Identification of a Phosphorylated Non-structural Form of the P Protein of Newcastle Disease Virus and Analysis of P Multimers

By Lawrence E. Hightower,* Peter L. Collins† and Glenn W. Smith

Microbiology Section, Biological Sciences Group, The University of Connecticut, Storrs, Connecticut 06268, U.S.A.

(Accepted 4 June 1984)

SUMMARY

Two phosphorylated and two non-phosphorylated variants of P protein isolated from Newcastle disease virions are known. Here, a fifth form of P was identified using two-dimensional polyacrylamide gel electrophoresis and peptide mapping. P form 5 was phosphorylated; however, unlike the four known variants of P, the new form was not a major protein in virions, which suggested an intracellular function. The subunit composition of four electrophoretically distinct, disulphide-linked multimers of P from virions was determined. Each homomultimer was composed of at least three molecules of a different one of the four virion-associated P variants.

The polymerase (P) or nucleocapsid-associated protein (NAP) of the avian paramyxovirus Newcastle disease virus (NDV) is encoded by the second gene in the transcriptional order of the genome (Collins et al., 1980) and is the second most abundant protein synthesized by infected chicken embryo (CE) cells (Chambers & Samson, 1980; Morrison & Simpson, 1980; Smith & Hightower, 1981b). Biochemical (Colonna & Stone, 1976; Chinchar & Portner, 1981; Hamaguchi et al., 1983) and genetic (Samson et al., 1981; Peeples & Bratt, 1982; Peeples et al., 1982) evidence indicates that P is involved in viral RNA synthesis along with the large nucleocapsid protein (L) and the major nucleocapsid protein (NP). A role for P in the spread of viral infection among cultured cells has been proposed as well (Madansky & Bratt, 1981); however, the molecular details of these processes and definite functional roles for the P protein remain unknown.

Purified virions yield four forms of P identified by two-dimensional PAGE and partial digest peptide mapping (Smith & Hightower, 1981b). The two most acidic forms are phosphorylated, but the majority of virion-associated P is not phosphorylated (Smith & Hightower, 1981b). P has been isolated from virions as 53 kilodalton monomers and 180 kilodalton disulphide-linked multimers (Smith & Hightower, 1981b). Forms of P with different isoelectric points have also been obtained from infected cells (Chambers & Samson, 1980); however, detailed comparisons of P variants from virions, infected cells, and cell-free protein-synthesizing systems programmed with viral mRNA have not been reported. The results of such comparisons, presented here, provided evidence of a fifth form of P having properties different from the known variants. A preliminary report of these results was presented at a symposium entitled 'Molecular Biology of Negative Strand Viruses' held at Hilton Head, South Carolina, September 11 to 17, 1983.

Forms of [35S]methionine-labelled P from virions of strain Australia Victoria (AV) were separated by isoelectric focusing (IEF) and PAGE (Fig. 1a). Forms 3 and 4 are the phosphorylated variants (Smith & Hightower, 1981b). A trace of a possible fifth form slightly more acidic and larger in apparent size than form 3 was detected (not visible in Fig. 1a). Analyses of radioactive proteins from mock-infected (Fig. 1b) and strain AV-infected (Fig. 1c) CE cells showed that spot 5 represented a newly synthesized protein in infected cells. The

* Present address: Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27514, U.S.A.

† Present address: Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27514, U.S.A.
Fig. 1. P variants from purified virions and infected cells. Virus stocks and secondary cultures of CE cells were prepared as before (Smith & Hightower, 1981b). The cells were infected with 5 p.f.u. of AV per cell and incubated at 40 °C in Eagle's MEM containing 5% calf serum (CS) (standard medium). At 6 h post-infection, cultures were rinsed twice with prewarmed MEM containing 1% (0.15 mg/l) of the normal concentration of methionine and 2% dialysed CS (label medium). The cultures were exposed to 100 μCi [35S]methionine (approx. 1000 Ci/mmol) per ml of the same medium for 10 min, washed, and incubated in standard medium supplemented with a tenfold excess of methionine (150 mg/l) for 1 h. Culture medium was then collected from two 60 mm plates and centrifuged to remove cellular debris (12000 g for 10 min) and to concentrate virions (83000 g for 1 h through a layer of 20% w/v sucrose). The resulting gels were prepared for fluorography (Laskey & Mills, 1975). A portion of each fluorogram from approx pH 5.4 to 6.5 and from 45 kdal to 62 kdal is shown in (a) (virions) and subsequent panels. Mock-infected (b, d) and infected (c, e) cultures in 35 mm plates were labelled with either 250 μCi 32P per ml standard medium without phosphate between 3 and 6 h post-infection (d, e) or with 25 μCi [35S]-methionine per ml label medium between 4 and 5 h post-infection (b, c). Cultures were solubilized directly in lysis buffer (O'Farrell, 1975) and analysed by IEF-PAGE and fluorography as described by Hightower & White (1981). The star marks the same position in each panel within a figure. A trace of P form 5 was visible at the position indicated in (a). Equal amounts of cell extract were loaded on the gels represented in (b) and (c), and in (d) and (e).

Horizontal streak in Fig. 1(c) was caused by the nucleocapsid protein which does not focus in this gel procedure. Spot 5 was identified as a fifth form of P protein by partial digest peptide mapping (Fig. 2).

To determine whether P form 5 was phosphorylated, mock-infected (Fig. 1d) and infected (Fig. 1e) cells were exposed to 32P, and the resulting radioactive proteins were analysed by IEF-PAGE. The 32P was incorporated into form 5 and also forms 3 and 4. Form 5 characteristically focused into an oblong spot suggesting either charge heterogeneity or reduced solubility in the IEF gel. The pi and Mr values of the various forms of P from virions were indistinguishable on
Short communication

Fig. 3. Kinetics of synthesis of P variants in vitro and in vivo. At 9 h post-infection, mRNA was extracted from infected CE cells, treated with actinomycin D (1 μg/ml) and translated in nuclease-treated rabbit reticulocyte lysates as described previously (Collins et al., 1980, 1982). Reaction mixtures containing 1 μM-methionine and 250 μCi[^35S]methionine per ml were incubated at 30 °C. Samples were taken at 8 min (a), 15 min (b), 30 min (c), 60 min (d), and 120 min (e) of incubation and the reactions were stopped by freezing in a methanol-dry ice bath. Equal amounts of lysate were mixed with 10 vol. of O'Farrell lysis buffer and analysed by IEF-PAGE followed by fluorography. Infected CE cells were exposed to 100 μCi[^35S]methionine per ml label medium for 1 min, washed, and incubated in standard medium containing tenfold additional methionine for either 10 min (f) or 30 min (g). The cultures were solubilized in gel sample buffer (Smith & Hightower, 1981b), mixed with 1 vol. each of lysis buffer containing 9 M-urea and twice the normal concentration of other components, and equal amounts of cell lysates were analysed by IEF-PAGE.

two-dimensional gels from those obtained for P variants from infected cells. The patterns of P microheterogeneity were highly reproducible; however, differences in the relative proportions of P variants from virions were obtained using different protocols for radioisotopic labelling. Relatively more form 3 accumulated in virions during the brief pulse-chase protocol used here compared to previous experiments using long radioisotopic labelling periods in which form 2 was second in abundance (Smith & Hightower, 1981b).

Forms of P made in reticulocyte lysates programmed with mRNA extracted from infected cells were also analysed by IEF-PAGE (Fig. 3a to e). After 8 min incubation with[^35S]-methionine, forms 1, 3 and 5 were detectable (Fig. 3a). Forms 2 and 4 were detectable by 60 min (Fig. 3d). Thus, two general processing schemes were apparent. Forms 1, 3 and 5 appeared immediately following translation, and therefore the phosphorylations of forms 3 and 5 were...
rapid. In contrast, form 2 (non-phosphorylated) and form 4 (phosphorylated) appeared much less rapidly and presumably were derived from one or more of the early forms. Form 3 was the only variant that decreased noticeably in relative abundance as the radioisotopic labelling period increased (Fig. 3 a to e) or during a chase following radioisotopic incorporation (Fig. 3, compare f with g). Therefore, form 3 is a candidate for a precursor role.

The most remarkable aspect of the cell-free synthesis of P was the similarity in microheterogeneity exhibited by P made in vivo and in vitro. Patterns of P extracted from infected cells after 10 min (Fig. 3 f) and 30 min (Fig. 3 g) chase periods following 1 min incubation with [35S]-methionine were almost indistinguishable from patterns of P made by 6 min (Fig. 3 a) and 60 to 120 min (Fig. 3 d, e) respectively in reticulocyte lysates. The main difference was the accumulation of more form 2 in infected cells.

If P microheterogeneity has functional significance, then major features of the pattern should be conserved among naturally occurring strains of NDV. Virulent strain AV and avirulent strain B1 were chosen for this comparison. The nucleocapsid protein of strain AV is slightly smaller than that of strain B1 and the P protein of strain B1 is slightly larger than its counterpart in strain AV (Collins & Hightower, 1982). Apart from these apparent size differences, there were strong similarities between the patterns of P made in cell-free systems programmed with mRNA from AV (Fig. 4 b) and B1 (Fig. 4 c) infected cells. Form 1 was the most abundant in both patterns and a fifth form was present in both. It is possible that spot 6 which was also present in both patterns was another form of P, but it was not analysed by peptide mapping owing to its low abundance.

The main cluster of forms 2, 3 and 4 characteristic of P from strain AV was also discernible from B1; however, the P forms in the latter cluster could be only partially resolved.

NDV P proteins can be extracted from virions as disulphide-linked multimers large enough to be trimers (Smith & Hightower, 1981 b). Although it is not certain that P molecules are normally disulphide-linked in virions, P multimers are also obtained after extraction in the presence of
iodoacetamide, indicating they are not formed by auto-oxidation during extraction (Smith & Hightower, 1981b). To determine the composition of P multimers, 2-mercaptoethanol was omitted from the IEF dimension only of a two-dimensional gel analysis to allow P proteins to focus as disulphide-linked molecules. Under these conditions, the net charge of P multimers was different enough from P monomers to allow separation (Fig. 5). The pattern of four P variants from [35S]methionine-labelled, purified virions analysed under normal reducing conditions is shown in (a). When radioactive P variants were separated under non-reducing conditions during IEF, the characteristic pattern of P monomers and four additional spots which constituted a duplicate of the monomer pattern with an acidic shift were obtained (b). Previously, it was reported that only a portion of P can be extracted from virions as P multimers (Smith & Hightower, 1981b). Our interpretation of this pattern, diagrammed in (c), was that the shifted spots were disulphide-linked homomultimers of P. Gel analysis of P proteins omitting 2-mercaptoethanol from both dimensions confirmed that the shifted proteins were in disulphide-linked trimers (data not shown). Since each spot underwent the same magnitude of acidic shift, we suggest that the subunits in all four types of homotrimer associate in a similar way. These observations are consistent with a recent report (Chinchar & Portner, 1981) that the disulphide-linked parts of P are in a highly basic, protease-resistant fragment of the molecule. Neutralization or shielding of basic amino acids in disulphide-linked multimers would explain the acidic shifts shown in Fig. 5(b). Our data also imply that modified forms of P such as the phosphorylated forms 3 and 4 either associate with the same modified forms or that all subunits in a P multimer undergo the same charge modifications after multimer formation.

At present, we can only speculate on the biological roles of the various forms of P in virions and infected cells. P is one of the last of the NDV proteins to be assembled into virions (Smith & Hightower, 1981a) and consequently, P forms are candidates for involvement in folding of the nucleocapsid and budding of virions from cells. All of the structural P forms are incorporated into virions at approximately the same rate (unpublished results) so kinetic analyses have not distinguished different roles in assembly for P variants. Based on its high abundance, we suggest that form 1 may have primarily a structural role in nucleocapsids. Form 5 was abundant in infected cells but not in virions so the possibility that this P form might be a precursor to others was considered; however, this possibility is inconsistent with the apparent intracellular stability of form 5 (Fig. 3f, g).

There is more to P microheterogeneity than phosphorylations. The difference between forms 1 and 2 does not involve phosphorylation and phosphorylated forms 3, 4 and 5 each have different pIs and apparent sizes. The similarities between P patterns in vivo and in vitro suggest that mechanisms at the level of translational initiation and termination or perhaps mRNA heterogeneity as well as post-translational modifications may contribute to P microheterogeneity, at least for the early appearing forms 1, 3 and 5. It would be very surprising if all of the modifications of P were post-translational events which could be carried out authentically in reticulocyte lysates, unless perhaps P modified itself. Further study of the molecular complexity of the P protein may yield clues to the assembly and biological activities of the nucleocapsids of paramyxoviruses.

This work was supported by U.S. Public Health Service Grant HL23588 and National Science Foundation Grants PCM78-08088 and PCM81-18285. We benefitted from the use of a Cell Culture Facility supported by U.S. Public Health Service Grant CA14733. P.L.C. was a National Institutes of Health predoctoral trainee.

REFERENCES


(Received 18 April 1984)