The Capsid Polypeptides of the 190S Virus of Helminthosporium victoriae

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SUMMARY

SDS-PAGE of the 190S virus of Helminthosporium victoriae, using a discontinuous buffer system, revealed two major capsid polypeptides of mol. wt. 88K (p88) and 83K (p83) and a minor polypeptide, p78. Peptide mapping by both limited proteolysis and selective chemical cleavage showed p83 and p78 to be closely related to p88. The origin of p83/p78 could not be explained by proteolysis of p88 during virus preparation and storage. In rabbit reticulocyte lysates, denatured dsRNA directed the synthesis of a single major translation product which was identical to capsid polypeptide p88 on the basis of coelectrophoresis, immunoprecipitation and peptide mapping. No translation products comparable in size to p83 or p78 were detected in vitro. These data indicated that the capsid of the 190S virus is encoded by a single gene and verified the classification of the virus as a member of the family Tottiviridae. Radioiodination of intact virus under conditions considered optimum for surface-specific iodination showed p88 to be more readily available for labelling than p83 or p78. Furthermore, when Western blots of capsid polypeptides were reacted with an antiserum to glutaraldehyde-stabilized virus (190S-G), p88 was more reactive to 190S-G antibodies than was p83/p78. These results suggest p88 is external to p83/p78 in the capsid.

INTRODUCTION

Two isometric dsRNA viruses, designated according to the sedimentation coefficients of their particles as the 190S and 145S viruses, are known to infect isolates of the plant pathogenic fungus Helminthosporium victoriae, the causal agent of Victoria blight of oats (Sanderlin & Ghabrial, 1978). These two viruses are of special interest because they are associated with a disease of their fungal host, whereas the majority of mycoviruses are avirulent (Ghabrial et al., 1979; Ghabrial, 1980; Ghabrial & Mernaugh, 1983). The relationship between the two viruses and their roles in disease development, however, are not known. To shed some light on this, studies were initiated to isolate and characterize the capsid polypeptides of the two viruses and to characterize the gene products of the individual dsRNAs. The present paper describes experiments with the 190S virus.

The 190S virus has isometric particles 35 to 40 nm in diameter and possesses a single species of dsRNA of mol. wt. 3.0 × 10^6. Two major polypeptides of mol. wt. 88K (p88) and 83K (p83), occurring in equimolar amounts, are resolved by SDS-PAGE of capsid protein using a continuous buffer system (Sanderlin & Ghabrial, 1978). These proteins, if unique in sequence, would require virtually the entire coding capacity of the genome (assuming no overlapping genes) and would not allow for the encoding of the virion-associated RNA polymerase, the activity of which has recently been demonstrated (Ghabrial & Havens, 1986). The virion-associated RNA polymerases of some dsRNA mycoviruses and of reovirus have been tentatively identified as minor capsid components (Buck, 1980; Joklik, 1985). We have therefore investigated the structural relationship between p88 and p83 and characterized the translation products of denatured dsRNA in vitro.
METHODS

Virus source and purification. *H. victoriae* isolate B-1 (ATCC 42019) was used as a source of 190S virus. Mycelium, harvested from 10-day-old stationary cultures of isolate B-1 grown on potato dextrose broth supplemented with 0.5% (w/v) yeast extract, was processed for virus purification as described by Sanderlin & Ghabrial (1978). In some experiments, the final purification step was equilibrium centrifugation in CsCl. For this purpose, samples of the 190S virus (1 ml), purified by sucrose density gradient centrifugation, were layered over 4 ml of 36% (w/w) CsCl solutions in 0.1 M-sodium phosphate buffer pH 7.0 and centrifuged for 17 h at 45000 r.p.m. in a Spinco SW50 rotor at 4 °C.

Preparation of dsRNA. Virus suspensions were made 1% in SDS and heated to 60 °C for 15 min. The mixture was then extracted with an equal volume of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and the dsRNA in the aqueous phase was precipitated by adding 2.5 volumes of ethanol.

SDS-PAGE of capsid polypeptides. Virus samples were dissociated by boiling for 3 min in an appropriate buffer (0.01 M-sodium phosphate buffer pH 7.2 or 0.125 M-Tris-HCl pH 6.8) containing 1% SDS and 1% 2-mercaptoethanol and analysed by electrophoresis in either (i) cylindrical or slab gels of 10% polyacrylamide using the continuous buffer system of Weber & Osborn (1969), or (ii) in slab gels of 10% polyacrylamide overlaid with a stacking gel of 4% polyacrylamide using the discontinuous buffer system of Laemmli (1970). Following electrophoresis, the gels were stained for 1 to 3 h in 0.125% (w/v) Coomassie Brilliant Blue R250 in acetic acid:methanol:water (1:5:4 v/v/v) and destained overnight in 7.5% acetic acid, 5% ethanol. Molecular weight markers used in this study were: myosin (200K), β-galactosidase (116K), phosphorylase B (93K), bovine serum albumin (66K), ovalbumin (45K), glyceraldehyde 3-phosphate dehydrogenase (36K), carbonic anhydrase (31K), trypsin inhibitor (22K) and lysozyme (14K).

Isolation and separation of capsid polypeptides. Individual capsid polypeptides were isolated by preparative SDS-PAGE using a discontinuous buffer system (Laemmli, 1970). The stacking gels (4% polyacrylamide) were made using a blank comb without slots so that relatively large amounts of protein could be layered onto the entire length of the gel. About 100 to 300 μg of dissociated virus in 200 μl of disruption buffer was used. After electrophoresis, staining and destaining, the protein bands were excised and cut into slices approximately 8 mm long. For peptide mapping by partial proteolysis, the gel slices were placed in 1 ml microfuge tubes containing sample equilibration buffer (0.01 M-Tris-HCl pH 6.8, 0.1% SDS, 1 mM-EDTA, 10% glycerol, 0.1% dithiothreitol) and processed as described by Cleveland et al. (1977).

Partial peptide mapping. Limited proteolysis using *Staphylococcus aureus* V-8 protease was as described by Cleveland et al. (1977) or by selective chemical cleavage using N-chlorosuccinimide (NCS)/urea as described by Lischwe & Ochs (1982). Gel slices containing the individual capsid polypeptides were transferred to the sample wells of a 15% polyacrylamide gel and 0.1 to 1.0 μg of *Staphylococcus aureus* V-8 protease was added per well. Electrophoresis was interrupted for 30 min after stacking the protein into the spacer gel to allow proteolysis to occur. For cleavage of the proteins at the tryptophanyl peptide bonds using NCS, the gel slices were incubated for 30 min at room temperature with 0.015 M-NCS in urea :water :acetic acid (1:1:1 w/v/v), washed with water followed by equilibration buffer (0.0625 M-Tris-HCl pH 6.8, containing 10% glycerol, 15% 2-mercaptoethanol and 3% SDS) and loaded on to the gel (Lischwe & Ochs, 1982). Following electrophoresis, staining with Coomassie Blue, and destaining, the gels were silver-stained using a Bio-Rad silver staining kit.

Translation in vitro. Nuclease-treated rabbit reticulocyte lysates were purchased from Promega-Biotec and used for cell-free protein synthesis. A standard reaction mixture of 50 μl containing 50 μCi [35S]methionine (>100 Ci/mmol) was mixed with 4 μg of dsRNA denatured by incubation with methylmercuric hydroxide (final concentration 12.5 mM) at room temperature for 20 min. Translation was performed at 30 °C for 60 min. In some experiments, the translation products were incubated for a further 2 to 3 h. Labelled translation products in 10 μl aliquots of reaction mixture were separated by SDS–PAGE using the Laemmli system and after staining, destaining and drying, the gels were autoradiographed using Kodak X-Omat film and an intensifying screen.

Immunoprecipitation. Immunoprecipitation of translation products was performed according to the procedure of Fransen et al. (1978) using an antiserum to intact 190S virus.

Radioiodination. Intact virus was iodinated using Iodogen (Pierce Chemical Company, Rockford, Ill., U.S.A.) under optimal conditions for specific iodination of surface proteins (Markwell & Fox, 1978). Virus, 100 μg in 100 μl of 0.05 M-Tris-HCl buffer pH 7.4, was reacted with 500 μCi of Na125I at 0 °C for 15 min in a 12 × 75 mm test tube which had been previously coated with 3 to 10 μg Iodogen. Similar procedures were used to iodinate disrupted virus except that (i) the virus was solubilized in 2% SDS prior to iodination, (ii) Iodogen was used at 10 to 100 μg per tube and (iii) the iodination reaction was at room temperature. The iodinated intact virus was made 2% with respect to both SDS and 2-mercaptoethanol, disrupted by heating at 100 °C for 3 min and analysed on 10% polyacrylamide gel using the discontinuous buffer system. Virus samples which were disrupted prior to iodination were adjusted to 2% with 2-mercaptoethanol and analysed by SDS–PAGE and autoradiography.

Antiserum preparation. Antisera to glutaraldehyde-stabilized 190S virus (190S-G) and untreated virus (190S-NG) were produced in rabbits by a series of intravenous and subcutaneous injections. Glutaraldehyde-stabilized
antigen was prepared by dialysing purified virus (1 mg/ml) from sucrose density gradients against 0.1 M-sodium phosphate buffer pH 7.0 containing 1% glutaraldehyde, for 24 h. A subsequent dialysis against phosphate buffer for 24 h served to remove unreacted glutaraldehyde. Final purification of 190S-G was by equilibrium centrifugation in CsCl. The two antisera had similar titres (1/1024) in a ring precipitin test with purified virus (0.1 mg/ml) in 0.1 M-sodium phosphate buffer pH 7.0.

Western blots. Electrophoretic transfer of viral proteins from SDS-polyacrylamide gels to nitrocellulose membranes was by the procedures of Towbin et al. (1979) as modified by Burnette (1981). After the transfer, the nitrocellulose was blocked in 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS) pH 7.2 (blocking buffer). The membrane was cut into strips representing identical blots and the strips were individually placed into plastic sealer bags. The primary antibodies used were a partially purified IgG fraction derived from antisera to 190S-G or 190S-NG. About 100 µg IgG diluted in 10 ml blocking buffer was added to each strip and incubated overnight at 4 °C. The strips were then removed from the bags, washed twice (10 min each) in blocking buffer and reacted with the secondary antibodies. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Ca., U.S.A.) was diluted 1:1000 in blocking buffer and added to the strips in plastic bags. These were incubated for 1 h at room temperature. The nitrocellulose was then washed three times in PBS, and then once for 10 min in PBS containing 1% (v/v) horse serum. The strips were then incubated for 1 h with Vectastain (Vectastain ABC kit, Vector Laboratories) according to the manufacturer’s instructions. The strips were washed for 5 min five times in PBS and stained with substrate solution (3,3'-diaminobenzidine, 50 mg dissolved in a solution prepared by mixing PBS, 98.9 ml; 2% CaCl₂, 1 ml and 30% H₂O₂, 0.1 ml). The strips were finally washed in water.

RESULTS

Separation and characterization of capsid polypeptides

SDS–PAGE of the 190S virus in either slab or cylindrical gels using the continuous buffer system revealed two major proteins, p88 and p83, which occurred in equimolar amounts (Fig. 1 a). Using the discontinuous buffer system, however, the capsid polypeptides were resolved into three components, p88, p83 and p78 (Fig. 1 b, c). In all cases, two major polypeptides present in equimolar amounts and one minor component were detected. In all the preparations p88 was a major polypeptide and in most, p83 was the second major polypeptide (Fig. 1 b). However, in some preparations, p78 was a major component and p83 was the minor polypeptide (Fig. 1 c). No preparation was ever found that contained a single major polypeptide and in no instance was p88 a minor component.

![Fig. 1. SDS–PAGE of the 190S virus capsid polypeptides. (a) Electrophoresis was performed in cylindrical 10% polyacrylamide gels in 0.1 M-phosphate buffer pH 7.2, containing 0.1% SDS and 0.1% 2-mercaptoethanol; (b) and (c) electrophoresis was carried out in preparative slab gels containing 10% polyacrylamide using the discontinuous buffer system (Laemmli, 1970).](image-url)
Partial peptide mapping

Efficient separation and isolation of the individual capsid polypeptides was achieved by preparative SDS–PAGE. Representatives of such preparative gels are shown in Fig. 1(b) and (c). The separated capsid proteins were shown to be free of other polypeptides by SDS–PAGE. Peptide mapping of p88, p83 and p78 by limited proteolysis using V-8 protease indicated that the three proteins had very similar peptide patterns (Fig. 2 and 3). A peptide with a mol. wt. of approx. 45K appeared to be unique to p88 (Fig. 2); this was more evident when lower amounts of enzyme were used (compare lanes 1 and 4). However, this 45K peptide could also be detected among peptides generated from p78 in experiments when a 10-fold range of enzyme concentration was used (Fig. 3, lane 7, arrow). Differences in the intensity of the 45K peptide band were most likely due to different stages of digestion being detected. A relatively large number of identical stable peptide bands, however, was evident (Fig. 2 and 3) and this supported the conclusion that the capsid polypeptides were similar.

Origin of p83 and p78

Results of peptide mapping of the 190S capsid polypeptides suggest that p78 and p83 may have originated from p88 by limited proteolysis during virus isolation, purification and/or storage after purification. In an attempt to confirm this, we investigated the effects of the age of fungal culture, the method of purification and the period of storage after purification on the relative proportions of the individual capsid polypeptides. Fungal cultures 4, 11 and 21 days old were tested as sources of virus. Variations in the purification method included (i) the omission of the chloroform emulsification step used for clarification of mycelial extracts and (ii) the use of polyethylene glycol (PEG) for concentrating clarified extracts because plant viruses purified using PEG have been reported to have a higher concentration of contaminating proteases than those purified using chloroform (Blevings & Stace-Smith, 1976). Purified or partially purified virus preparations were stored at 4 °C for periods ranging from a few days to several weeks prior to analysis by SDS–PAGE. Furthermore, clarified extracts were dialysed for 3 days at 4 °C prior to purification. In all samples tested, p88 was a major component and the SDS–PAGE polypeptide profiles were unaltered. Thus, neither the age of culture, the method of purification, nor the period of storage prior to or following purification influenced the ratio of p88 to p83/p78 (data not shown).

Translation of denatured dsRNA in vitro

Efficient denaturation of the dsRNA from 190S virus was obtained by treatment with methylmercuric hydroxide. When examined by agarose gel electrophoresis, denatured dsRNA migrated as a single species of ssRNA of mol. wt. $1.5 \times 10^6$ to $1.6 \times 10^6$ (Fig. 5), which is half the size of the virus dsRNA. Translation of denatured dsRNA in the rabbit reticulocyte lysate resulted in the synthesis of a single major polypeptide of mol. wt. 88K (Fig. 6). This was identical to the p88 capsid protein as determined by coelectrophoresis with p88 capsid protein by specific immunoprecipitation using an antiserum to the 190S virus (Fig. 6) and by the similarity of the products of partial proteolytic digestion of native p88 capsid protein and those of the 88K translation product (Fig. 7a). The positions of the three capsid proteins, visualized by Coomassie Blue staining, were marked with radioactive ink and compared with the radiolabelled translation products. No in vitro translation products comparable in size to p83 or p78 were detected (Fig. 7b). Minor bands observed in such autoradiographs probably represent premature termination of translation. No translation products were detected in the absence of exogenous mRNA or when undenatured dsRNA was added to the reticulocyte lysate system. No proteolysis of the 88K polypeptide occurred when the translation products were incubated for a further 3 h at 30 °C.
Fig. 2. Comparative peptide mapping of 190S virus capsid polypeptides using *S. aureus* V-8 protease and SDS–PAGE. Gel slices containing p88 and p83 were added to sample wells of a 15% polyacrylamide slab gel along with 1.0 μg (lanes 1 and 2) or 0.5 μg (lanes 3 and 4) of *S. aureus* V-8 protease. Following electrophoresis, the gel was stained with silver. Positions of migration of standard protein markers are indicated to the right. Positions of migration for undigested p88 and p83 and for V-8 protease are indicated to the left. Lanes 1 and 4, p88; lanes 2 and 3, p83.

Fig. 3. Comparative peptide mapping of p88 and p78 cut from SDS gels and re-electrophoresed with V-8 protease. Lane 1, protein standards with molecular weights indicated to the left; lanes 2 to 4, p88 digested with 0.1, 0.5 or 1.0 μg V-8 protease, respectively; lanes 5 to 7, p78 digested with 1.0, 0.5 or 0.1 μg V-8 protease, respectively; lanes 8 to 10, undigested p88, p78 and p83, respectively. Arrows indicate a peptide with mol. wt. approx. 45K.

Fig. 4. SDS–PAGE of peptides generated by NCS cleavage of 190S virus capsid polypeptides. Untreated p78, p83 and p88 (lanes 1 to 3, respectively); treated p78, p83 and p88 (lanes 4 to 6, respectively). Positions of molecular weight standards are indicated to the left.
Selective radioiodination of capsid polypeptides

To investigate the topological relationship between p88 and p83/p78, we surface labelled intact virions with $^{125}$I using Iodogen at a range of concentrations considered optimum for surface-specific iodination of viral proteins (Markwell & Fox, 1978). Following dissociation of labelled virions, the capsid polypeptides were analysed by SDS–PAGE. The results showed p88 to be more heavily labelled than p83 and p78 (Fig. 8, lanes 3 to 6). Selective labelling of p88 in
Fig. 7. (a) Peptide mapping of translation products of denatured 190S virus dsRNA. Gel bands containing translation products of molecular weight 88K were cut from dried gels and added to sample wells of a 15% polyacrylamide gel along with gel slices containing unlabelled p88 and 1.0 μg *S. aureus* V-8 protease. The positions of unlabelled peptides stained by Coomassie Blue were marked by radioactive ink; the labelled peptides were detected by autoradiography. (b) Comparison of *in vitro* translation products of denatured dsRNA and capsid polypeptides of 190S virus using SDS–PAGE. Lane 1, positions of capsid polypeptides p88, p83 and p78 (visualized by Coomassie Blue staining) were marked by radioactive ink; lane 2, labelled translation products of denatured dsRNA in the rabbit reticulocyte lysate; lane 3, positions of protein standards marked with radioactive ink and the molecular weights indicated to the right; lane 4, translation products of unfractionated brome mosaic virus RNA.

Intact virions was more pronounced at an Iodogen concentration of 3 μg than at 10 μg per reaction tube (Fig. 8, lanes 3 to 6). The distribution of label incorporated by the individual capsid polypeptides was also assessed by gamma-counting the radioactivity in bands excised from dried gels. The ratio of the amount of label in p88 to that in p78 ranged from 1:5 to 2:5. On the other hand, when samples of the same virus preparation were dissociated and then radiiodinated and analysed by SDS–PAGE, label distribution was strikingly different and showed equal labelling of p88 and p78 (Fig. 8, lanes 1 and 2). Similar results were obtained in three experiments and the data suggest that p88 is external in capsid arrangement to p83/p78.

Serological reactivity of capsid polypeptides

To test further the possibility that p88 is external in capsid arrangement, the reactivity of capsid polypeptides with an antiserum to 190S-G was compared to that with an antiserum raised against 190S-NG using Western blotting. The results shown in Fig. 9 (lane 2) indicated that p88 was more reactive with anti-190S-G than either p83 or p78 and, as a result, the Western blot profile was different from the Coomassie Blue staining pattern (lane 3). The capsid polypeptides reacted equally well to antiserum to 190S-NG and the Western blot and Coomassie Blue patterns were similar (lanes 1 and 3). The results of the immunological study thus supported the hypothesis that p88 is externally located on the surface of the virions and suggest that p88 is serologically distinguishable from p83/p78.
Fig. 8. SDS-PAGE of $^{125}$I-labelled capsid polypeptides. Dissociated (lanes 1 and 2) or intact (lanes 3 to 6) virus was labelled with $^{125}$I using Iodogen, then disrupted (in the case of the intact virus) and analysed using SDS-PAGE. The amount of Iodogen per reaction vessel and amounts of viral proteins (in parentheses) layered per well were: lane 1, 10 µg (20 µg); lane 2, 10 µg (10 µg); lane 3, 10 µg (20 µg); lane 4, 10 µg (10 µg); lane 5, 3 µg (20 µg) and lane 6, 3 µg (10 µg).

Fig. 9. Comparison of the reactivities of the 190S virus capsid polypeptides to antibodies to 190S-G and to 190S-NG using Western blots. Aliquots of the same virus sample were analysed by SDS-PAGE, transferred to nitrocellulose membranes and reacted with antibodies to 190S-NG (lane 1) or antibodies to 190S-G (lane 2). Lane 3 shows the Coomassie Brilliant Blue staining profile of an aliquot from the same virus sample which was not processed for Western bloting.

DISCUSSION

SDS-PAGE of purified 190S virus from *H. victoriae* revealed two major polypeptides, p88 and p83, occurring in equimolar amounts and a minor polypeptide, p78. Peptide mapping by partial proteolysis or by selective chemical cleavage at tryptophan residues indicated that p83 and p78 were closely related to p88. The origin of p83/p78 could not be explained by proteolysis during virus isolation and purification. The smaller polypeptides may be derived from p88 by proteolysis in *vivo* or, alternatively, p83 and/or p78 may represent modified forms of p88 (e.g. phosphorylated or adenylated derivatives). The finding that p83 and p78 could be resolved from each other on SDS gels using the discontinuous, but not the continuous, buffer system lent some support to this latter possibility. Evidence that modification of charge can affect electrophoretic mobility in the presence of SDS has been reported (Tung & Knight, 1971). Post-translational cleavage and/or modification of p88 could serve to extend and modulate its properties and functions. In addition to their structural roles, the capsid polypeptides may be involved in maintaining dsRNA in the proper conformation for replication or transcription (Buck, 1979) and in switching-on of transcriptase activity and the release of RNA transcripts. Furthermore, there is no direct evidence at present that p88, p83 or p78 do not themselves function as transcriptase/replicase.

The occurrence of two or more closely related capsid polypeptides appears to be a common feature of dsRNA mycoviruses with undivided genomes. The group III virus, designated 87-1-H, from *Gaewannomyces graminis* (Jamil & Buck, 1986) and the virus from the yeast *Yarrowia lipolytica* (El-Sherbeini et al., 1987) have recently been reported to possess two or more related capsid polypeptides of comparable size to those of the 190S virus. The larger of the two capsid polypeptides of *G. graminis* 87-1-H virus, unlike p88 of *H. victoriae* 190S virus, appears to convert to the smaller polypeptide when virus preparations are stored at 4 °C for 1 month or longer (Jamil & Buck, 1986).
The results of translation of the dsRNA from the virus 190S in vitro have shown that the principal product was a single polypeptide identical to the capsid polypeptide p88. No polypeptides comparable in size to p83 or p78 were detected. Our data differ somewhat from those of El-Sherbeini et al. (1987) who demonstrated that translation of denatured dsRNA of Y. lipolytica virus in vitro yielded four polypeptides, the two largest of which were identical to the two capsid proteins. The origin of this polypeptide heterogeneity, however, is not clear (El-Sherbeini et al., 1987).

Results of peptide mapping and translation in vitro indicate that the capsid of the 190S virus is encoded by a single gene and verify the earlier tentative classification of the virus as a member of the family Totiviridae (Buck et al., 1984; Brown, 1986).

Radioiodination and immunological studies have suggested that p88 is external to p83/p78 in capsid arrangement. The 190S virus particles may have double-shelled capsids with the inner core composed of p83/p78 and the outer capsid of p88. This model is similar to the basic structure of viruses in the Reoviridae (Joklik, 1983) and to the structure of the polyhedral virus particles from the basidiomycete Lentinus edodes (Ushiyama & Nakai, 1982). The L. edodes virus is a dsRNA mycovirus of comparable size to the 190S virus (Ushiyama et al., 1977). Combined ultrastructural and biochemical analyses are under way to verify this proposed structure for the 190S virus particles.

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