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Genetically Related Protein Variants Specifically Associated with Fruiting Body Maturation in *Neurospora*

(development/perithecium/electrophoresis)

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ABSTRACT **Electrophoretic analysis of fruiting body extracts from *Neurospora* reveals a characteristic protein, apparently absent in vegetative structures and ascospores, and which increases markedly in relative concentration after fertilization. Different wild-type species and strains studied have electrophoretic variants of this protein, two of which are shown to be controlled by members of an allelic pair.**

Long known as a favorable object for biochemical genetic research, *Neurospora* has been utilized increasingly in attempts to gain insight into development by a consideration of biochemical and genetic events in relation to morphogenesis. Mutations that cause morphological variations have been shown to give rise to concomitant changes in protein composition (1), and in certain instances, to particular enzymatic alteration, e.g., of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (2). Studies of other kinds have correlated the regulation of particular areas of metabolism with particular aspects of differentiation, as in the genesis of macroconidia, which is associated with a sharp reduction in the rate of glycolysis, as compared to that in the vegetative mycelium, and with a channeling of cellular metabolism into new pathways (3). And in a comparison of wild-type and aconidial strains, the process of conidiation has been correlated with an increase in the activity of specific enzymes (4). Thus far, however, one knows few if any instances in *Neurospora* where a molecule under strict genetic control is phase-specific for a particular aspect of development. Such instances would be most useful in determining whether biochemical changes associated with development were mere correlates or had a more profound cause-and-effect relationship. In this report, a study of several naturally occurring genetic variants of *Neurospora* implicates a possibly phase-specific protein in the sequence of events involved in the differentiation of fruiting bodies.

MATERIALS AND METHODS

Strains of Neurospora. The strains used in these studies are all wild types. Some of them are designated by their geographic origin rather than by a species designation. These strains are: the standard laboratory St. Lawrence strains of *N. crassa* 74*A* and 77*a;* Costa Rica 205-2*A* and 205-5*a*, obtained from S. R. Freiberg; Philippine Islands 3 (P.I.-3-3*A* and P.I.-3-5*a*), collected by F. T. Orillo and H. L. Everett; *N. sitophila* 540-34*A* and 2*a*, obtained from J. R. S. Fincham; Honduras 3*A* and 1*a* from R. H. Stover and S. R. Freiberg; a homokaryotic strain of *N. tetrasperma* from Borneo, originally isolated as strain T-220, provided by J. H. Warcup; *N. tetrasperma* strains 1270 (85*A*) and 1271 (85*a*), derived from an isolate by B. O. Dodge and obtained from the Fungal Genetics Stock Center; and *N. terricola*, a true homothallic, from S. E. Gochenaur.

Culture and Harvesting Techniques. Maintenance of stocks and of cultures for biochemical work with vegetative mycelia and conidia was at 25° on the minimal medium of Beadle and Tatum (5), supplemented with 2% agar where solid medium was required.

Crosses were made at 25° on Difco Cornmeal agar or on the crossing medium of Westergaard and Mitchell (6), adjusted to pH 5.7. The latter medium was solidified with 2% agar for crosses on slants or in petri dishes. For crosses in liquid medium, 250-ml Erlenmeyer flasks were supplied with 100 ml of liquid Westergaard–Mitchell medium to which dialysis parchment wicks were added before autoclaving. Appropriate material was harvested from *Neurospora* growing on the wicks.

Preparation and Treatment of Protein Extracts. For biochemical analysis, protoperithecial mycelia were harvested and extracted in 0.1 M sodium phosphate buffer (pH 7.0) by grinding with a mortar and pestle. Perithecia were treated in the same way after separation from the surrounding mycelium and repeated washing in buffer. Vegetative mycelia grown in liquid to prevent conidiation, filtered conidia, and ascospores were extracted in the same manner but with the addition of washed sea sand to facilitate grinding. The ground extracts were centrifuged at 14,000 rpm (20,000 × *g*) for 1 hr in an I.E.C. model B-20 centrifuge, Type 870 rotor.

Separation of the acidic proteins (i.e., those that migrate to the anode under the conditions used) by polyacrylamide gel electrophoresis was carried out according to the method of Davis (7), on 7.5% (w/v) acrylamide gels. After electrophoresis the gels were stained for proteins with Coomassie Brilliant Blue (8). When protein quantitation was needed, gels were stained with Fast Green, scanned at 625 nm with a Gilford densitometer, and relative amounts of protein were estimated as described by Gorovsky *et al.* (9). Protein determination was done by the Lowry *et al.* method (10). Molecular weight was determined by the sodium dodecyl sulfate–gel method of Shapiro *et al.* (11).

RESULTS

Polyacrylamide gel electrophoresis of perithecial extracts revealed a deeply staining, concentrated proteinaceous band,

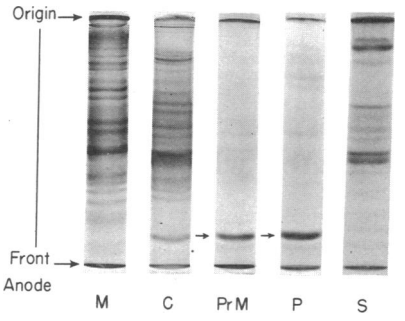

FIG. 1. Electrophoretic separation of the soluble proteins of *N. tetrasperma* (Dodge). *M*, mycelium; *C*, conidia; *PrM*, protoperithecial mycelium; *P*, perithecia; *S*, ascospores. The *arrow* indicates the perithecial protein. The fast migrating protein in the conidial extract does not react with antibodies raised against the perithecial protein and is therefore presumably not homologous with the perithecial protein that migrates to essentially the same position.

as shown in Fig. 1 for *N. tetrasperma* (Dodge). Perithecial extracts of all *Neurospora* strains tested showed the presence of such a major protein, but under identical conditions on the gel, its relative electrophoretic mobility varied with the strain. The protein from *N. tetrasperma* (Dodge), *N. sitophila*, Costa Rica, and Philippine Islands showed the same mobility as that of *N. crassa*, while the *N. tetrasperma* (Borneo), *N. terricola*, and Honduras proteins differed both from that of *N. crassa* and from one another, as shown by electrophoresis of mixed extracts. Fig. 1 also shows that the protein appears to be primarily associated with developmental events occurring in sexual reproduction in *Neurospora*, inasmuch as it is characteristic of fruiting bodies, both fertilized and unfertilized, while electrophoresis of extracts of the vegetative mycelium, conidia, and ascospores does not reveal its presence. Attempts to extract the protein from the vegetative mycelium after sonication, freeze–thawing, or Triton-X or urea treatment have not proven fruitful.

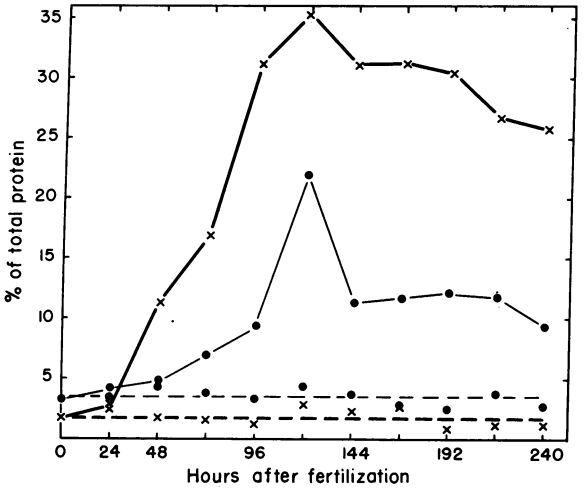

FIG. 2. Relative concentration of the major perithecial protein in fruiting bodies collected at intervals after fertilization (0 hr). The *solid line curves* represent values for fertilized perithecia, and the *broken line curves* values for unfertilized protoperithecia. Essentially similar curves were obtained when the protein concentration was expressed on a "per perithecium" basis. ×, *N. crassa*; ●, *N. tetrasperma*.

The relative concentration of the perithecial protein, expressed as percent of total soluble acidic protein measurable on the gel (9), was followed over the course of time in both pre- and post-fertilization fruiting bodies. The results, shown in Fig. 2 for *N. crassa*, reveal that a rapid increase in the relative concentration of the protein follows fertilization (indicated by 0 hr), with a maximum being reached at 120 hr. A similar post-fertilization increase is shown for *N. tetrasperma* (Dodge), even though in this species the relative concentration of the protein in unfertilized fruiting bodies appears to be initially higher than in *N. crassa*. The rapid increase after fertilization is followed by a slow decrease in relative concentration, never, however, leading to a complete disappearance of the protein, which persists in perithecia after spore discharge even as they remain in old cultures. The relative concentration of the protein followed over the same course of time in unfertilized protoperithecia is shown to remain essentially constant at the low prefertilization level.

As mentioned earlier, several intra- and interspecific differences in the migration of the perithecial protein have been observed. Such an electrophoretic difference is shown for Honduras and Philippine Island strains (P.I.) in Fig. 3-a. That the difference is real is demonstrated by the electrophoretic pattern of a mixture of perithecial extracts from the two strains.

The difference between the major perithecial proteins of P.I. and Honduras has been exploited to study the mode of inheritance of these proteins. Honduras 3*a* was crossed to P.I. 3-3*A*. No whole asci were produced, but progeny cultures were established from randomly isolated ascospores. For these cultures, and in subsequent steps in the genetic analysis, the electrophoretic mobility of the perithecial protein was determined in extracts obtained from perithecia from crosses in which the culture to be tested was used as protoperithecial parent and P.I. as conidial (male) parent. Among the progeny from the cross P.I. x Honduras, cultures that exhibited a Honduras-type protein were backcrossed to P.I. In the backcross, fertility was sufficient that tetrad analysis was possible. A 1:1 segregation of the Honduras-type protein and the P.I.-type protein was observed (Fig. 3-b). The variant proteins in Honduras and Philippine Island strains appear, therefore, to be determined by members of an allelic pair. The

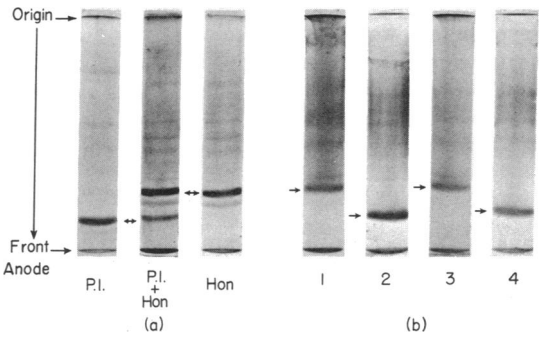

FIG. 3. Inheritance of two electrophoretic variants of the perithecial protein. (*a*) Electrophoretic separation of the perithecial soluble proteins of the parental strains: Philippine Islands (*P.I.*) and a culture exhibiting the Honduras-type protein (*Hon*). *P.I.* + *Hon* represents a mixture of the two perithecial extracts. (*b*) Electrophoretic separation of the perithecial soluble proteins of cultures derived from a tetrad obtained from a cross of the two parental strains in (*a*).

determinants of the variant proteins segregate from the mating type alleles, *A* and *a*, and are not allelic with them.

After elution of the protein band from gel slices, the sodium dodecyl sulfate–gel method of Shapiro *et al.* (11) was used to determine an approximate molecular weight of 20,000 for the perithecial protein.

DISCUSSION

The electrophoretic patterns of perithecial extracts show that a major perithecial protein is characteristic of the fruiting bodies of all of a significant number and variety of *Neurospora* species and strains tested. The major protein is not identical for all species and strains, as evidenced by the different mobilities on polyacrylamide gels, but its variant forms are apparently homologous in structure and function. Such homology is supported by two lines of evidence. Firstly, the observed single-gene control of at least two electrophoretically different proteins demonstrates molecular structural homology; additional genetic experiments will be required to establish whether all of the variant proteins are allelically determined, as has already been shown for the Honduras and Philippine Islands proteins. Secondly, the similar rapid increase in relative concentration of the protein after fertilization, as studied closely both in *N. crassa* and in *N. tetrasperma*, suggests functional homology. This increase in the protein, up to a level at which it constitutes a substantial fraction of the total soluble proteins of perithecia, indicates a major structural function—as yet unidentified—in the active developmental events that occur after fertilization. The nature of the rapid increase is also unresolved, but pulse-labeling experiments should allow an answer as to whether fertilization triggers *de novo* synthesis of this protein or simply its release from a previously bound form. As indicated before, although a membrane-bound form of the protein is possibly present in vegetative mycelia it has not been demonstrable by the chemical and mechanical treatments used. On the other hand, the decrease in relative concentration of the protein after 120 hr has been correlated with the formation of exudate at the perithecial ostioles before spore discharge. Polyacrylamide gel electrophoresis of the exudate has revealed the presence of the major protein that is characteristic of perithecial extracts.

Localization of the protein, either in one of the cell types or in the noncellular matrix within the perithecium, may provide clues to the function of the protein and to an explanation of its initial increase and later its decrease in relative concentration. Mutants lacking this protein, or abnormal in its behavior, will be sought. In any case, the perithecial protein under consideration appears to represent an instance of a macromolecule that is phase-specific in reference to a morphogenetic sequence. Its appearance in perithecia and disappearance in the vegetative mycelia may reflect a gene being turned on and off as a matter of control of developmental processes.

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