A developmental mutation (*npfL1*) resulting in cell death in *Physarum polycephalum*

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In *Physarum*, microscopic uninucleate amoebae develop into macroscopic multinucleate plasmodia. In the mutant strain, RA614, plasmodium development is blocked. RA614 carries a recessive mutation (*npfL1*) in a gene that functions in sexual as well as apogamic development. In *npfL*⁺ apogamic development, binucleate cells arise from uninucleate cells by mitosis without cytokinesis at the end of an extended cell cycle. In *npfL1* cultures, apogamic development became abnormal at the end of the extended cell cycle. The cells developed a characteristic rounded, vacuolated appearance, nuclear fusion and vigorous cytoplasmic motion occurred, and the cells eventually died. Nuclei were not visible by phase-contrast microscopy in most of the abnormally developing cells, but fluorescence microscopy after DAPI staining revealed intensely staining, condensed nuclei without nucleoli. Studies of tubulin organization during *npfL1* development indicated a high frequency of abnormal mitotic spindles and, in some interphase cells, abnormally thick microtubules. Some of these features were observed at low frequency in the parental *npfL*⁺ strain and may represent a pathway of cell death, resembling apoptosis, that may be triggered in more than one way. Nuclear fusion occurred during interphase and mitosis in *npfL1* cells, and multipolar spindles were also observed. None of these features were observed in *npfL*⁺ cells, suggesting that a specific effect of the *npfL1* mutation may be an incomplete alteration of nuclear structure from the amoebal to the plasmodial state.

Introduction

The life cycle of the acellular slime mould *Physarum polycephalum* includes two vegetative growth phases – uninucleate, haploid amoebae and multinucleate, syncytial plasmodia – which differ greatly in their patterns of gene expression and cellular organization (Gull *et al.*, 1985; Sweeney *et al.*, 1987). The transition from amoeba to plasmodium is normally a heterothallic (sexual) process controlled by the multiallelic mating-type locus *matA*; for plasmodium formation to occur, fusing amoebae must carry different alleles of *matA* (Burland,

1986). Time-lapse cinematography of sexual plasmodium formation has shown that nuclear fusion occurs shortly after amoebal fusion and the resulting diploid zygote enters an extended cell cycle at the end of which it becomes binucleate by mitosis without cytokinesis (Bailey *et al.*, 1990). In apogamic strains, a haploid amoeba develops into a haploid plasmodium; such strains result from *gad* mutations which map at *matA* (*gad* = greater asexual differentiation; Adler & Holt, 1977). In the apogamic strain CL, binucleate plasmodium formation is preceded by an extended cell cycle of similar length to that observed in sexual development (Anderson *et al.*, 1976; Bailey *et al.*, 1987). During the extended cell cycle in both sexual and apogamic development, uninucleate cells become committed to plasmodium formation (Youngman *et al.*, 1977; Burland *et al.*, 1981) and many of the changes in cellular organization that accompany development are initiated (Blindt, 1987; Bailey *et al.*, 1990; Solnica-Krezel *et al.*, 1990, 1991).

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Abbreviations: IMT, intermitotic time; DAPI, 4,6-diamidine-2-phenylindole; MTOC, microtubule organizing centre.

Interphase amoebae possess a cytoplasmic microtubular network which radiates from a nucleus-associated microtubule organizing centre (MTOC). This MTOC is closely associated with a pair of centrioles and is attached to the nucleus (Havercroft & Gull, 1983). At mitosis, the nuclear membrane breaks down, and the MTOC and centrioles duplicate, divide and migrate to form the poles of the spindle. In contrast, plasmodial nuclei undergo synchronous anastral mitoses inside intact nuclear membranes (Havercroft & Gull, 1983) and the interphase microtubules of plasmodia are cytoplasmic with no obvious association with the nuclei (Salles-Passador *et al.*, 1991). Thus the alteration from amoebal to plasmodial microtubule organization involves changes in the numbers of MTOCs per cell, the arrangement of the cytoplasmic microtubules and the type of mitosis. The time course of these changes has been studied in the apogamic strain CL (Gull *et al.*, 1985; Blindt, 1987; Solnica-Krezel *et al.*, 1990, 1991). The earliest stage at which a plasmodial spindle has been observed is at the mitosis at which a binucleate cell is formed. Although most cells form plasmodial spindles at this mitosis, a significant number form other types of spindles (Solnica-Krezel *et al.*, 1991). Extra MTOCs that are not nucleating the spindle are frequently observed at this mitosis in CL (Blindt, 1987; Solnica-Krezel *et al.*, 1991). These MTOCs are presumed to be the remnants of the amoebal MTOCs which do not function in intranuclear plasmodial mitosis. Most developing uninucleate cells possess a single MTOC but binucleate cells display variability in the number of MTOCs they possess (Blindt, 1987; Solnica-Krezel *et al.*, 1990).

The distribution of two phase-specific tubulin isotypes has been studied in CL. The $\alpha 3$ tubulin isotype is an acetylated form of the $\alpha 1$ -isotype and, in amoebae, is found only in the centrioles during interphase and in the spindle poles during mitosis. The $\alpha 3$ -isotype is not detectable in plasmodia. The loss of the $\alpha 3$ -isotype begins during the extended cell cycle, but in some cells it does not completely disappear until the quadrinucleate stage (Solnica-Krezel *et al.*, 1990). In contrast, the $\beta 2$ -isotype is not present in amoebae and is first expressed shortly after cells become committed to development, although there is variability in the time at which individual cells first show this marker of development (Solnica-Krezel *et al.*, 1990).

The apogamic strain CL arose as a result of a *gad* mutation (*gadAh*) in a *matA2* strain and the cellular events leading to plasmodium formation in this strain have been well studied (Anderson, 1979; Blindt *et al.*, 1986; Bailey *et al.*, 1987; Solnica-Krezel *et al.*, 1990, 1991). Other independently isolated *gad* mutants have been characterized genetically; for example, the apogamic strain RA376 arose as a result of a mutation

(*gadA111*) in a *matA3* strain (Shinnick & Holt, 1977; Anderson *et al.*, 1989). However, the cellular events of plasmodium development in these other *gad* strains have not been reported.

From apogamic *gad* strains, a number of *npf* (no plasmodium formation) mutants have been isolated in which plasmodium formation is blocked. Since *npf* strains proliferate as amoebae but cannot complete development, the mutations they carry identify genes essential for plasmodium formation but not required for vegetative amoebal growth. In strains where the *npf* mutation maps at *matA*, there is no evidence that development is initiated (Anderson & Dee, 1977; R. W. Anderson, unpublished observations). Mutations that map at loci other than *matA* give rise to strains in which development is initiated but halts before a mature plasmodium forms (Anderson *et al.*, 1986). Such strains exhibit a variety of abnormal phenotypes. One of these strains, RA614, is the subject of the present work.

Amoebal growth was normal in RA614 but this strain failed to complete the transition from amoeba to plasmodium. First we verified that the mutant phenotype resulted from a single gene mutation. We then used a variety of techniques to determine the earliest stage at which development became detectably abnormal, and the sequence of events leading to the terminal phenotype. Since RA614 was isolated from the apogamic strain RA376, and the cellular events of plasmodium formation in the parental strain had not previously been elucidated, development was studied in RA376 also.

Methods

Media. SDM (semi-defined medium), DSDM agar (agar containing SDM), and LIA (liver infusion agar): Blindt *et al.* (1986). LIA + citrate (liver infusion agar with citrate buffer): Bailey *et al.* (1987). SM-1: Dee & Anderson (1984). SM-4: 33 ml Solution A (20 g Difco bacto tryptone, 6 g Difco yeast extract, 18 g glucose plus water to 1 l) and 6 ml Solution B (36.8 g citric acid monohydrate, 95.5 g trisodium citrate dihydrate plus water to 1 l) to 1 l molten 1.5% agar. SBS (standard bacterial suspension): Burland *et al.* (1981). FKB (formalin killed bacterial suspension): Dee (1986).

Culture conditions. Apogamic plasmodium development is temperature-sensitive. Studies of apogamic development were carried out on DSDM or LIA + citrate at 21–22 °C, the permissive temperature for development. Experiments with vegetative amoebae were carried out on LIA at 28.5–29.5 °C, the nonpermissive temperature for apogamic development in the strains used. Amoebal stocks were also maintained at 28.5–29.5 °C. Genetic analysis of RA614 was carried out on SM-1 or SM-4 as given below.

Loci. These loci are described in detail in Anderson *et al.* (1989). *matA*: mating-type locus controlling zygote differentiation. Heterozygosity at this locus is essential for plasmodium formation. *matB*, *matC*: mating-type loci affecting the frequency of zygote formation. *fusA*, *fusC*: plasmodial fusion compatibility loci. *whiA*: plasmodial colour gene. Plasmodia expressing the recessive *whiA1* allele are white; wild-type plasmodia are yellow. *gadA*: *gadA* mutations allow amoebae to

develop into plasmodia without mating; they are tightly linked to *matA* and dominant to *gadA*⁺. *npf* (no plasmodium formation): Mutations at these loci block plasmodium development.

Strains. CH818: *matA2 gadAh npfF1 npfL*⁺ (Anderson, 1979). CH938: *matA4 gadA*⁺ *npfL*⁺ *matB2 matC1 fusA1 fusC1 whiA1* (Kawano *et al.*, 1987). LU867: *matA2 gadAh npfA1 npfL*⁺ (Anderson & Dee, 1977). RA376: *matA3 gadA111 npfL*⁺ *matB3 matC2 fusA2 fusC2 whiA*⁺ (Anderson *et al.*, 1989). RA614: *matA3 gadA111 npfL1 matB3 matC2 fusA2 fusC2 whiA*⁺ arose as a result of mutation in RA376.

Mutagenesis of RA376 and isolation of *npf* derivatives. RA376 amoebae were spread, 10⁵ per plate, on SM-1 agar containing 0.2% caffeine. After 48 h incubation at 29.5 °C, the cultures were irradiated for 10 s at 30 cm from a mercury vapour lamp (T/M5/369E, Thermal Syndicate) and reincubated for 48 h. The cells from three plates were resuspended in 3 ml distilled water and plated on SM-4 agar with SBS at a density which gave well-separated colonies. After 7 d incubation at 21 °C, well-formed plasmodia were apparent in all but 35 of 3306 colonies examined. Amoebae were recovered from these colonies, purified and retested; RA614 was one of several isolates that consistently showed abnormalities in plasmodium development. Culturing and genetic analysis of RA614 amoebae were as described in Anderson *et al.* (1989).

Cell assays. Replicate LIA + citrate or DSDM plates were inoculated with 5 × 10⁴ amoebal cysts and 0.1 ml of FKB suspension diluted 1:2 with water. The plates were incubated at 22 °C. At various times after inoculation, cells were harvested from two plates. A drop of the cell suspension was allowed to dry into the surface of an agar-coated slide and about 300 cells were examined by phase-contrast microscopy to determine the number of nuclei per cell. In some experiments, the number of cells able to undergo the amoeba-flagellate transformation was determined (Blindt *et al.*, 1986).

Time-lapse cinematography. Filming was carried out in cavity slides using the equipment and conditions described by Bailey *et al.* (1987) and an initial cell density of 4 × 10⁵ cysts ml⁻¹. One frame was exposed every 30 s with an exposure time of 0.2 s. Intermitotic time (IMT) was measured from nucleolar disappearance in one cell cycle to nucleolar disappearance in the next cell cycle. In the text, all times are given in hours as mean ± standard deviation. Cell area was measured as described in Bailey *et al.* (1987). At the magnification used in this study, each arbitrary unit of area represents 7.30 µm² on the slide culture. In the text, area measurements are given in these arbitrary units as mean ± standard deviation. It should be noted that area measurements from different films cannot be compared directly because of variation between slide cultures in the extent of cell flattening. Unless otherwise stated, all statistical analyses were performed by Student's *t*-tests.

Two films were made of development in RA614 and one of development in RA376. One of the RA614 films (Film 1) and the RA376 film showed cultures recorded from 48–114 h after inoculation with cysts and FKB. Actively growing RA614 cells, plus extra FKB, were used to inoculate the culture recorded in Film 2; this film started immediately after inoculation and continued for 66 h.

Flow cytometry. Cultures were inoculated at a density of 5 × 10⁵ cysts per plate and incubated at 22 °C (developing cultures on LIA + citrate plates) or 29.5 °C (amoebal controls on LIA plates). After a period of growth, the cells were harvested and stained for flow cytometry (Bailey *et al.*, 1987). Before staining, a sample was removed and the number of nuclei in each cell was determined by phase-contrast microscopy. The stained cells were examined on a FACS 420 flow cytometer linked to a Consort 30 accessory computer (Becton-Dickinson Immunocytometry Systems). The intensity of fluorescence emitted by each cell is directly proportional to its DNA content (Crissman & Tobey 1974; Taylor & Milthorpe 1980).

Fluorescence microscopy. Cultures were set up as for flow cytometry. After 3 d of growth at 22 °C, the cells from two plates were used to determine the number of nuclei visible in a sample of cells. The cells on the remaining plates were fixed and stained with antibodies and DAPI (4',6-diamidino-2-phenylindole) as described in Bailey *et al.* (1990) or as in Diggins-Gilcinski *et al.* (1989) with modifications for the α3-tubulin-specific antibody 6-11B-1 as described in Solnica-Krezel *et al.* (1990). The stained coverslips were then mounted in antifade (*p*-phenylenediamine; Sigma) as described in Solnica-Krezel *et al.* (1990).

Results

Comparison of wild-type and mutant phenotypes

Amoebal cultures of RA376, the wild-type parent strain, gave rise to small plasmodia (1–2 mm diameter) after 3–5 d; these plasmodia were highly motile and possessed a characteristic fan shape with veins. Phase-contrast microscopy of RA376 cultures revealed that small developing plasmodia were irregularly shaped with a prominent slime layer and contained many food vacuoles, contractile vacuoles and nuclei (Fig. 1*a*). By a combination of cell fusions and mitoses unaccompanied by cytokinesis, these small plasmodia rapidly grew into yellow macroplasmodia with prominent veins. These characteristics of RA376 development are indistinguishable from development in the *gadAh* apogamic strain CL (Bailey *et al.*, 1987).

In cultures of the mutant strain, RA614, amoebal proliferation was normal, but developing cells appeared as amorphous blobs and macroscopic plasmodia never formed; the maximum diameter reached by developing cells was less than 1 mm. Developing cells of RA614 were unusually rounded, surrounded by an abnormally thick slime sheath, possessed no veins, and their cytoplasm appeared denser than normal (Fig. 1*b*). The developing cells contained food vacuoles and contractile vacuoles, but in many cases nuclei could not be observed. These initial observations could not indicate the stage at which development became abnormal in RA614 nor whether the mutation affected plasmodium development or plasmodial growth.

Genetic analysis

To determine whether abnormal plasmodium development in RA614 was caused by a single gene mutation, the segregation of the mutant 'blob' phenotype was studied in the meiotic progeny of a cross between RA614 and a heterothallic strain that was wild-type except for a plasmodial colour mutation (Table 1*a*). Among 49 progeny analysed, 24 began apogamic plasmodium formation at the permissive temperature, 21 °C, and had therefore inherited the *gadA111* allele from RA614.

Fig. 1. Phase-contrast micrographs of apogamic amoebae allowed to develop for a few days on a bacterial lawn. (a). RA376: a normally differentiating microplasmodium (P) is visible in contact with two amoebae (A). (b) RA614: a large cell (C) showing terminal mutant phenotype. Bar, 10 μ m.

Apogamic development in 12 of these progeny gave rise to cells of the mutant phenotype, and 12 formed wild-type plasmodia (Table 1a). This 1:1 segregation of plasmodial morphologies indicated that the developmental abnormalities shown by RA614 were due to a mutation at a locus that was unlinked to *gadA*; this mutation was designated *npfL1*.

All the progeny were classified for *matA* allele (Table 1a) by mixing them with *npfL*⁺ tester strains at 29.5 °C, a temperature that was non-permissive for apogamic development, but permissive for mating. As expected, since *gadA* and *matA* were known to be tightly linked (Anderson *et al.*, 1989), all the *gadA111* progeny carried the *matA3* allele from RA614, and all the *gadA*⁺ progeny carried the *matA4* allele from the wild-type parent. The 25 *gadA*⁺ *matA4* progeny were then mixed with a *matA3 npfL1* strain and cultured at 29.5 °C. Sexual plasmodium formation began in each of these mixtures, but cultures containing eight of the 25 progeny gave rise only to cells

of the mutant phenotype, showing that the *npfL1* mutation affected sexual, as well as apogamic, development. Seventeen progeny formed wild-type plasmodia in these mixtures, confirming that *npfL1* and *npfL*⁺ were segregating among the *gadA*⁺ *matA4* progeny, and indicating that the wild-type allele was dominant. The deviation from 1:1:1:1 (Table 1a) amongst the four progeny classes of the cross between RA614 and the wild-type strain was not significant (Chi-square test; $p > 0.3$), confirming that the *npfL* gene was unlinked to *matA* and *gadA*.

The progeny of the cross were also segregating for several additional markers known to be unlinked to *matA* and to each other (*matB*, *matC*, *fusA*, *fusC*, *whiA*; Methods). All progeny were classified for these markers, but no significant deviation from free recombination with *npfL* was detected in any case (Table 1b; $p > 0.05$, Chi-square tests). Further crosses were analysed between RA614 and strains carrying *npfA1* (LU867; Anderson & Dee, 1977) or *npfF1* (CH818; Anderson, 1979), two other developmental mutations known to be unlinked to *matA*. No significant deviation from free recombination was found with either mutation (Table 1b; $p > 0.05$, Chi-square test). Thus the *npfL1* mutation carried by RA614 was not linked to any of the genes tested.

The amoeba-flagellate transformation and binucleate cell formation

In the parent strain RA376, as in CL (Blindt *et al.*, 1986; Bailey *et al.*, 1987), commitment occurred about halfway through the extended cell cycle, very close to the time that ability to undergo the amoeba-flagellate transformation was lost and several hours before binucleate cell formation (data not shown). Since the assay for commitment depends upon the formation of macroscopic motile plasmodia, it was not possible to determine the time of commitment in the *npfL1* mutant strain. It was possible, however, to assay cells in developing cultures of the mutant strain for loss of ability to undergo the amoeba-flagellate transformation and for the presence of binucleate cells. In developing cultures that contained only uninucleate cells, most cells (94%) were able to transform into flagellates. When 0.2% of cells had reached the binucleate stage, the proportion of cells able to undergo the amoeba-flagellate transformation had declined to 87%; the proportion continued to decline, reaching 41% when 3% of the cells were binucleate. The decline in the proportion of cells able to undergo the amoeba-flagellate transformation suggested that many uninucleate cells had initiated plasmodium development. The presence of binucleate cells in the cultures indicated that some cells had passed the developmental stage at which commit-

Table 1. Genetic analysis of RA614

(a) Classification of progeny from the cross RA614 × CH938

Clonal cultures at 21 °C	Plasmodium development in crosses at 29.5 °C with			Number in class	Deduced genotype
	<i>npfL</i> ⁺ <i>matA3</i>	<i>npfL</i> ⁺ <i>matA4</i>	<i>matA3 npfL1</i>		
+	–	+	ND	12	<i>gadA111 matA3 npfL</i> ⁺
L	–	+	ND	12	<i>gadA111 matA3 npfL1</i>
–	+	–	+	17	<i>gadA</i> ⁺ <i>matA4 npfL</i> ⁺
–	+	–	L	8	<i>gadA</i> ⁺ <i>matA4 npfL1</i>

+, Wild-type; –, no plasmodia formed; L, RA614-like; ND, not done.

(b) Recombination of *npfL* and other loci

Cross	Locus	Recombinants:parentals
RA614 × CH938	<i>matB</i>	28:21
	<i>matC</i>	27:22
	<i>fusA</i>	29:20
	<i>fusC</i>	30:19
	<i>whiA</i>	25:24
RA614 × LU867*	<i>npfA</i>	14:12
RA614 × CH818†	<i>npfF</i>	10:17

* Only scored in *matA2 gadAh npfA*⁺ progeny (26/99).† Only scored in *matA2 gadAh npfF*⁺ progeny (27/99).

ment normally occurs and were presumably committed to development.

To identify the stage of development at which the *npfL1* lesion first affected plasmodium formation, the proportions of cells with different numbers of nuclei were determined by phase-contrast microscopy of RA614 cultures at various times after inoculation. In developing cultures of the apogamic strain CL, binucleate cells were first detected at about 48–60 h of development; the number of binucleate cells rose rapidly for a short time and then cells with more than two nuclei were detected (Blindt *et al.*, 1986). Developing CL plasmodia readily fused to give large plasmodia that appeared some hours after the first multinucleate cells were observed (Bailey *et al.*, 1987). In developing *npfL1* cultures, all the cells were uninucleate for the first 60 h of incubation (Fig. 2). After this time, some binucleate cells were detected but they never exceeded 5% of the population. No cells with more than 2 nuclei were detected but a new cell-type was observed in which nuclei were not visible by phase-contrast microscopy. These apparently anucleate cells were first detected at 78 h and their numbers increased rapidly to 30% at 100 h. At the end of the experiment (168 h), nuclei were not visible in 36% of the cells, 5% were binucleate and the remainder were uninucleate (Fig. 2). These results confirmed that development was initiated in *npfL1* cells but became abnormal before the end of the binucleate cell stage with the accumulation of large numbers of apparently anucleate cells.

Fig. 2. Cell types during development in RA614. The percentages of uninucleate, binucleate and apparently anucleate cells in developing cultures were determined at different times after inoculation as described in Methods. Binucleate cells (□) and anucleate cells (■) are shown; the remaining cells were uninucleate.

Origin of abnormal cells

To investigate the origin of the apparently anucleate cells during development of RA614, time-lapse cinematography was used to trace individual cells through successive cell cycles. Two films were made of development in RA614 (Methods). For comparison, cinematographic

Table 2. Time-lapse cinematographic data

Times are given as the mean (h) $\pm$ SD; the number of observations *n* is given in parentheses.

Measurement	RA376	RA614	
		Film 1	Film 2
Amoebal IMT	13.4 $\pm$ 3.0 h (<i>n</i> = 24)	13.1 $\pm$ 1.8 h (<i>n</i> = 38)	10.3 $\pm$ 3.3 h (<i>n</i> = 20)
Uninucleate to binucleate IMT	29.7 $\pm$ 4.2 h (<i>n</i> = 10)	32.8 $\pm$ 1.4 h (<i>n</i> = 3)	24.5 $\pm$ 2.7 h (<i>n</i> = 7)
IMT for cells that divided after long cell cycle	31.3 h (<i>n</i> = 1)	29.0 h (<i>n</i> = 1)	28.6 $\pm$ 5.4 h (<i>n</i> = 3)
Amoebal area at birth	10.6 $\pm$ 1.6 (<i>n</i> = 23)	14.9 $\pm$ 3.0 (<i>n</i> = 38)	—
Amoebal area at mitosis	23.4 $\pm$ 5.6 (<i>n</i> = 23)	34.0 $\pm$ 6.0 (<i>n</i> = 38)	—
Area at start of long cell cycle	15.6 $\pm$ 5.0 (<i>n</i> = 10)	16.8 $\pm$ 2.7 (<i>n</i> = 4)	—
Area at end of long cell cycle	58.0 $\pm$ 21.6 (<i>n</i> = 10)	71.3 $\pm$ 20.3 (<i>n</i> = 4)	—

analysis was also carried out on the parental strain, RA376. Amoebal proliferation appeared normal in both strains; amoebal area approximately doubled in each cell cycle and amoebal IMTs were of similar length in RA376 and RA614 (Film 1; Table 2). The mean *npfL1* (RA614) amoebal IMT measured from Film 2 was significantly shorter than that measured from Film 1 ($p < 0.005$), presumably due to the difference in the growth state of the cells used to set up the slide cultures (Methods).

Most of the developing cells in both films of RA614 eventually developed the characteristic abnormal phenotype of *npfL1* which began with the disappearance of the nuclei from view, the cessation of locomotion and the rounding up of the cells; these features often appeared within a few minutes. At about the same time, the cells began to accumulate contractile vacuoles and the cytoplasm began to move vigorously, appearing as 'boiling' under rapid playback. This vigorous cytoplasmic motion often continued for over 24 h, during which time the cells became smaller and some eventually burst. Although the abnormal *npfL1* cells failed to locomote, they ingested passing amoebae and underwent apparently normal plasmodial fusions with developing cells that were still locomoting. In addition, they developed the prominent slime layer and rhythmic pulsations characteristic of plasmodia. Since no plasmodia ever formed in RA614 cultures, we assume that all cells would have eventually developed the characteristic abnormal phenotype and died.

Although the terminal phenotype shown by developing RA614 cells was always essentially the same, there was wide variation in the history of the cells that developed this phenotype. Some of these pathways are illustrated in Fig. 3. As in all apogamic strains studied, including RA376, development involved an extended cell cycle about 2.3 times the length of an amoebal cell cycle during which the developing cells continued to grow until they were about twice the area of amoebae at mitosis (Table 2). Fourteen RA614 cells entered this long cell cycle, at the end of which 10 became binucleate (Fig.

Fig. 3. Representative RA614 cell pedigree from time-lapse cinematography. Time is given in hours after the start of filming. NV, nuclei vanish; F, cell fusion; NF, nuclear fusion; B, approximate time of onset of boiling phenotype. $\odot$, Uninucleate cell; $\bullet\bullet$, binucleate cell; $\circ$, cell in which no nuclei are visible; $\rightarrow$, time of nucleolar disappearance at start of mitosis; $\rightarrow$, cell left field of view.

3, Cells A, D) and 4 divided to give two daughter cells each (a total of 8 daughter cells; Fig. 3, cell C); in RA376, nine cells became binucleate and one cell divided. All RA614 and RA376 cells that entered the extended cell

Table 3. Nuclear morphology during development

Cell type	Percentage in RA376 (<i>npfL</i> ⁺)		Percentage in RA614 (<i>npfL1</i>)	
	Phase contrast	DAPI	Phase contrast	DAPI
Uninucleate	88.2	85.7	78.1	77.9
Binucleate	7.8	4.0	5.5	6.1
More than four nuclei	1.4	3.3	0.0	1.8
Mitotic	0.9	4.0	0.8	3.0
One condensed nucleus	0.0	1.7	0.0	3.3
Two condensed nuclei	0.0	1.3	0.0	6.1
Four condensed nuclei	0.0	0.0	0.0	1.2
No visible nucleus	1.7	0.0	15.6	0.6
Total cells scored	632	299	385	329

cycle arose from apparently normal amoebal divisions and were not significantly different in area, at birth, from cells that continued to cycle vegetatively (Table 2; both strains, $p < 0.005$).

Seven of the cells that became binucleate after the extended cell cycle could be traced further. Of the 8 daughter cells resulting from division at the end of the long cell cycle, five became binucleate after an approximately amoebal length cell cycle (Fig. 3, Cell C1); one RA376 daughter cell also became binucleate in this manner. In addition, the fates of five cells that entered the field of view as uninucleates and later became binucleate could be traced. Thus, the fates of a total of 17 RA614 cells observed to become binucleate could be traced. In 10 binucleate cells, the nuclei vanished in interphase (NV in Fig. 3; Cells A, D) and the cells exhibited the characteristic 'boiling' phenotype of the mutant (B in Fig. 3). In another three binucleate cells, the nuclei vanished after the start of mitosis and the cells developed the characteristic *npfL1* phenotype. The nuclei of the remaining four binucleate cells underwent nuclear fusion in interphase, giving rise to uninucleate, presumably diploid, cells (Fig. 3, cell C1). In two of these cells, the nuclei subsequently vanished in interphase. The other two uninucleate diploid cells became binucleate by mitosis without cytokinesis before the two nuclei in each cell vanished and the cells began to show the abnormal characteristics of *npfL1*. Two RA376 binucleate cells also developed the vigorous cytoplasmic motion characteristic of RA614 after their nuclei vanished in interphase.

Three RA614 cells entered the field as binucleate cells and underwent mitosis without cytokinesis to give rise to quadrinucleate cells. All of these subsequently developed the characteristic *npfL1* phenotype. In one of the three quadrinucleate cells, the nuclei vanished at mitosis (Fig. 3) and the characteristic phenotype developed. In the other two quadrinucleate cells, the four nuclei entered mitosis synchronously some hours after the previous

mitosis, but instead of eight nuclei being formed, only one or two giant, presumably polyploid, nuclei were observed. These nuclei eventually vanished and the characteristic phenotype developed.

The films showed that development in the apogamic strains CL, RA376 and RA614 was similar until the end of the extended cell cycle. In RA614, development became abnormal close to the end of the extended cell cycle and all the developing cells eventually developed the characteristic 'boiling' phenotype, although the pathways leading to this phenotype were varied. Nuclear fusion at mitosis and in interphase was observed only in RA614 but other abnormalities seen in RA614 also occurred in a few cells of RA376. Nuclear disappearance in interphase occurred in two developing RA376 cells and these subsequently developed the same terminal phenotype as RA614.

Nuclear morphology during development

Filming indicated that the disappearance of nuclei was an early event in the abnormal development of RA614. In order to determine whether the nuclei had fragmented or were present in a form that was not detectable by phase-contrast microscopy, the nuclei in samples of cells from both RA614 and RA376 were examined by phase-contrast microscopy and by DAPI fluorescence microscopy. The DAPI-stained cells were also stained with a β -tubulin antibody to visualize microtubules.

For both strains, the different microscopical techniques made little difference to the proportions of uninucleate or multinucleate cells (Table 3). The frequency of mitotic cells detected by fluorescence microscopy was slightly higher than by phase-contrast microscopy (Table 3) since the condensed chromosomes of mitotic cells were visualized more clearly by DAPI staining. By phase-contrast microscopy, some cells of each strain appeared anucleate; the frequency of such

Fig. 4. Fluorescence micrograph of two DAPI-stained RA614 cells. One has a single normal interphase nucleus, the other has two condensed nuclei. DAPI also stains the DNA in mitochondria and in ingested bacteria in the cytoplasm. Bar, 5 μ m.

cells was much higher in RA614 (*npfL1*) than in RA376 (15.6% compared to 1.7%; Table 3). By DAPI-fluorescence microscopy, about 1% of RA614 cells were anucleate but no anucleate RA376 cells were observed (Table 3); to avoid possible confusion between genuine anucleate cells and debris on the slides, structures observed by DAPI-fluorescence were not classified as anucleate cells unless tubulin staining confirmed the presence of microtubules. By fluorescence microscopy, cells with condensed nuclei were detected in each strain; such cells were more frequent in *npfL1* cultures than in *npfL+* cultures (Table 3; 10.6% compared with 3.0%). Condensed nuclei were 25–50% the area of normal nuclei, did not exhibit nucleoli and stained uniformly and very brightly with DAPI (Fig. 4). These nuclei were distinctly different from mitotic nuclei, in which condensed chromosomes were observable (Fig. 6). Condensed nuclei were not visible by phase-contrast microscopy. These results suggested that many of the cells scored as anucleate by phase-contrast microscopy were those that contained condensed nuclei when viewed by fluorescence microscopy. Nuclear ‘disappearance’ in interphase during filming would therefore be due to nuclear condensation.

DNA content during development

The nuclear fusions observed during filming of *npfL1* cells suggested that some *npfL1* uninucleate cells were not haploid, as is usual in apogamic strains, but diploid. Our studies of nuclear morphology in RA614 indicated that most of the cells that appeared anucleate by phase-contrast microscopy contained two or more condensed nuclei (Table 3). Both these observations led to a

Fig. 5. Cellular DNA contents in RA614 (*npfL1*) estimated by flow cytometry. Channel number is proportional to DNA content (see Methods). For each sample, 10^4 cells were counted. (a) Vegetative amoebae at 29.5 °C. The main peak at Channel 50 contains 90% of cells. The remaining cells had higher DNA contents. (b) Developing population at 22 °C. The peaks at Channel 50 and Channel 100 contain 53% and 27% of cells respectively. The remaining cells had higher DNA contents.

prediction that, in a developing culture of RA614, the frequency of cells with a 4C (binucleate haploid G2) DNA content would be greater than the frequency of binucleate cells scored by phase-contrast microscopy. We therefore used flow cytometry to measure the cellular DNA contents in RA614 cultures.

Phase-contrast microscopy of an amoebal culture incubated at 29 °C for 3 d indicated that it contained only uninucleate and encysted cells. Flow cytometry of the same population (Fig. 5a) showed a distribution typical of haploid amoebal cultures (Dee *et al.*, 1989). The single main peak around Channel 50 in Fig. 5(a) corresponds to the 2C (haploid G2) DNA content. Phase-contrast microscopy of a developing culture incubated at 22 °C for 8 d indicated that it contained 65% uninucleate cells, 1% binucleate cells and 33% apparently anucleate cells. Although only 1% of the RA614 cells were binucleate, flow cytometry (Fig. 5b) showed, in addition to the 2C

Fig. 6. Abnormal mitotic spindles in RA614 uninucleate developing cells. (a–d) Cells stained with β -tubulin antibody. (a'–d') The same cells double stained with DAPI. DAPI also stains the DNA in mitochondria and in ingested bacteria in the cytoplasm. Bar, 5 μ m. Arrows indicate chromosome masses. (a, a') Mitotic cell with a cross-shaped spindle. There is a central mass of chromatin as well as smaller clusters at each arm of the cross. (b, b') The chromosomes in this cell are divided into three portions with one residing at each arm of the spindle. (c, c') Most of the chromosomes in this cell lie in or near the main group. (d, d'). This 'star' type of mitotic spindle was the most common abnormality observed. The chromatin forms a central mass and the microtubules radiate from there.

peak, a clear peak (27%) at the 4C position. Thus, as predicted, there was an excess of cells with the 4C DNA content: presumably G2 cells with either a single diploid nucleus or two haploid nuclei.

Microtubule organization during development

We examined microtubule organization in RA614 to determine whether the *npfL1* mutation affected the alteration from amoebal to plasmodial microtubule organization. Cells were doubly stained with tubulin antibodies to visualise microtubules and with DAPI to show DNA.

Mitotic cells. The following types of spindles were recognised in uninucleate cells. Class I (amoebal): bipolar spindles with asters and a metaphase plate or two sets of separating chromosomes. Class II (plasmodial): bipolar spindles lacking asters, but having a metaphase plate or two sets of separating chromosomes. Class III: bipolar spindles that could not definitely be placed in Class I or Class II. These three classes of spindles were

considered to be normal. Class IV: star microtubular arrays in which microtubules radiated in the form of a star and the condensed chromosomes formed a mass in the centre of the microtubular array (Fig. 6d, d'). Class

Table 4. Frequencies of binucleate cells and abnormal uninucleate mitoses in RA614

Binucleate cells (%)	Mitotic cells counted	Mitotic uninucleate cells	
		Percentage Class IV (star)	Percentage Class V
0.0	399	0.0	0.2
0.0	565	0.0	0.0
0.0	1651	0.0	0.0
1.2	690	21.4	2.6
4.0	1027	34.0	4.0
6.2	609	17.3	3.2
7.6	584	43.5	17.5
12.4	457	51.8	11.8
14.8	132	43.2	16.8
16.7	637	52.7	6.3
18.9	327	45.8	14.7
30.0	67	49.2	26.9

Fig. 7. Interphase microtubules in RA614 cells. (*a–e*) Cells stained with β -tubulin antibody. (*a'–e'*) DAPI staining in the same cells. DAPI also stains the DNA in mitochondria and in ingested bacteria in the cytoplasm. Bar, 5 μ m. $\blacktriangleright$ MTOC. (*a, a'*) Normal uninucleate cell with microtubules radiating from a single MTOC. (*b, b'*) Quadrinucleate cell with condensed nuclei and abnormally thick microtubules which radiate from two MTOCs. (*c, c'*) Anucleate cell with a few thick microtubules but no MTOC. (*d, d'*) Five MTOCs can be seen in this cell which has three condensed nuclei. (*e, e'*) Thick microtubules in a cell with two condensed nuclei.

V: uninucleate mitotic cells in which there were more than two spindle poles and generally several chromatin masses (Fig. 6a–c). Classes IV and V were considered to be abnormal. It was assumed that correct chromosome separation could not occur in these two classes of spindles, and that cells containing them would die.

Uninucleate cells presumed to be at the end of the extended cell cycle, selected from a culture containing 6% multinucleate cells, were scored for spindle type and for the presence of extra MTOCs. The cells were selected because all were twice the size of vegetative amoebae and some contained ingested cells. Of the 102 cells selected, 51 contained a normal spindle (Classes I, II, III) of which 20 were plasmodial (Class II). The presence of Class II (plasmodial) spindles indicated that the *npfL1* mutation had not blocked the switch to the plasmodial type of mitosis. Extra MTOCs were observed in 1 of 12 Class I (amoebal) spindles; 9 of 20 Class II (plasmodial) and 4 of 19 Class III spindles. In the apogamic strain CL, extra MTOCs in mitotic cells are associated with developmental changes in microtubular organization (Blindt, 1987; Solnica-Krezel *et al.*, 1991). Of the 102 cells selected, 51 contained an abnormal spindle (Classes IV and V; Fig. 6). Several of the Class IV (star) spindles (8/44) possessed extra MTOCs but none of the Class V spindles did. Star spindles, both with and without extra MTOCs, have also been observed in developing uninucleate cells of CL (Solnica-Krezel *et al.*, 1991). Class V spindles have not been reported in CL; we suggest they occur during mitosis of the polyploid cells that form by nuclear fusion in the mutant (see Discussion).

The frequency of abnormal spindles in all uninucleate cells was determined for RA614 cultures at different stages of development (Table 4). The proportion of binucleate cells served as a measure of the stage of development of the culture. Among mitotic uninucleate cells, the proportion with abnormal spindles increased as the frequency of binucleate cells increased. In cultures that lacked binucleate cells, and thus were in early development, few abnormal mitotic figures were observed and all of these were of the Class V type (Table 4). The proportion of abnormal mitoses, particularly of the Class IV type, increased rapidly as binucleate cells began to appear, reaching 75% when 30% binucleate cells were present.

Interphase cells. In developing cultures of RA614 that contained no multinucleate cells, 98% of cells were positive for the $\alpha 3$ -tubulin isotype. As the number of multinucleate cells in the developing cultures increased, the number of $\alpha 3$ -tubulin positive cells decreased; in a culture containing 16% multinucleate cells only 73% of cells were $\alpha 3$ -tubulin positive. In developing *npfL1* cultures containing no multinucleate cells, less than 1%

of cells were $\beta 2$ -tubulin positive. As the number of multinucleate cells increased so did the number of $\beta 2$ -tubulin positive cells, reaching 27% when 6% of cells were multinucleate and 55% when 22% of cells had more than one nucleus. These observations indicated that developing cultures of RA614 initiated the same changes in $\alpha 3$ - and $\beta 2$ -tubulin isotype expression that accompanied development in CL (Solnica-Krezel *et al.*, 1990).

Almost all uninucleate cells of RA376 and RA614 with a normal nucleus possessed a single MTOC (RA376: 97%, $n = 260$; RA614: 86%, $n = 282$; Fig. 7a, a'). Amongst cells with two normal nuclei, both strains showed variation in MTOC number. The frequencies in RA376 ($n = 156$) and RA614 ($n = 44$) respectively were as follows: 0 MTOC 13%, 14%; 1 MTOC 64%, 70%; 2 or more MTOCs 23%, 16%. These observations reinforce the conclusion from studies of mitotic cells that the developmentally associated microtubule reorganization is initiated in RA614.

Many of the cells of both strains with condensed nuclei, or no nucleus, possessed abnormally thick microtubules (2–3 times as thick as normal microtubules; Fig. 7c, c'; e, e'). It was not possible to tell whether these thick structures were composed of bundles of microtubules of normal thickness or single, abnormally thick microtubules. Most of the anucleate cells did not have a MTOC (RA376: 11/19; RA614: 15/16; Fig. 7c, c') and the rest had only one. For both strains, the majority (55–66%) of the cells with condensed nuclei possessed one or more MTOCs (Fig. 7e, e'); this is a lower frequency than observed for cells with one or two normal nuclei. This difference may, however, be a consequence, rather than a cause of cell degeneration. A few cells with more than two condensed nuclei were observed (Fig. 7b, b'; d, d') in *npfL1* cultures.

Discussion

Genetic analysis indicated that the *npfL1* mutation carried by RA614 was a single gene mutation unlinked to any of the marker genes tested. Abnormal development of strains carrying *npfL1* ended in cell death; this is the first report of such a developmental mutant in *Physarum polycephalum*. Our study of the events leading to cell death in RA614 showed that the *npfL1* mutation did not affect amoebal proliferation, and that development in the mutant was identical to that in the *npfL1*⁺ parent until the mitosis at the end of the extended cell cycle. After the end of the long cell cycle in developing cells of RA614, a number of developmental events occurred. Events typical of plasmodium development included disappearance of the amoeba-specific $\alpha 3$ -tubulin isotype; appear-

ance of the plasmodium-specific $\beta 2$ -tubulin isotype; the presence of extra MTOCs at mitosis in some developing uninucleate cells; the appearance of plasmodial (Class II) mitotic arrays; and acquisition of plasmodial fusion behaviour and the ability to ingest amoebae. Abnormal events included nuclear fusion at mitosis and in interphase; the appearance of abnormal mitotic spindles (Classes IV and V) and abnormally thick microtubules; the appearance of cells with condensed nuclei and the development of cells that rounded up and showed the 'boiling' phenotype. All developing cells in the *npfL1* cultures eventually died. The stage of plasmodium formation at which normal development ceased was variable. In *npfL*⁺ strains, there was similar variability in the timing of developmental changes in tubulin isotype expression, microtubular organization and cell behaviour, suggesting that variability was a normal part of plasmodium development (Blindt, 1987; Bailey *et al.*, 1987, 1990; Solnica-Krezel *et al.*, 1990, 1991). Our film of RA376 showed that there was also variability in the events at the end of the extended cell cycle; some developing cells divided rather than becoming binucleate and later became binucleate without passing through another long cell cycle. It was not surprising, therefore, that the time at which developmental abnormalities were first observed in RA614 was also variable.

Even after *npfL1* cells became visibly abnormal, some facets of normal plasmodium behaviour were observed, such as plasmodial fusions and ingestion of amoebae. These observations suggest that plasmodium development involved a number of pathways, some of which could continue even though other pathways were blocked in the absence of the *npfL*⁺ gene product. This may make it difficult to deduce where the primary lesion lies. Many of the features of abnormal development in RA614 were also shown by a minority of RA376 cells. Events occurring exclusively in RA614 were nuclear fusion at mitosis and in interphase, and the formation of multipolar Class V spindles.

Since both amoebal and plasmodial nuclei possess a MTOC that organizes the spindle (Havercroft & Gull, 1983), fusion of nuclei at mitosis or in interphase would result in polyploid nuclei with multiple MTOCs (one from each of the fusing nuclei), and some or all of the MTOCs could remain active. Akhavan-Niaki *et al.* (1991) demonstrated that the presence of multiple MTOCs in amoebae or plasmodia led to the formation of spindles with three or more poles. A few of the multipolar spindles (Class V) seen in RA614 were tripolar with the DNA equally distributed between the poles; this type of spindle is characteristic of diploid amoebae (Lemoine *et al.*, 1984; Bailey *et al.*, 1990) which occur at a low frequency even in vegetative cultures of amoebae. Most multipolar spindles seen in RA614, however, occurred in

developing cells and had more than three spindle poles with the DNA unequally divided between the poles (Fig. 6). This type of spindle has not been reported before and appears to be specific to development in *npfL1*. It seems likely that the multipolar spindles were a consequence of the nuclear fusions that were specific to developing *npfL1* cells.

Nuclear fusion at mitosis in RA614 could have occurred by spindle fusion. In the ATS23 temperature-sensitive mutant (Burland *et al.*, 1981), cytokinesis failed in amoebae grown at the restrictive temperature, resulting in multinucleate amoebae that later underwent nuclear fusion at mitosis, presumably as a result of spindle fusion. Spindle fusion would explain the observation that, during filming, two RA614 cells entered mitosis with four nuclei but reconstructed only two giant nuclei. However, such fusions could only occur in cells undergoing astral amoebal mitosis and not in cells undergoing intranuclear plasmodial mitosis and thus would not normally occur in multinucleate plasmodia. Our observations indicated that some mitotic multinucleate *npfL1* cells formed amoebal spindles in which asters radiated from the spindle poles; it seems likely that spindle fusion would occur in these cells. Thus, a characteristic of some developing *npfL1* cells may be an incomplete alteration to the plasmodial type of spindle.

Nuclear fusion at interphase normally occurs only in sexual interactions. When two amoebae carrying different *matA* alleles fused, nuclear fusion followed, in interphase, about two hours after amoebal fusion (Bailey *et al.*, 1990). Since ability to undergo interphase nuclear fusion occurred only in *matA*-heteroallelic fusion cells, fusion may have been switched on by the presence of two unlike *matA* alleles in the same cell and was presumably switched off by the time of binucleate plasmodium formation. If, as a result of the *gad* mutation, a corresponding period of nuclear-fusion competence occurred in apogamic development, there would not usually be any consequence because the cells would be uninucleate at that time. If, however, the *npfL1* mutation prolonged the period of nuclear-fusion-competence, the observed interphase nuclear fusions would occur. The *npfL1* mutation may thus affect a developmentally associated alteration in nuclear structure.

Although nuclear fusion and multipolar spindles were observed only in RA614, a number of other abnormal features (nuclear condensation and the terminal 'boiling' phenotype) seen in developing RA614 cells were also observed at low frequency in RA376. Mutation in the *npfL* gene was thus not the only possible cause of these events. These features are also characteristic of cells dying by apoptosis (Kerr *et al.*, 1972), a process of cell death observed during morphogenesis in many eukaryotes (Giorgi & Deri, 1976; Robertson & Thomson,

1982) and in cells exposed to a number of cytotoxic agents and during tumour growth (Sorensen *et al.*, 1990; Martin *et al.*, 1990). There are typically three phases of apoptosis (Wyllie, 1988); firstly, chromatin condensation, nucleolar disintegration and a reduction in nuclear and cell size, with an increase in cytoplasmic density and extensive blebbing of the cytoplasm; secondly, cells may fragment into small membrane-bound apoptotic bodies which, in tissues, are phagocytosed and degraded by neighbouring cells; thirdly, the residual nuclear and cytoplasmic structures disintegrate as the cells swell and the membranes rupture.

Sanderson (1976) showed by time-lapse cinematography that mastocytoma cells in apoptosis exhibit cytoplasmic 'boiling' and an increased cytoplasmic density remarkably similar to that shown by *npl/L1* cells. Some terminally differentiated RA614 cells burst after the onset of cytoplasmic 'boiling'. The condensed nuclei seen in our study appeared similar to nuclei isolated from apoptotic cells (Arends *et al.*, 1990); both are about half the area of normal nuclei, lack nucleoli and stain intensely. In the later stages of apoptosis, nuclear fragmentation occurs (Wyllie, 1988). The anucleate cells we observed after DAPI staining could have resulted from nuclear fragmentation in cells with condensed nuclei.

Many cells with condensed nuclei were characterized by the presence of unusually thick microtubules. Although microtubules have not been studied in detail in apoptotic cells, it is interesting to note that some apoptotic cells contain bundles of cytoplasmic fibrils of unknown composition (Wyllie *et al.*, 1984) and that apoptosis can be induced by treatment with microtubule-disrupting agents but not by disruption of microfilaments (Martin & Cotter, 1990). The similarities between apoptosis and the changes observed during abnormal development in RA614 and RA376 suggest that cell death by an apoptosis-like mechanism may occur as a consequence of the breakdown of normal plasmodium development.

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