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Effect of a Virus on Accumulation of a Tissue-Specific Cell-Surface Protein of the Fungus *Cryphonectria (Endothia) parasitica*

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Hypovirulence and decreased sporulation of the plant pathogenic fungus *Cryphonectria (Endothia) parasitica* is caused by double-stranded (ds)RNAs. These symptoms of dsRNA infection are correlated with down-regulation of at least nine major fungal polypeptides. One of the regulated polypeptides was purified to homogeneity and antibody to it was prepared. This polypeptide (cryparin) has a -glycine-serine- repeating sequence near the amino-terminal end that is typical of structural proteins and has properties of a lectin. Antibody-staining showed that this 18.6-kDa polypeptide is specific to aerial hyphae and fruiting bodies and that it accumulates in large amounts on hyphal cell surfaces. The dsRNA affects accumulation of this protein, both in the fungal hyphae and in the growth medium. Cryparin is similar in physical properties to those of the putative phytotoxin cerato-ulmin produced by the Dutch elm disease fungus. Toxicity of cryparin is not detectable, but the striking similarities between the physical properties and locations of accumulation of cryparin and cerato-ulmin in fungal fruiting structures suggest either conservation of structure or convergent evolution in function of these two proteins.

Additional keyword: mycovirus.

Cryphonectria parasitica (Murrill) Barr is the fungal pathogen responsible for chestnut blight, the disease that devastated North American and European chestnut stands (Van Alfen 1988). Cytoplasmically transmissible, but non-infective dsRNAs affect virulence expression and sporulation by this fungus. These dsRNA-containing hypovirulent strains of the fungus are only weakly virulent toward their hosts and they sporulate more poorly than noninfected strains (Van Alfen 1988). The dsRNAs appear to be the genomes of fungal viruses (Rae *et al.* 1989; Shapira *et al.* 1991). Although apparently lacking capsids, an RNA polymerase has been found to be associated with them (Hansen *et al.* 1985). The genomic organization of two of the dsRNAs suggests that they are strains of a single virus (Hiremath *et al.* 1986; Tartaglia *et al.* 1986). These viruses are responsible for the biological control of chestnut blight in Europe and parts of North America (Griffin 1986).

All strains of the dsRNA studied to date cause similar hypovirulent and low sporulation symptoms in the fungus, but there may also be other dsRNA strain-specific symptoms exhibited by infected fungi, such as reduced pigmentation or slower growth (Elliston 1985). The effect of the dsRNAs at the molecular level is to down-regulate specific fungal poly(A)$^+$ RNAs and polypeptides (Powell and Van Alfen 1987a, 1987b). Such regulation of specific host gene products by dsRNA viruses is unique. Most fungal viruses have no detectable effects on their hosts (Buck 1986). The report of Rogers *et al.* (1987) on the dsRNA-associated effects on mitochondrial cytochrome oxidase aa_3 in *Ophiostoma ulmi* (Buisman) Nannf., however, is another example of a specific effect of dsRNA in fungi.

As part of our efforts to understand the molecular mechanisms of dsRNA symptom induction in this fungus, we have characterized one of the fungal polypeptides regulated by the virus. Isogeneic strains of *C. parasitica* differing only in the presence or absence of dsRNA, were used to compare polypeptide accumulation (Powell and Van Alfen 1987a, 1987b). We describe here a fungal protein that is regulated by the dsRNA. The NH_2-terminal end of this cell-surface protein is high in glycine, and it has properties of a lectin. The protein is found in aerial hyphae and fruiting bodies. The function of the protein is not known, although its properties suggest it is a structural protein associated with fungal development.

MATERIALS AND METHODS

Strains and culture conditions. *C. parasitica* strains used and general culture conditions were described by Powell and Van Alfen (1987a). Strains EP155/2 and UEPI are isogeneic except for the presence of dsRNA in UEPI. Nuclear markers, mtDNA, restriction fragment length polymorphisms, and searches for cryptic cytoplasmic genes were used to confirm the isogeneic nature of the two strains (Gobbi *et al.* 1990). Liquid cultures of the fungus were grown in a medium containing potassium L-aspartate as the carbon source (Hansen *et al.* 1985). The cultures were harvested 4–6 days after reaching stationary phase of growth. Under our conditions (25 C, 100 rpm, shake culture) stationary phase is reached after 4 days. Liquid cultures were inoculated as previously described (Hansen *et al.* 1985). Time course studies were done using uniformly inoculated individual culture flasks for each sampling time.

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Isolation of cryparin. The fungus grown in liquid culture was collected by filtration through cheesecloth, frozen, and lyophilized. Lyophilized fungus was homogenized in a blender for 30 sec with 2 ml of 60% ethanol per 100 mg of fungus. The homogenate was left at room temperature for 30 min and then centrifuged at 5,000 × *g* for 15 min. The supernatant was dialyzed against distilled water at 4 C. The dialysate was centrifuged at 10,000 × *g* for 15 min and then filtered through a 0.22-μm Millipore filter. The filtrate was lyophilized and then dissolved in 60% ethanol (10 mg/ml) and 0.8 ml was applied to a reverse-phase C4 high-performance liquid chromatography (HPLC) column. Detection was by UV absorption at 280 nm. A linear gradient of from 0 to 80% acetonitrile in 0.1% aqueous TFA was used. The peak containing cryparin was collected. The HPLC-purified cryparin was lyophilized and stored at −20 C until used for composition studies and antibody formation.

Antibody formation. Polyclonal antibody was prepared in rabbits against HPLC-purified cryparin. The immunization schedule of the rabbits followed that used in previous studies (Carpenter *et al.* 1987). Antibodies were isolated from serum using a column of Baker-bond ABx (J. T. Baker, Inc., Phillipsburg, NJ) following the procedure supplied by the manufacturer. The serum IgG was eluted from the column using 500 mM KH_2PO_4, pH 6.8.

Composition studies. Amino acid analysis was carried out on a Beckman 119CL analyzer using a citrate buffer system supplied by the manufacturer. The analysis was done at the Medical Center, University of Utah, Salt Lake City. The peptide sequencing was done by W. R. Gray, Department of Biology, University of Utah, Salt Lake City, using a Beckman model 890 sequencer. Carbohydrate analysis was by Schiff's base or dansyl hydrazine staining of gels (Eckhardt *et al.* 1976), and by anthrone reagent (Spiro 1966).

Hemagglutination assay. Serial dilutions of cryparin were added to red blood cell suspensions. A final concentration of 1.0% red blood cells in PBS-BSA (50 mM K_2HPO_4, pH 7.2, 0.1 M NaCl, 1 mM NaAzide, 0.1% bovine serum albumin) was used. The tubes containing the mixtures were mixed vigorously and allowed to stand for 15 min at 25 C. The tubes were mixed again and then 100 μl each was added to a well of a V-bottom microtiter plate. The plates were incubated 2 hr at room temperature before reading. Agglutination inhibitors, when assayed, did not change the final concentrations of red blood cells or cryparin. Crude cell wall preparations were made by repeated homogenization of the fungus with 60% ethanol and removal of the ethanol by centrifugation. To test the effects of the cell wall preparation on the hemagglutination activity of cryparin, 250 mg wet weight of the crude cell wall preparation was combined with 1 ml of PBS-BSA containing 1 mg of cryparin. The mixture stood for 15 min at 25 C and was then centrifuged at 5,000 × *g* for 15 min. The supernatant was used in the assay as cryparin, as described above.

Electrolyte leakage assay. Two pieces of apple fruit (1 × 23 mm each) were placed in a beaker containing 0, 2, 4, 6, 10, 15, or 20 μg/ml of cryparin in water. The volume of each was 50 ml. The solutions were shaken for 2.5 hr at room temperature then washed three times in 25 ml of water over a 10-min period. The apple tissue was then shaken for 3 hr in water before conductivity of this last wash water was determined with a conductivity meter (Damann *et al.* 1974).

Histochemistry. Tissue samples, including agar substrate, were cut from colonies and fixed for 12 hr in 3% glutaraldehyde in pH 6.8, 0.2 M phosphate buffer at 5° C. The tissue was rinsed in buffer and covered with 50% glycol methylacrylate monomer at 5° C. Monomer concentration was increased dropwise to 95% and infiltration occurred overnight. Polymerization of monomer was in polyethylene trays sealed with Parafilm on crushed ice. Blocks were stored at 5° C until 3-μm sections were cut with glass knives. Sections for structural observation were stained for 10 sec in 0.05% toluidine blue. Antibodies prepared against cryparin were used for fluorescein isothiocyanate (FITC)-immunofluorescent staining at a concentration of 80 μg/ml (Carpenter *et al.* 1987). Preimmune serum did not react with antigens of *C. parasitica*.

RESULTS

Purification and characterization. Cryparin was extracted from lyophilized fungal tissue on the basis of its

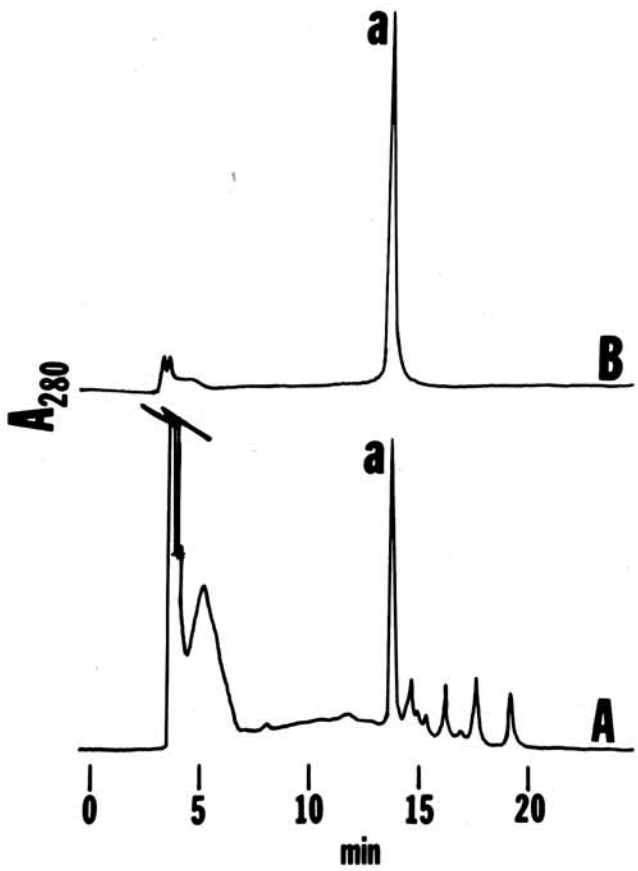

Fig. 1. High-performance liquid chromatography (HPLC) chromatograms. **A,** HPLC chromatogram of cryparin (peak a) and contaminating compounds. Lyophilized cryparin was dissolved in 60% ethanol (10 mg/ml) and and 8 ml was applied to a reverse-phase C4 HPLC column. Detection was by UV absorption at 280 nm. A linear gradient of from 0 to 80% acetonitrile in 0.1% aqueous TFA was used. **B,** Cryparin (peak a) collected from the HPLC purification procedure described in A was reapplied to the column and eluted again as described.

hydrophobic properties. The major detectable contaminating molecules, after 60% ethanol extraction, were pigments. The HPLC purification eliminated the pigments. Figure 1 illustrates the single HPLC peak that resulted from reinjection of the HPLC fraction containing cryparin. The HPLC-purified cryparin fraction contained a single protein band as detected by SDS-PAGE.

Cryparin was also detected in culture filtrates of the fungus. An enrichment for cryparin in culture filtrates could be made by causing the filtrate to foam, either by shaking vigorously or by shaking the filtrate under reduced air pressure. When the foam was collected by aspiration, similar to the procedure reported for the phytotoxin cerato-ulmin (Richards and Takai 1973), a single protein was detected by SDS-PAGE having the same mobility as cryparin. Microscopic examination of the air bubbles resulting from shaking of aqueous cryparin showed structures similar to those reported for cerato-ulmin (Russo *et al.* 1982). In the case of cerato-ulmin, these structures were "microscopic air bubbles, stabilized by a semi-flexible skin of cerato-ulmin" (Russo *et al.* 1982). The microstructures from *C. parasitica* did not form in ethanol solutions greater than 40% or in 0.1% Triton X-100, which is further evidence of the similarity of properties of cryparin and cerato-ulmin (Russo *et al.* 1982).

The amino acid analysis of cryparin is presented in Table 1. The protein is rich in glycine and serine, with approximately 19% of the amino acids being glycine and 15% serine. The molecular mass estimated by amino acid analysis is a multiple of 18.6 kDa. By SDS-PAGE, the molecular mass estimates ranged from 23.5 kDa on 15% acrylamide gels to 30.4 kDa on 10% gels. Such anomalous behavior on gels is typical of glycopeptides. Carbohydrate, however, was not detected when gels were stained with Schiff's base or dansyl hydrazine (Eckhardt *et al.* 1976), nor were detectable amounts of carbohydrate found by colorimetric assay of purified cryparin using the anthrone reagent (Spiro 1966). Amino acid analysis supported experimental evidence of cryparin's hydrophobicity. It has a polarity index (Capaldi and Vanderkooi 1972) of 38.8, a typical value for hydrophobic proteins.

Table 1. Amino acid analysis of cryparin

Residue	nmole	nmole Ratio[a]	Number of residues
Ala	2.80	1.00	16
Arg	0.37	0.13	2
Asx	1.82	0.65	10
Cys	3.03	1.08	17
Glx	2.77	0.99	16
Gly	6.42	2.29	37
Ile	0.66	0.24	4
Leu	2.99	1.07	17
Lys	0.45	0.16	3
Phe	0.37	0.13	2
Pro	1.70	0.61	10
Ser	4.87	1.74	28
Thr	3.46	1.24	20
Tyr	0.69	0.25	4
Val	1.47	0.53	8
Assuming 16 Ala residues, Total mol wt		18,617	

[a] Ratio of nmoles to those of Ala.

The amino terminal sequence of cryparin is presented in Figure 2. The glycine- and serine-rich nature of the polypeptide is clearly shown in the sequence, with the first 23 amino acids being either glycine or serine. Serine occurs in this portion of the protein primarily in the repeating sequence -gly-ser-.

Liquid culture production of cryparin. Liquid cultures of the fungus reached stationary growth phase 4 days after inoculation (Fig. 3A). Cryparin began to accumulate in large amounts in strain EP155/2 (which lacks dsRNA) about 3–4 days after inoculation, after which the amount detectable in mycelium by gel electrophoresis decreased (Fig. 3B). In culture medium, cryparin was detectable in large amounts at day 3, then decreased until after day 6, when the amount in the culture fluid increased again (Fig. 3C). The amount of cryparin in the dsRNA-containing strain UEP1 was significantly less than that produced by EP155/2.

Histochemical staining of cryparin. Antibody against cryparin was used for histochemical staining of this polypeptide. The specificity of the antibody to cryparin was determined by western blots (Fig. 4). Preimmune serum did not bind to any proteins of the mycelium. Sections of fungal colonies growing on agar were examined by using FITC-bound antibody specific to cryparin to determine where the polypeptide accumulates. As illustrated in Figure 5, *C. parasitica* formed morphologically distinct hyphal types and aggregations of hyphae on agar culture. Large-diameter, sparse hyphae penetrated the agar, but an interwoven hyphal mat, with mostly vertically oriented hyphae, was formed at the agar surface. Narrower, more cytoplasmically dense aerial hyphae that locally differentiate into pycnidia (asexual spore-containing bodies) formed above the mat. Cryparin accumulated only in the uppermost aerial hyphae and pycnidia. Aerial hyphae form closely behind the growing front of the colony and cryparin was detected on these aerial hyphae located near the growing edge of the colony. In naturally infected bark of American chestnut, cryparin was only detected on fruiting bodies (Fig. 5H). No cryparin was detected in mycelial fans, fungal structures that are important in the penetration of host tissue by the fungus (Hebard *et al.* 1984).

Cryparin observed in sectioned hyphal tissue was always associated with cell surfaces (Fig. 5G). Cryparin coated the surfaces of cell walls of aerial hyphae and appeared to be present in the cell walls. The ease by which cryparin

Fig. 2. N-terminal amino acid sequence of cryparin.

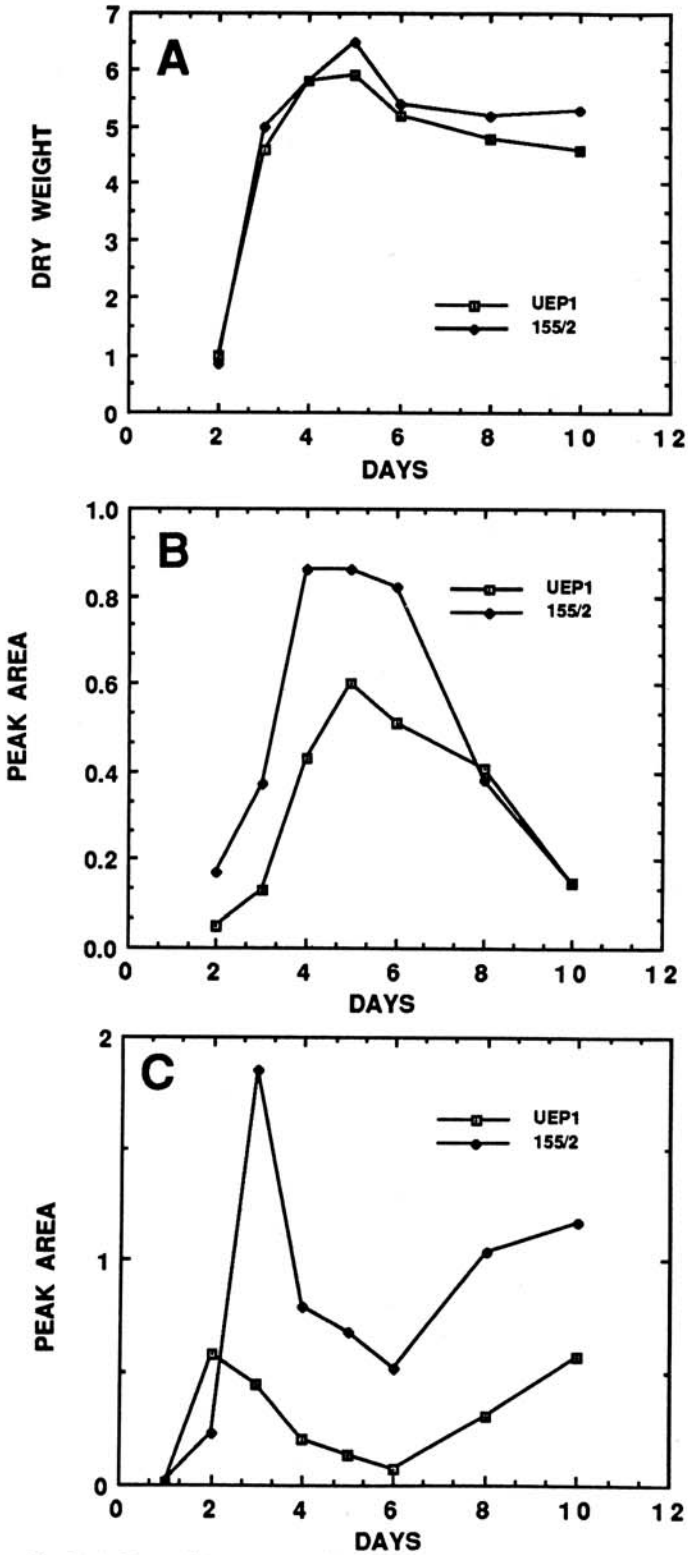

Fig. 3. A, Dry weight accumulation as a function of time in liquid culture of mycelium of a virulent (EP155/2) and isogenic hypovirulent strain (UEP1) of *Cryphonectria parasitica.* **B,** Accumulation of cryparin with time in the mycelium of EP155/2 and UEP1. Cryparin was extracted with 60% ethanol, as described in Materials and Methods. PAGE gels were stained with Coomassie blue and the cryparin bands scanned with a densitometer to obtain peak areas for each time-point. **C,** Accumulation of cryparin in culture media of EP155/2 and UEP1 as a function of time. The methods for extracting and quantifying cryparin were described in B.

could be extracted from only slightly homogenized hyphae and its presence in the culture fluid also suggested that cryparin was located on the cell surface.

Biological properties of cryparin. We assayed the purified polypeptide to determine its ability to agglutinate red blood cells. Cryparin, at concentrations greater than 4 μg/ml, agglutinated 1% suspensions of red blood cells of rabbit, slightly agglutinated those of rat and chicken, but not those of ox, horse, human A, or guinea pig. This selective agglutination is typical of lectins (Lis and Sharon 1986). We have not yet identified a specific sugar or sugar linkage that inhibits the agglutination. The following sugars were tested at 200 mM concentrations for inhibition of agglutination: galactose, galactosamine, lactose, glucose, arabinose, mannose, xylose, fucose, *N*-acetylglucosamine. When crude cell wall preparations of the fungus, from which cryparin had been extracted, were added, agglutination was inhibited.

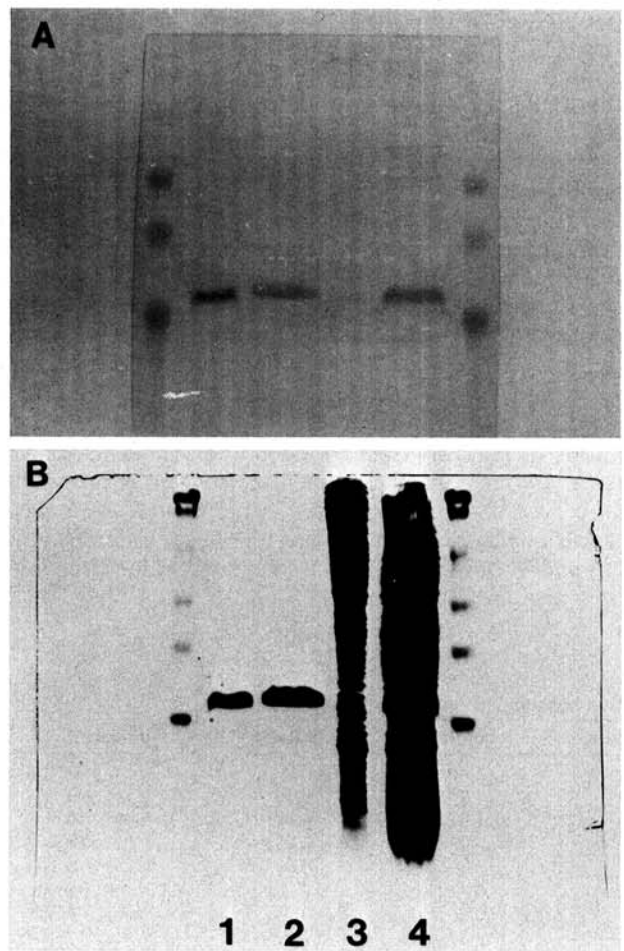

Fig. 4. A, Western blot, using antibodies (2 μg/ml) to cryparin, of a 10% SDS-PAGE gel (shown in B). Lane 1 is 60 μg of high-performance liquid chromatography-purified cryparin. Lane 2 is cryparin that was ethanol-extracted from lyophilized fungus. Lane 3 is residue remaining after ethanol extraction of cryparin from lyophilized fungus. Lane 4 is from lyophilized fungus before extraction of cryparin. The gel was stained with Coomassie blue and then a contact print of the gel made onto photographic paper. The outer lanes are molecular weight standards (21.5, 31.0, 45.0, 66.2, and 92.5 KDa). The standards were stained with specific antibodies on the Western blot (A).

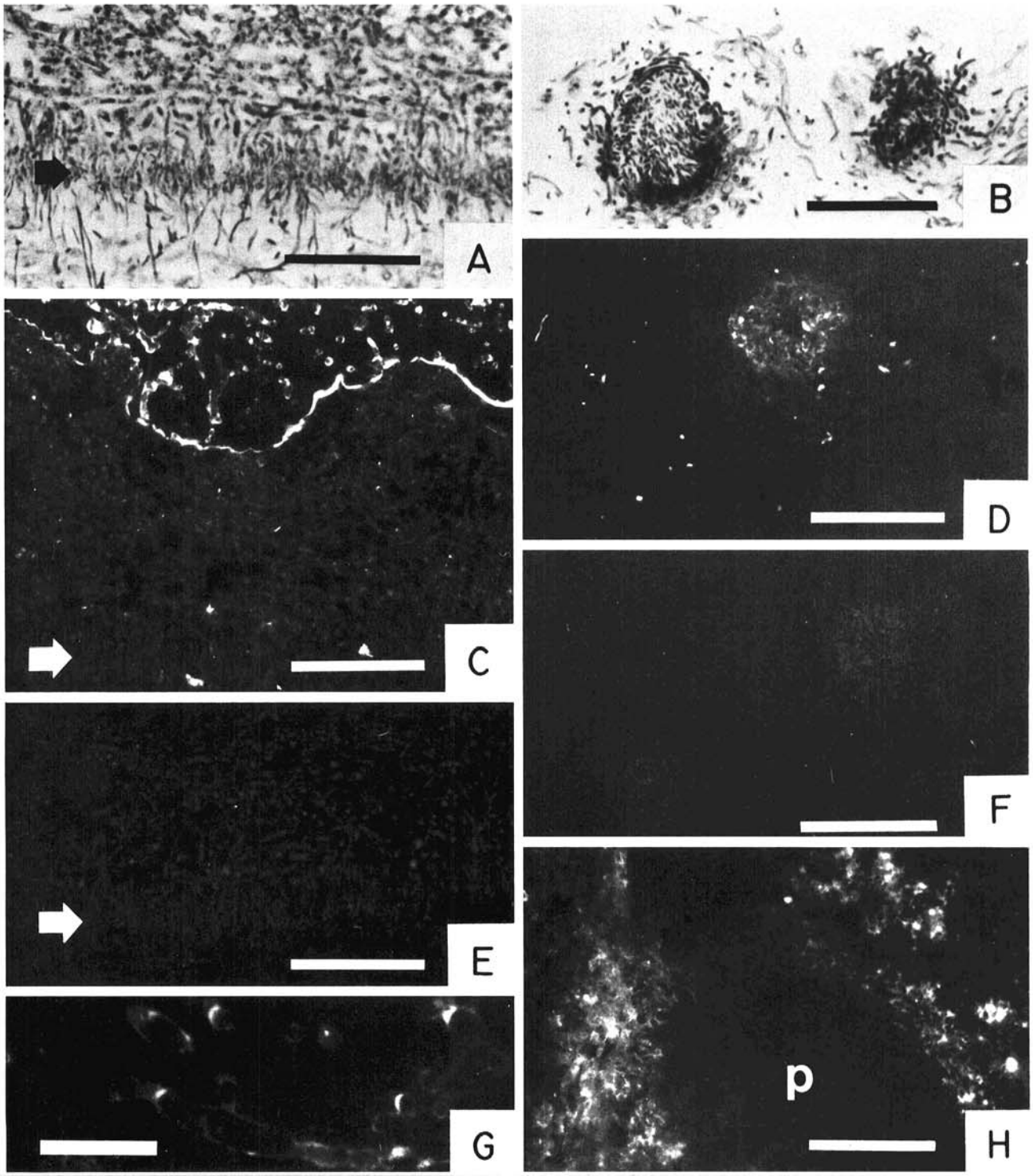

Fig. 5. Histological and immunofluorescent staining of hyphae and fruiting bodies of *Cryphonectria parasitica* from a 6-day-old agar culture (A–G) or naturally infected chestnut trees (H). **A, B,** Toluidine blue stained cross section near center of 9-cm-diameter colony. A, colony at agar boundry and, B, pycnidia near top of aerial hyphae. Arrows at A, C, and E at agar surface. **C,** Immunofluorescent detection of cryparin in aerial hyphae. **D,** Immunfluorescent detection of cryparin in pycnidium. **E,** Immunfluorescent staining control near section in C and **F** control near section in D. **G,** Immunfluorescent staining of aerial hyphae showing localized cell-wall and extracellular accumulation. **H,** Immunofluorescent staining of putative perithecial wall and perithecium (p) from naturally infected American chestnut tree. The bars represent 50 μm in A–F, and H, and 10 μm in G. Antibodies used in the straining procedure are those described in legend to Figure 4. The treatments (C, D, and G) and the controls (E and F) were from closely adjacent serial sections from the same block of tissue.

The similarity of the properties of cryparin and the phytotoxin cerato-ulmin prompted us to test the phytotoxicity of cryparin. Apple fruit tissue was used for the assay because apple fruits are routinely used to distinguish virulent from hypovirulent strains of *C. parasitica* (Fulbright 1984). An electrolyte leakage assay (Damann *et al.* 1974) was used to assess the effects of concentrations of up to 20 μg/ml of cryparin on cell membrane functions. Cryparin had no significant effects on electrolyte leakage from the apple tissue compared with water controls.

DISCUSSION

A number of lectins have been described that are present on cell surfaces of eukaryotic microbes or are secreted into the growth environment. The best-characterized microbial lectins, discoidin I and II produced by *Dictyostelium discoideum* Raper, are typical of these soluble lectins (Barondes 1984). Cryparin appears to be a similar molecule because it is developmentally regulated and accumulates both on the cell surface and within the growth medium (Figs. 3 and 5). Although cryparin has the ability to agglutinate specific red blood cell types, and in this respect can be considered a lectin, we have not yet identified the specific sugars or linkages that are recognized by this molecule. The red blood cell agglutination by cryparin, however, can be inhibited by a crude preparation of the fungal cell wall, providing evidence of lectinlike properties.

The amino-terminal sequence of cryparin has a -glycine-serine- repeating structure that is similar to the -glycine-X- repeating structure of the protein found in spider's web. Likewise, the glycine-rich nature of cryparin is typical of plant cell-wall proteins that have recently been described (Condit and Meagher 1986; Keller *et al.* 1989). These characteristics of the amino-terminal end of cryparin suggest that it has a structural role in the cell wall, which together with its ability to agglutinate some insoluble component of the cell, suggests that the molecule may be involved in cell-cell adhesion.

In liquid cultures of the fungus, cryparin is produced by both the dsRNA-infected and noninfected strain but the amount of cryparin produced by the dsRNA-infected strain is much less than that from the virulent strains. On agar media, cryparin was produced only in aerial hyphae. The aerial hyphae were one of three hyphal types identified in sections of the fungal colony: the feeding hyphae that penetrated the agar, a dense mat of hyphae on the surface of the agar, and the aerial hyphae found above this mat. The pycnidia originated primarily in the aerial hyphae. Aerial hyphae are formed just behind the margin of the colony, and cryparin was detected in these newly formed aerial hyphae. Although these histochemical studies are preliminary, they did show that cryparin was most abundant, both in culture and in infected trees, in the fruiting bodies of the fungus (Fig. 5).

The question of whether cryparin is involved in virulence expression of *C. parasitica* has not been resolved by these studies. Based only on the differential expression of this protein in virulent and hypovirulent strains, it would be unwise to suggest that cryparin could be involved in virulence. The similarity in physical properties between cryparin and the putative phytotoxin cerato-ulmin, however, requires that the role of cryparin in virulence expression by the fungus be carefully considered. The electrolyte leakage assay indicated that cryparin is not generally toxic to membranes. Because apple fruits can distinguish virulent from hypovirulent strains of *C. parasitica*, use of apple fruits in this assay was appropriate (Fulbright 1984). Wilt assays, as used for many of the studies with cerato-ulmin, are not appropriate for reasons described in previous studies from this laboratory (Van Alfen and McMillan 1982). The question of whether cerato-ulmin is important in virulence of *O. ulmi* has been addressed primarily by correlation between toxin production and pathogenicity of the fungus (Takai 1980). That the question is still debated clearly shows that correlative approaches to addressing the question are not satisfactory.

Although the roles for cryparin and cerato-ulmin in the biology of the fungi producing these proteins are not known, the similarities of the two proteins in physical properties and their accumulation on cell-surfaces of sporulation structures (Takai and Hiratsuka 1980) suggests that convergent evolution of function may have occurred. The N-terminal sequences of the two proteins are different (Stevenson *et al.* 1979) so there is no evidence that they are conserved in sequence. Cell surface lectins have been reported to be present in a number of different filamentous fungi, but their functions are still a matter of speculation (Chabasse *et al.* 1988; Kellens *et al.* 1989; Ishikawa and Oishi 1989). Expression of genes encoding a number of small hydrophobic proteins has also been found to be associated with fruiting bodies of the basidomycete *Schizophyllum commune* Fr.:Fr. (Schuren and Wessels 1990). The evolution of abundantly expressed small hydrophobic proteins associated with fungal fruiting bodies in a number of different fungi suggests a similar function in each case. Future studies are needed to evaluate the role of these molecules in the biology of the fungus. The most direct approach to understanding the role of these proteins in the biology of the different fungi will come from site-specific mutagenesis experiments (Van Alfen 1989).

The down-regulation of specific major fungal polypeptides by the dsRNAs that cause hypovirulence of *C. parasitica* (Powell and Van Alfen 1987b) suggests that the viruses are affecting normal regulatory functions of their fungal host. The characterization of this viral-regulated polypeptide is an important step in our studies of how fungal gene expression is affected by the viruses responsible for hypovirulence.

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