were scraped into 0.5 ml of the buffer without NP40, pelleted by centrifugation for 10 s in an Eppendorf microcentrifuge (type 5412) and washed once by vortexing in the same buffer.
Fig. 1. Analysis (by NEPHGE in the first dimension and PAGE in the second dimension) of CEF cells labelled from 2 to 4 h post-infection with $^{35}$S-methionine (a, b) or $^{32}$P (c to f). (a) An FP/R-infected whole cell-extract; (b) a mock-infected whole cell extract; (c, d) cytoplasmic fractions; (e, f) nuclear fractions. (c, e) From FP/R-infected cells; (d, f) from mock-infected cells. Virus proteins are indicated by capital letters and were identified from the extract run only in the second dimension. Nomenclature follows the standard conventions. Letters a to g are $^{35}$S-methionine-labelled cellular proteins which could be aligned as markers on the autoradiographs of infected and mock-infected cells. Actin is denoted by a; three novel virus-induced basic proteins are grouped as v. The small arrow in (c, d) indicates a cellular basic phosphoprotein migrating ahead of NP.
Phosphorylated virus proteins could not be clearly resolved by polyacrylamide gel electrophoresis (PAGE) as they were obscured by the presence of host cell material (data not shown). Consequently, we turned to a two-dimensional analysis based on charge separation in the first dimension (non-equilibrium pH gradient gel electrophoresis (NEPHGE), O'Farrell et al., 1977) and size in the second (PAGE). Initially it was necessary to establish the positions to which virus proteins migrated using [35S]methionine-labelled FP/R-infected and mock-infected cells. This was achieved using 50 μCi [35S]methionine (800 Ci/mmol; The Radiochemical Centre, Amersham) in 0.5 ml MEM containing 1% of the usual concentration of methionine. Labelled cells were pelleted, resuspended in 0.5 ml 1/10 PBS, sonicated, treated with 50 μg/ml RNase A and 20 μg/ml DNase I (both from bovine pancreas; Sigma) for 30 min at 37 °C, lyophilized and dissolved in 50 μl 0.5% (w/v) SDS (20 min at 70 °C). Samples of 10 μl were made up to 9 M-urea, mixed with lysis buffer (O'Farrell, 1975) and loaded on to the anodic end of a tube gel containing 3.5% ampholines of pH 3.5 to 10 and 0.5% ampholines of pH 9 to 11 (LKB). Gels were pre-electrophoresed for 15 min at 200 V, 30 min at 300 V and 15 min at 400 V as this procedure improved the analysis. Samples were then loaded and electrophoresed for 4 h at 400 V. Subsequently, the gels were extruded, equilibrated for 1 h in 0.0625 M-tris–HCl pH 6.8, 2.3% (w/v) SDS and 5% (v/v) β-mercaptoethanol and set on top of a 3-6% (w/v) acrylamide stacker gel over a 10 to 30% linear acrylamide gradient slab gel (Cook et al., 1979). An aliquot of the sample
Fig. 2. PAGE of $^{32}$P-labelled NP immunoprecipitated with monoclonal anti-NP from (a) nuclear fractions of FP/R-infected CEF cells; (b) cytoplasmic fractions of FP/R-infected CEF cells. Radiolabel (250 μCi) was added for 15 min at 2.5 h post-infection in 0.5 ml phosphate-free MEM. Cells were then rinsed in complete medium and incubated from 0 to 120 min as shown. V is a marker track of [$^35$S]methionine-labelled FP/R-infected CEF cells. Lanes 1 to 6 represent 0, 15, 30, 60, 90 and 120 min after pulse respectively.

subjected to NEPHGE was co-electrophoresed in the second dimension as a marker (Fig. 1a, b).

The relative positions of FP/R proteins were in good agreement with data obtained with other influenza strains (Raghow et al., 1978; Leavitt et al., 1979; Horisberger, 1980). However, there are major differences between our data and that of Privalsky & Penhoet (1978) who resolved NP but found M too basic to be focused. Our data and the extensive data of Leavitt et al. (1979), using a total of 20 type A strains, show M and NP to have a very similar charge. It thus seems likely that the basic protein which Privalsky & Penhoet (1978) term 'NS1' is M and the slightly smaller acidic protein (which is unlabelled in their Fig. 2), is NS1. We agree with their identification of phosphate-labelled NS1 (Privalsky & Penhoet, 1978, Fig. 4) although the NS1 induced in FP/R-infected CEF cells was not phosphorylated (see below). Another feature of interest in Fig. 1(a) is the multiplicity of charged forms of M, the uncleaved HA precursor and HA1. However, HA2 migrated as a single species. The significance of this polymorphism is unknown although it could represent different extents of glycosylation of HA polypeptides. There was poor recovery of the P2 protein. We do not know which of the proteins with a mol. wt. < 23000 in Fig. 1(a) is the
non-structural protein 2 (NS2). Three hitherto unreported basic proteins were found in infected cells (labelled v in Fig. 1a) and their significance is unknown.

Two-dimensional analysis of nuclear and cytoplasmic extracts from $^{32}$P-labelled mock-infected and FP/R-infected cells is presented in Fig. 1(c to f). NP was the only viral phosphoprotein detected even when greater amounts were analysed, and was present in both cytoplasm (Fig. 1c) and nucleus (Fig. 1e). Notice the presence in the cytoplasm of a host protein of similar mol. wt. to NP, but slightly more basic, and the characteristic sets of phosphoproteins found in nuclear and cytoplasmic fractions. The absence of phosphorylated NS1 from FP/R-infected CEF cells was confirmed by PAGE analysis of the immunoprecipitate prepared from $^{32}$P and $[^3H]$leucine double-labelled cells using an antiserum specific for NS1 (data not shown). Presumably there is variation in phosphorylation of NS1 according to virus strain and/or cell type (Privalsky & Penhoet, 1978).

One of the possible functions of phosphorylation of NP is to control its migration into (Breitenfeld & Schäfer, 1957) and out of the nucleus (Flawith & Dimmock, 1979). This was explored by determining the kinetics of accumulation of phosphorylated NP in the cytoplasmic and nuclear compartments. NP was precipitated completely by the addition of excess mouse monoclonal anti-NP IgG (ref. no. C1-AF 27P), purified from ascitic fluid by protein A–Sepharose chromatography (Pharmacia). Immunoprecipitates were collected by the addition of fixed Staphylococcus aureus (Kessler, 1975) and then eluted and analysed by single-dimension PAGE. Fig. 2 shows that similar amounts of phosphate-labelled NP appeared with similar kinetics in both cytoplasmic and nuclear fractions. In further experiments (data not shown) in which cells were double-labelled with $^{32}$P and $[^3H]$leucine, we were also able to show that the specific extent of phosphorylation ($^{32}$P : $^3$H) of NP was the same in both locations between 2.5 and 4.5 h post-infection.

Thus, the overall level of phosphorylation did not appear to be a factor in the association of NP with the nucleus. However, since NP forms a heterogeneous collection of structures [free protein, structural RNP (RNA$^-$), replicative RNP (RNA$^+$); Caliguiri & Gerstein, 1978; P. Rees & N. J. Dimmock, unpublished results], significant changes in phosphorylation in one of these species may be concealed and cannot yet be ruled out; neither do we have information about the intramolecular distribution of phosphate groups in nuclear and cytoplasmic NPs.

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