Proteins of Palyam serogroup viruses

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The replication and polypeptide synthesis of Abadina virus, a member of the Palyam serogroup of orbiviruses, was studied. The first virus-specified proteins could be demonstrated 2 to 4 h post-infection (p.i.) by immunoprecipitation. The rate of synthesis increased rapidly until 12 h p.i. after which it remained fairly constant until 18 h p.i. when it began to decline. Host cell protein synthesis shutoff was incomplete. Twelve virus-induced polypeptides were identified in infected cell lysates, ranging in Mr from 36K to 143K. Three small polypeptides (Mr, 15K to 20K) identified in homologous immunoprecipitation studies are also thought to be virus-specified. Nine structural proteins were identified, four being major components of the purified virion. Partial proteolysis was used to demonstrate homology between some proteins. Pulse–chase experiments provided no evidence for a precursor–product relationship between any of the Abadina virus proteins. A non-structural protein was found to be phosphorylated.

Introduction

The Palyam serogroup of orbiviruses comprises at least 15 viruses in six antigenic complexes (Knudson et al., 1984; Boiro et al., 1986; Whistler & Swanepoel, 1988; Goto et al., 1988; Whistler et al., 1989). They are widely distributed in Africa, Asia and Australia and are presumed to be vector-borne since they have commonly been isolated from haematophagous arthropods such as mosquitoes, midges and ticks. Until comparatively recently, no pathogenic role had been ascribed to the viruses, but increasing evidence is emerging that they may be involved in producing abortion and teratology in cattle (Swanepoel & Blackburn, 1976; Whistler & Swanepoel, 1988; Goto et al., 1988; Whistler et al., 1989). There have been preliminary studies on characterization of the RNA genomes of the viruses (Knudson et al., 1984; Bodkin & Knudson, 1985, 1986; Whistler & Swanepoel, 1988), but information on proteins is lacking. The present paper reports identification and preliminary characterization of virus-induced proteins in infected cells and structural proteins of the virion. Glycosylation, phosphorylation and possible post-translational modification of proteins were also examined.

Methods

Vero cells (Yasumura & Kawakita, 1963) were grown as monolayers using Eagle’s minimal essential amino acid medium with Earle’s salt solution (EMEM) (Eagle, 1959) and 10% foetal calf serum. After cultures were infected, the serum content of the medium was reduced to 2%. Hyperimmune mouse ascitic fluids (MIAFs) were raised against a 10% suspension of infected mouse brain as described by Sartorelli et al. (1966).

Growth cycle. A single-step growth curve of Abadina virus was investigated in 25 cm² flasks containing confluent Vero cell monolayers. Cultures were inoculated with Abadina virus at a multiplicity of 20 fluorescent focus-forming units/cell (f.f.u./cell) adsorbed at 37 °C for 1 h. The inoculum was removed, 4 ml maintenance medium added and the cultures were incubated at 37 °C until required for labelling. An hour before the start of the different 2 h labelling periods (indicated in Results), the medium was removed, clarified and kept at 4 °C for titration of cell-free virus. The cells were rinsed with methionine-free EMEM, and 4 ml of the medium was added per flask. The cells were reincubated at 37 °C for 1 h and, at the indicated times, 50μCi [35S]methionine (Amersham) was added and the cultures were incubated at 37 °C for 2 h. When hypertonic shock was used to suppress host cell protein synthesis, NaCl was added to a final concentration of 150 mm, 15 min prior to the addition of radiolabel. At the end of the labelling period, cells were scrapped from the surface and collected by centrifugation at 1500 r.p.m. for 10 min. They were resuspended in 0.5 ml phosphate-buffered saline pH 7.2 (PBS) and split into three fractions: 0.05 ml fractions for assay of cell-associated virus were subjected to three cycles of freezing and thawing and virus from these and the cell-free fractions were titrated in fluorescent focus assays as described by Whistler & Swanepoel (1988); 0.1 ml fractions for direct analysis of proteins by PAGE were prepared by adding sample buffer (Laemmli, 1970); the remaining 0.35 ml fractions were used for immunoprecipitation analysis.

Preparation of radiolabelled structural proteins. Monolayer cultures of cells were infected with Abadina virus as above and subjected to hypertonic shock followed by labelling at 8 h p.i. with 25 μCi/ml [35S]methionine. When maximum c.p.e. occurred, cells were collected...
and virus was purified from cell lysates according to the method of Huismans et al. (1987a).

Preparation of radiolabelled intracellular viral proteins. Virus infection and radiolabelling were performed as above. After a 16 h incubation with radiolabel, the cells were washed three times with PBS and lysed in Laemmli sample buffer. Mock-infected host cell proteins were prepared in the same manner.

Pulse–chase experiments. Virus infection and radiolabelling were performed as described. The cells were pulse-labelled for 15 min with $25 \mu$Ci/ml $[^{35}S]$methionine at 8 h p.i. and then harvested immediately or chased for various lengths of time with maintenance medium, plus excess NaCl. The intracellular viral proteins were examined using the PAGE system of Laemmli (1970).

Glycosylation inhibition studies. Virus was grown and radiolabelled as described, except that from 2 h p.i. tunicamycin (Boehringer Mannheim) was added to all media, at a concentration of 2 $\mu$g/ml. Cells were harvested at 24 h.

Phosphoprotein detection. Monolayer cultures of Vero cells were mock-infected or infected with Abadina virus. At 4 h p.i. the medium was removed and the monolayer was washed and re-fed with phosphate-free MEM. After a further 4 h incubation carrier-free $[^{32}P]$orthophosphate (Amersham) was added to 10 $\mu$Ci/ml. Cells were lysed at 24 h p.i. in Laemmli sample buffer.

Immunoprecipitation. Cells were resuspended in NET/BSA buffer (150 mM-sodium chloride, 5 mM-EDTA, 50 mM-Tris–HCl pH 7.4, 0.5% Nonidet P40, 1 mg/ml bovine serum albumin) at a concentration of 1 x 10$^5$ cells/ml and kept on ice for 30 min. Nuclei and other insoluble materials were removed by centrifuging at 3000 r.p.m. for 15 min. The supernatant was made 0.1% in SDS, sonicated in a bath for 5 min and 0.01 ml of the required MIAF was added for every 1 ml of supernatant. Mixtures were incubated for 4 to 6 h at room temperature (RT) and Protein A–sepharose CL4B beads (Pharmacia) were added to 2 mg/ml. The mixtures were then incubated at RT for 1 h and at 4 °C overnight. The beads were washed five times with NET/BSA buffer, resuspended in Laemmli sample buffer and cooled at 100 °C for 10 min. The beads were pelleted and the sample buffer was recovered. Specificity of reaction was checked using normal ascitic fluid in place of the MIAF.

Polyacrylamide gel electrophoresis. Proteins were separated on a 10% resolving gel with a 4% stacking gel, using the discontinuous Tris–glycine buffer system of Laemmli (1970). After electrophoresis gels were fixed in 45% methanol and 7% acetic acid, dried and exposed to $\beta$-max film (Amersham) at $-70$ °C. $M_r$ values were estimated using $^{14}C$-labelled marker proteins (Amersham).

Peptide mapping. Peptides of viral proteins were analysed according to Cleveland et al. (1977).

Results

Growth cycle

Using an infection rate of 20 f.f.u. of Abadina virus per cell, a single-cycle growth curve was obtained with virus yield reaching a maximum at 10 to 12 h p.i. (Fig. 1). More than 90% of the infectious virus remained cell-associated, even at late stages of the growth cycle. At 24 h p.i. approximately 50% of the cells remained attached and by 36 h p.i., c.p.e. had occurred in greater than 90% of the cells.

Analysis of cell lysates at different 2 h intervals from 0 to 36 h p.i. (results not shown) revealed that up until 12 h p.i. the synthesis of viral polypeptides was almost entirely obscured by that of the host cell; the latter was not completely suppressed until late in the replication cycle. Suppression of cellular protein synthesis with excess NaCl enabled the detection of viral proteins as early as 4 to 6 h p.i. Following immunoprecipitation with Abadina MIAF, however, at least seven virus-induced polypeptides could be discerned at 2 to 4 h p.i. (Fig. 2). The synthesis of the viral polypeptides appeared to increase to a maximum at 12 h p.i. and remained constant to 18 h p.i. after which it decreased.

Identification of structural and non-structural proteins

Comparison of Abadina virus-infected cell lysates radiolabelled from 8 to 24 h.p.i. with host cell proteins revealed the presence of at least 10 virus-induced proteins (Fig. 3). Identification of structural proteins was by comparison of the proteins of sucrose gradient-purified virus with those of infected cell lysates (Fig. 3). Seven of the virus-induced proteins were identified as being structural proteins. The protein designated P8 in Fig. 3 was present when cell lysates were concentrated by immune precipitation (results not shown) but was not detected otherwise. A virus-encoded protein slightly smaller than the structural protein P9 was identified in cell lysates and whether these two proteins are homologous needs to be clarified; if they are, the slight difference in $M_r$ requires investigation. Thus, Abadina virus has at least 12 virus-induced proteins, nine of which are structural. The structural proteins were designated P1 to P9 in order of decreasing $M_r$ and the three non-structural proteins were designated NS1, NS2a and NS2b. Of the nine structural proteins, four were major components of the virion (P2, P3, P4 and P7) and the remaining five were minor components. It must be
noted, however, that minor proteins P8 and P9 were not present in all purified virus preparations. Their absence appeared to have no effect on virus infectivity as preparations with or without these proteins were similarly infectious when inoculated onto cell monolayers.

**Pulse-chase studies**

Brief pulse-labelling followed by prolonged chases (up to 5 h) showed that all the major virus-coded proteins are completely stable and are not products of post-translational cleavage of a high $M_r$ precursor. None of the bands on the polyacrylamide gels appeared to change significantly in intensity or position throughout the chase period (results not shown).

**Glycoprotein and phosphoprotein studies**

No differences in viral protein banding pattern were noted with or without tunicamycin treatment (Fig. 4a), indicating that none of the viral proteins undergo N-linked glycosylation. One protein band that was enhanced in the infected cell lysate with tunicamycin proved to be of host cell origin. The possibility of O-linked sugar addition was not investigated.

Growth of Abadina virus in the presence of $^{32}$Porthophosphate revealed that viral protein NS2a or NS2b is a phosphoprotein (Fig. 4b). The viral phosphoprotein was identified as an NS2 protein by comparison with $^{35}$Smethionine-labelled viral proteins, but as these two proteins have similar $M_r$ values and the phosphoprotein migrated as a broad band, it could not be identified as
either one or the other. NS2a and NS2b have been shown to be structurally related by peptide mapping (results not shown). Mapping of $^{35}$S-labelled NS2b and the $^{32}$P-labelled protein confirm the identity of the phosphoprotein as an NS2 protein (Fig. 4c).

Discussion

The major problem encountered in measuring the rate and extent of synthesis of Abadina virus-specified proteins is that the virus failed to depress host cell synthesis until late in the infection cycle and even then shutoff was incomplete, as observed also with Kemerovo serogroup viruses (Black et al., 1985). In several systems labelling of viral proteins after hypertonic shock of cells has been shown to be highly efficient in suppressing host cell background (Nuss et al., 1975; Frugulhetti et al., 1987), and this was true when Abadina virus was labelled in the presence of 150 mm-NaCl. Viral proteins could be detected 8 h earlier than without hypertonic shock. However, immunoprecipitation revealed that Abadina polypeptides appear to be synthesized synchronously and can first be detected 2 to 4 h p.i. There is no evidence for early or late polypeptide classes.

Abadina virus was shown to have 12 virus-induced proteins. Comparison of proteins from other viruses in this serogroup by homologous immune precipitation of cytoplasmic extracts revealed that all had similar profiles of 12 virus-induced proteins and on several occasions three additional small polypeptides ($M$, 15K to 20K) were detected (results not shown). Similar numbers of induced polypeptides have been reported for other viruses of the genus. Spence et al. (1985) reported the presence of 13 virus-specified polypeptides in cells infected with the Kemerovo serogroup Mill Door/79 virus. Twelve virus-specified polypeptides were identified by in vitro translation of BT serotype 1 virus (Mertens et al., 1984) and 11 for EHDV (Mecham & Dean, 1988). Failure to detect all of the virus-induced polypeptides in the present time-course experiments could indicate that short labelling periods were inadequate to demonstrate proteins that are synthesized in small quantities, particularly if the proteins incorporate low levels of methionine.

It is postulated that each of the 10 genomic segments of orbiviruses codes for a single protein (Gorman et al., 1973).
and the present detection of 12 virus-induced proteins suggests the occurrence of either post-translational modification, incomplete translation or the presence of more than one open reading frame. As protein processing was not demonstrated in pulse-chase experiments, it is possible that these proteins are produced by translation of the viral mRNA from multiple initiation sites. The non-structural proteins NS2a and NS2b also showed very similar peptide maps. One of these proteins is phosphorylated and it is possible that the other is the non-phosphorylated form. The reason for the phosphorylation is unknown, but phosphorylation and dephosphorylation are commonly found as mechanisms controlling the function of cellular proteins (Uy & Wold, 1977; Greengard, 1978). A phosphorylated, non-structural protein with an affinity for single-stranded RNA has been reported in BT virus-infected cells (Huismans et al., 1987b).

Protein P2 of BT virus carries the dominant serotype-specific antigens (Huismans & Erasmus, 1981), and it appears likely that P2 of the Palyam serogroup may carry the main determinant of serotype specificity since this protein was precipitated only by heterologous sera raised using closely related viruses and showed the greatest variation in size in comparative electrophoresis of the serotypes. Similar size variation occurred in P2 of BT virus (De Villiers, 1974). Furthermore, peptide maps generated for P2 from different serotypes had no evident structural homologies, making it the only viral protein showing divergent structure among the serotypes examined (results not shown).

It is evident that the Palyam group viruses have many
features of protein synthesis and virus structure in common with other members of the orbivirus genus but for a fuller understanding of these viruses, it is necessary that the coding assignments of the genome segments should be established.

References


(Received 23 October 1989; Accepted 13 February 1990)