Clipboard

Het up mould unleashes a sporekiller prion

Those who have not heard of *Podospora anserina* are missing out on the fascinating story of the prion encoded by its *het-s* gene. *P. anserina* is a haploid coprophilous fungus obtainable from the dung of herbivores such as deer and horse. It is closely related to the better studied (and arguably prettier) *Neurospora crassa*. Sexual crosses in Podospora involve the fusion of two nuclei that differ in mating type (+ and –). Karyogamy is followed by meiosis and a post-meiotic mitosis which generate eight haploid nuclei that are partitioned among the four ascospores that are produced per ascus. The partitioning of nuclei (two per ascospore) ensures that each ascospore receives both alleles of any marker that has undergone a crossover with its centromere. An obligatory crossover occurs between the mating type locus and the centromere of linkage group I, therefore each ascospore receives one + and one – nucleus. Since the resulting cultures are heterokaryotic for mating type they can be self-fertile. About 1–2% of asci have five spores, with two small initially uninucleate ascospores occupying the space that would otherwise have held a single binucleate ascospore. When a small ascospore germinates it produces a homokaryotic culture, i.e. a culture in which all nuclei are identical. All cultures, regardless of whether they are homokaryotic or heterokaryotic, can form both the female and male reproductive structures (respectively, the ascogonia and the microconidia). Fertilization occurs when an ascogonial cell fuses with a microconidium of the opposite mating type. Homokaryotic cultures are self-sterile because they can mate only with cultures of the opposite mating type, which are perforce derived from other ascospores. Since homokaryotic cultures are obligate out-crossers they are suited for genetic analyses.

1. Heterokaryon incompatibility and segregation distortion

The *het-s* locus is defined by the alleles *het-s* and *het-S*, that encode the corresponding HET-s and HET-S proteins. The HET-s protein can adopt an insoluble, proteinase K-resistant conformation, or a soluble, proteinase-sensitive conformation. If a culture expressing the soluble form ("recipient") makes contact (anastomoses) with one expressing the insoluble form ("donor") then the conformation of HET-s in the recipient culture changes into the insoluble form and this change is propagated rapidly throughout the recipient syncitium. The change does not require new protein synthesis and does not depend on the mixing of nuclei of the donor and recipient cultures (Coustou *et al* 1997). In inducing this change the insoluble form of HET-s behaves like a prion.

The HET-S protein exists in only one conformation (soluble). Fusion of a *het-S* mycelium with a *het-s* mycelium expressing the prion (or "reactive") form of HET-s results in cell death and the formation of a characteristic "barrage" in the fusion zone. The barrage prevents further mixing of mycelial contents (and gives the locus its name, *heterokaryon incompatibility-s*). However, no cell death or barrage is seen if the *het-S* mycelium fuses with a *het-s* culture expressing the soluble, non-prion ("non-reactive") form of HET-s. The reactive phenotype is designated as [Het-s], the non-reactive one as [Het-s*], and the phenotype of *het-S* mycelia as [Het-S]. Thus, even though both are genotypically *het-s*, the [Het-s] and [Het-s*] phenotypes are distinguishable based on whether or not they form a barrage when confronted with [Het-S] mycelia. Although the [Het-s*] phenotype is stable, at a low frequency it can convert spontaneously into [Het-s]. As noted above, the efficiency of conversion is increased to 100% by contact with a [Het-s] mycelium. The reverse transition (i.e. [Het-s] to [Het-s*]), was observed in a subset (~ 10%) of the protoplasts prepared from a [Het-s] mycelium. The convertants probably represent protoplasts that failed to receive any prion particle from the parent syncitium.

Progeny from crosses made between [Het-s] and [Het-s*] cultures inherit the phenotype of their maternal (i.e. ascogonial) parent. This suggests that the prion is excluded from the male gamete, the microconidia. In [Het-s*] × [Het-S] crosses the *het-s* and *het-S* alleles show 1 : 1 segregation in the progeny, but in [Het-s] × [Het-S] crosses the outcome depends on which mycelium serves as the female parent. If the [Het-S] culture is the female (in which case the cross is written as [Het-S] × [Het-s], in the gallant tradition of "females first"), the alleles segregate 1:1. However, in [Het-s] × [Het-S] crosses about 30% of the asci are two-spored because of the abortion of ascospores inheriting the *het-S* allele (Dalstra *et al* 2003). The surviving ascospores from the two-spored asci are of the genetic constitution [*het-s* + *het-s*]. Abortion of [*het-S* + *het-S*] and [*het-S* + *het-s*] ascospores (the latter class is produced by second division segregation for *het-s*) is attributed to the triggering of the incompatibility reaction due to the inheritance of the prion from the maternal parent and the *het-S* allele from the paternal parent. Any ascospore that inherits the *het-S* allele is always found to be issued from asci in which the sibling *het-s* ascospores were phenotypically [Het-s*]. This is presumed to reflect the dilution of the prion from the ascogenous hyphae. Thus the *het-s* allele can act as a spore-killing segregation distorter. In other words, it behaves as a selfish gene. It is worth noting that the segregation distortion would have been less pronounced had the *het-s* locus been located more distally on the chromosome, where it would be subjected to second division segregation with a higher probability. The essentials of the *het-s*/*het-S* heterokaryon incompatibility and spore abortion effects were discovered by Jean Bernet in his Ph. D. research and published in 1965 (cited in Dalstra *et al* 2003). Unfortunately, I was not able to locate an English translation or the original paper in French.

2. Incompatibility is triggered by differences in only a few amino acid residues

The discovery of a neutral wild strain that can form heterokaryons with both [Het-s] and [Het-S] mycelia made it possible to initiate the molecular analysis of [Het-s]/[Het-S] incompatibility. The phenotype of the neutral strain differed from [Het-s*] in that it was incapable of converting to [Het-s] following contact with a [Het-s] mycelium. The neutral strain was presumed to carry a null allele (*het-s*^{X}). The *het-s* allele was cloned by transformation of the neutral strain with a library of DNA from a *het-s* strain, allowing any potentially [Het-s*] transformants to convert to [Het-s] by contact with a [Het-s] mycelium, and then testing for the acquisition of incompatibility in confrontation with a [Het-S] strain. Transformants in which the HET-s open reading frame (ORF) was overexpressed from a transgene (*pGPDs*) using the strong promotor of the *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase gene were found to have an increased frequency of spontaneous [Het-s*] to [Het-s] conversion (Coustou *et al* 1997).

The *het-S* allele was cloned using the *het-s* gene to probe a library of DNA from a *het-S* strain, transforming the hybridizing clones into the neutral strain, and examining the transformants for the acquisition of incompatibility with [Het-s] mycelia. The nucleotide sequences of the cloned genes revealed that the HET-S and HET-s proteins differed in 13 of their 289 amino acid residues. Comparison with *het-s* allele sequences from the related species *P. comata* suggested that [Het-s]/[Het-S] specificity was correlated with differences at only eight of the residues. Studies with hybrid genes and site specific mutagenesis further established that among these differences a single amino acid change (H33P) could cause the HET-S protein to take on the specificity of HET-s (Deleu *et al* 1993). Conversely, two changes together (D23A and P33H) could switch the specificity of the HET-s protein to that of HET-S. If HET-s was altered in only a single residue (P33H) the mutant showed a bizarre new phenotype called [Het-S^{S}]. Cultures with this phenotype initially display [Het-S] specificity (i.e. incompatibility with [Het-s]) but later convert spontaneously to [Het-s] and this conversion can be induced by cytoplasmic contact with a *het-s* strain.

3. Mutants that "escape" from *het-s*/*het-S* incompatibility

Two other *het-S* mutants (F25S and Y38H) also were found to possess the [Het-s] specificity. They were obtained in a screen for UV-induced "escape" from the self-incompatibility phenotype of a *het-s*/*het-S* strain that was constructed by transforming a *het-S* strain with the *pGPDs* transgene which

overexpresses HET-s (Coustou *et al* 1999). Another mutant recovered from this screen, *het-S* E86K, lost reactivity in incompatibility tests with both *het-S* and *het-s* strains but nevertheless possessed the ability induce the [Het-s*] to [Het-s] transition following contact with a [Het-s] strain.

Another interesting mutant was *het-S* P175S. Subcultures of this mutant could differ in phenotype; some were phenotypically [Het-s], others were [Het-S] and still others showed a slow growth phenotype and produced a barrage reaction with both [Het-s] and [Het-S] testers. This mutant differed from the one described above as [Het-S^S] in that during vegetative propagation it appeared to switch back and forth between [Het-s] and [Het-S]. Two other *het-S* mutants (K201R and Q240*) displayed a neutral phenotype that could switch spontaneously to [Het-S]. This phenotype was designated as [Het-S*] because of its analogy to [Het-s*]. Finally one mutant allele, *het-s* R165G, conferred a growth defect that resembled an attenuated form of *het-S*/*het-s* incompatibility. This mutant encodes a glycine in the 165th position of HET-s. Interestingly, glycine is the 165th residue in HET-S. It was suggested that homomultimers of the mutant protein might resemble HET-s/HET-S heteromultimers. That HET-s and HET-S can form both homo- and heterodimers was established by using a yeast two-hybrid assay (Coustou *et al* 1997).

4. Purified recombinant HET-s protein forms amyloid-like aggregates

A recombinant histidine-tagged HET-s protein was expressed in *Escherichia coli*, affinity purified under denaturing conditions and allowed to renature and form aggregates. Microprojectile-borne injection of the aggregated protein into [Het-s*] mycelia was able to induce its conversion to [Het-s]. But the conversion could not be induced by freshly renatured but unaggregated HET-s protein (Maddelein *et al* 2002). Electron microscopic examination showed that the aggregated HET-s protein formed amyloid-like fibrils that were resistant to digestion by proteinase K whereas the unaggregated protein was proteinase K-sensitive (Dos Reis *et al* 2002). If the fibrils were sonicated and mixed in a 1 : 10 ratio with soluble protein then the conversion of the latter into the fibrillar form was greatly accelerated. The aggregation of soluble recombinant HET-s protein into amyloids was also inducible by cell extract from a [Het-s] strain, but not by extracts of a *het-s*-knockout strain. In contrast, amorphous aggregates of HET-s that were formed either by trichloric acid precipitation or by heat denaturation could neither effect the [Het-s*] to [Het-s] conversion nor accelerate the aggregation of soluble recombinant HET-s protein into the proteinase K resistant amyloid form. Thus the fibrillar HET-s aggregate behaved like an infectious prion. The fibrillar form also showed additional characteristics of amyloids in that the fibers could bind Congo red and showed birefringence under polarized light. Circular dichroism and Fourier Transform Infra Red spectroscopy provided evidence for an increase in antiparallel *b*-sheet structural content (Dos Reis *et al* 2002). The proteinase K-resistant core fragment corresponded to the residues 218–289 (Balguerie *et al* 2003). The *het-s* 218–289 segment was expressed as a histidine-tagged protein in *E. coli*. The recombinant protein also could form amyloid-like aggregates *in vitro* and they could promote the *in vivo* propagation of the HET-s prion. Chimeric proteins in which the *het-s* 218–289 segment was fused to the complementary N-terminal segment of HET-S did not form amyloids *in vitro* and imparted an [Het-S] phenotype *in vivo*. Thus the N-terminal domain of HET-S acted as an inhibitor of the transition to the prion state (Balguerie *et al* 2003). It would be interesting to work out why mutations (e.g. H33P) cause loss of this inhibitory activity.

5. What triggers cell death?

Null mutants of *het-s* display no detectable phenotype. Moreover the [HET-s] prion has no effect other than its involvement in *het-s*/*het-S* reactivity and in the promotion of the [Het-s*] to [Het-s] conversion as noted above. So what is the molecular basis for the triggering of incompatibility and cell death by the prion in the presence of HET-S protein? The short answer is that we do not know. Any explanation has to contend with the fact that although the presence of HET-S protein is required for incompatibility and sporekilling, somewhat contrarily, overexpression of HET-S suppresses incompatibility and sporekilling (Coustou *et al* 1999; Dalstra *et al* 2003). It has been suggested that

high-molecular-weight aggregates of the prion (as expected in a [Het-s] culture) may be innocuous but lower-molecular-weight oligomers might be toxic. The HET-S protein might "poison" the aggregation seed of the HET-s protein and cause shorter oligomers to accumulate (Balguerie *et al* 2003). HET-S overexpression might trap HET-s in heterodimers and thus prevent the accumulation of these toxic oligomers.

6. Conclusion

het-s/*het-S* incompatibility is one of only a handful of fungal heterokaryon incompatibility systems that have begun to be molecularly analysed (for a review see Saupe 2000). The spore killer phenotype also is one of only a few segregation ratio distortions that has been studied in any detail. Finally the [HET-s] prion is one of only three examples known in non-mammalian systems (for a review see Wickner *et al* 1999). Clearly the *het-s*/*het-S* story intersects with several exciting areas of research that offer many opportunities for young scientists.

References

Balguerie A, Doe Reis S, Ritter C, Chaignepain S, Coulary-Salin B, Forge V, Bathany K, Lascu I, Schmitter J-M, Riek R and Saupe S J 2003 Domain organization and structure-function relationship of the HET-s prion protein of *Podospora anserina*; *EMBO J*. **22** 2071–2081

Coustou V, Deleu C, Saupe S and Begueret J 1997 The protein product of the *het-s* heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog; *Proc. Natl. Acad. Sci. USA* **94** 9773–9778

Coustou V, Deleu C, Saupe S J and Begueret J 1999 Mutational analysis of the [Het-s] prion analog of *Podospora anserina*: a short N-terminal peptide allows prion propagation; *Genetics* **153** 1629–1640

Dalstra H J P, Swart K, Debets A J M, Saupe S J and Hoekstra R F 2003 Sexual transmission of the [Het-s] prion leads to meiotic drive in *Podospora anserina*; *Proc. Natl. Acad. Sci. USA* **100** 6616–6621

Deleu C, Clave C and Begueret J 1993 A single amino acid difference is sufficient to elicit vegetative incompatibility in the fungus *Podospora anserina*; *Genetics* **155** 45–52

Dos Reis S, Coulary-Salin B, Forge V, Lascu I, Begueret J and Saupe S J 2002 The HET-s prion protein of the filamentous fungus *Podospora anserina* aggregates *in vitro* into amyloid-like fibrils; *J. Biol. Chem*. **277** 5703–5706

Maddelein M -L, Dos Reis S, Duvezin-Caubet S, Coulary-Salin B and Saupe S J 2002 Amyloid aggregates of the HET-s prion protein are infectious; *Proc. Natl. Acad. Sci. USA* **99** 7402–7407

Saupe S J 2000 Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes; *Microbiol. Mol. Biol. Rev*. **64** 489–502

Wickner R B, Taylor K L, Edskes H K, Maddelein M L, Moriyama H and Roberts B T 1999 Prions in *Saccharomyces* and *Podospora* spp.: protein-based inheritance; *Microbiol. Mol. Biol. Rev*. **63** 844–861

DURGADAS P KASBEKAR
Centre for Cellular and Molecular Biology,
Hyderabad 500 007, India
(Email, kas@ccmb.res.in)