Genesis of a spatial pattern in the cellular slime mold
Polysphondylium pallidum

(lateral inhibition/reaction-diffusion/prepatterns/morphogenesis/symmetry breaking)

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ABSTRACT  The branches in Polysphondylium pallidum whorls are arranged in a radial pattern. We have used a pattern-specific monoclonal antibody to study branch formation and characterize the origin of this pattern. A quantitative spatial analysis of antibody staining reveals that the branching pattern arises from a random distribution. This distribution passes through a series of intermediate stages to yield a radial prepattern. The origins and evolution of this prepattern are satisfactorily accounted for by models that produce spatial patterns by short-range autocatalytic and longer-range inhibitory forces.

The mature fruiting body of Polysphondylium pallidum is composed of a central cellulose stalk along which 1–10 or more sets of branches are arrayed at regular intervals along the central stalk. Each branch terminates in a sorus of spores. Formation of the radial branch pattern begins with the segregation of a whorl mass from the base of the sorogen (Fig. 1). Soon after separating from the sorogen, one to eight tips form in a radial pattern about the equator of the whorl (Fig. LA). Each tip organizes the formation of a branch so that the initial whorl mass is equally partitioned, with each branch containing a similar number of spores (1). The number of tips that eventually arises is a function of the surface area of the whorl (2). It is not related to the number of tips present on neighboring whorls (1), and consequently the formation of tips represents a true symmetry breaking event in which the spheroidal symmetry of the whorl is transformed, by the initiation of tips, into the radial symmetry of the branch pattern.

In this report, we analyze the spatial distribution of tip-specific antigens over the equatorial surface of a series of whorls prior to and after the formation of tips. We use Fourier analysis to quantify the spatial distribution of antigen and compare our results to a simple model. Using this unbiased measure of the spatial pattern, we show that initially the spatial prepatternning of tip-specific antigens is randomly distributed about the equator of the whorl. This random prepattern subsequently evolves into a periodic radial prepattern. The essential features of our results are captured by models that form prepatterns of spatio-temporal information through local autocatalysis and long-range lateral inhibition (3–5).

MATERIALS AND METHODS

Strain. PN500 was used throughout (1).

Antibody Strains. Sorogens were methanol-fixed and incubated overnight with anti-Pg101 diluted in phosphate-buffered saline (PBS) containing 2% bovine serum albumin, as described (6). The samples were washed with PBS and incubated overnight with affinity-purified biotin-conjugated sheep anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA). Specimens were washed with PBS, incubated for 20 min with Texas-red-conjugated streptavidin (Amersham), washed in PBS, and mounted in a solution of glycerol/PBS (9:1) containing 1.0 mg of p-phenylenediamine per ml (7).

Quantitative Analysis of Antigen Distribution. Anti-Pg101-stained whorls and sorogens were immersed in glycerol and mounted in a pulled 20-μl capillary (Corning 7099-S; inner diameter, ~85 μm). The capillary was placed on a microscope slide in a drop of glycerol, covered with a coverslip, and the free end was attached to a micromanipulator. Whorls were photographed every 22.5° as the specimen was rotated about the axis of the central stalk. Eighteen photographs of the proximal whorl surface were taken through a Zeiss ×25 oil/glycerol/water immersion lens using a constant shutter speed. A photomontage of the equatorial image was constructed and scanned with a Perkin-Elmer PDS photodigitizer. Image density was measured using a 20 × 20 μm slit. This typically resolved the negative into 170 × 300 picture elements and 256 grey levels. To map the antigen density, each picture element was assigned a value of 0, 1, 2, or 4 corresponding to background, dim, bright, and very bright pixels. The initial density distribution is the sum of these values for each column of the digitized image. To compute the power spectrum, the density distribution was smoothed with a running average. Window size was the average cell diameter (3.5 μm for these fixed preparations). Any trend in the density data was removed by subtracting the best-fit linear regression line prior to a direct Fourier transformation of the smoothed density profile (8). The highest detectable Fourier frequency is limited by the Nyquist theorem (8), which in this case is ~7 μm, or a frequency equal to half the number of cells on the equator of the whorl. The power spectrum was normalized according to Shimshoni (9).

RESULTS

Tip Prepatterning. The monoclonal antibody anti-Pg101 detects an antigen (Pg101) present at high levels in tips and distributed as a shallow gradient along the anterior–posterior axis of the sorogen (6). Whorls stained with anti-Pg101 first display a band of increased fluorescence on their equatorial surface (Fig. 1B b–e). This band is detected well in advance of tip formation, usually before the whorl detaches from the sorogen. The fluorescence intensity of the equator is greater than the intensity observed in both the off-equatorial surface of the whorl, and the most basal section of the sorogen. Over time, the band fragments into a series of densely clustered fluorescent spots. These antigen clusters are restricted to the surface of the whorl, and each cluster generally represents the position where a new tip will form (6). Anti-Pg101 was

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chosen for this analysis because it detects tip-specific anti-
gens at a very early stage of prepatter formation, and
because the staining is punctate. Thus, quantification of the
anti-Pg101 staining pattern depends primarily on the spatial
density of antigen spots and not graded differences in
fluorescence intensity.

The equatorial distribution of anti-Pg101 specific antigen
was recorded by repeatedly photographing stained whorls
rotated in thin capillaries (see Materials and Methods). This
method and our analysis are illustrated in Fig. 2, where the
equator of a stained thorn with four visible regularly spaced
tips is shown. Due to a slight misalignment of the whorls in
the capillary, three of the tips are viewed end on, while the
fourth is viewed from the side. Each tip is brightely labeled,
and there is very little staining on the intertip surfaces.

The distribution of Pg101 was analyzed by first reducing
the pattern of stain to a plot of the relative antigen density
(ordinate) versus distance in radians about the equator
(abcissa) using a digitized copy of the original photomontage
(Fig. 2b). A direct Fourier transformation of the relative
density distribution was used to calculate a power spectrum
(Fig. 2c). This transformation decomposes the density dis-
tribution into a series of sine and cosine waves, each with an
integer number of wavelengths about the circumference.
When summed, these waves accurately describe the original
distribution (Fig. 2b, dotted line). As expected for a thorn
with four visible tips, the fourth Fourier frequency is the
strongest component in the power spectrum. This frequency
coresponds to a period of 45.5 μm. There is also significant
power in the fifth Fourier frequency (36.4 μm). Power in this
frequency is required to account for the imperfect spacing of
tips and the small patch of fluorescence to the left of one tip.
Using a weighted average of these periods, we estimate the
average intertip spacing on this thorn to be 41.7 μm. This
wavelength is likely to be an overestimate of the spacing
originally present in the prepatter, since the arc separation
between tips increases as the growing tips elongate.

Spectral Analysis. Using these methods we analyzed the
spatial distribution of anti-Pg101 stain on the equator of 15
whorls. Fig. 3 illustrates the patterns for 10 of them. These
whorls can be arranged in three groups. Whorls 1–3 (Fig. 3
a–c) have visible tips (whorl 1 is also shown in Fig. 2). The
remainder do not. Whorls 4–6 exhibit some degree of
periodicity (Fig. 3 d–f), while no strong periodic patterns are
present in whorls 7–10 (Fig. 3 g–i). Whorl 11 was stained with
Hoechst nuclear stain (Fig. 3e) to indicate the number and
distribution of cell nuclei. This thorn was at a developmental
stage comparable to the thorn in Fig. 1B, and to whorls 4–10
in Fig. 3. During the formation of a Pg101 prepatter, there is
no apparent correlation between antigen clusters and the
density of nuclei. The power spectra for whorls 4–7, 9, and
10 are shown in Fig. 4. Table 1 summarizes the major features
of the spectral analysis for each of these whorls.

These results demonstrate that when an equatorial band
of Pg101 is first observed on the incipient thorn, the band is
composed of randomly distributed punctate stain. With time
this equatorial band evolves into a periodic prepatter.
Whorls with visible tips always exhibit the densest antibody
labeling in their tip regions with reduced but variable amounts
of stain on the intertip surfaces (Fig. 3 a–c). Spectral analysis
of whors 1–3 emphasizes their periodic nature (Table 1).

Prior to tip formation whorls also display a periodic
antibody staining pattern. The periodic prepatter is rather
diffuse and extends around the entire circumference when
first detected (Fig. 3f). Over time this diffuse prepatter
appears to sharpen (Fig. 3 d and e). Spectral analysis of
whorls 4–6 (Fig. 4 a–c) reveals an average periodicity of 39.8
μm (Table 1), which is not significantly different from the
periodicity observed in more advanced whorls with visible
tips (P > 0.5 by a two-way t test of the means).

When we first detect a bright equatorial band of Pg101, the
spatial patterns are much more variable. Whorls 7–10 in Fig.
3 illustrate the range of patterns we have observed. Whorl 7
has two regions of localized anti-Pg101 stain and a third
diffuse area of stain. In its power spectrum (Fig. 4d) the
fourth Fourier frequency is just beneath the 5% significance
level. It appears that this thorn was still regulating its
prepatter, which may ultimately have developed four or
possibly three antigen clusters. Whorl 8 has a uniform level
of anti-Pg101 stain about its circumference underlying at least
two and possibly three patches (Fig. 3h). Unlike the other
whorls in Fig. 3, these Pgl101 patches are clustered on one
side of the whorl. This clustering gives rise to a power
spectrum with a significant amount of power in a higher
harmonic, the sixth Fourier frequency (29 μm). The other
striking aspect of this power spectrum, which is qualitatively
similar to Fig. 5b (τ = 80), is that 52% of the power is
uniformly distributed over the first to fifth and seventh to
ninth Fourier frequencies. Although the power spectrum of
whorl 8 exhibits a strong sinusoidal component, the Pgl101
pattern on the whorl (Fig. 3h) shows that this periodicity does
not extend about the entire circumference. It therefore seems
likely that a period of 29 μm reflects the inhomogeneities
initially present in whorl 8 rather than the equilibrium
pattern.

Whorls 9 and 10 do not exhibit obvious periodic patterns,
although some local regions of dense stain are apparent. The
spatially random nature of this stain is clear in the power
spectra for these whorls (Fig. 4 e and f). The power spectrum
for whorl 9 is broadly distributed among the lower Fourier
frequencies with no statistically significant single frequency.
The spectrum for whorl 10 is similar, although there is
somewhat greater power in the higher frequencies.

Circumference (Radians)

FIG. 2. Anti-Pgl101-specific stain on a whorl with well-developed
tips. (a) Immunofluorescent photomontage of the equatorial whorl
surface. Four brightly stained tips are present. (b) Solid line is the
density distribution of anti-Pgl101 stain about the circumference of
the whorl. Dotted line is a reconstruction of the original density
distribution using the Fourier frequencies from the power spectrum.
(c) Normalized power spectrum of b. An asterisk indicates the
Fourier frequency with power significantly above the random
expectation (9) (P > 0.05). Over 50% of the power is contained in the
fourth and fifth Fourier frequencies. These two frequencies largely
delimit the number and position of the antigen peaks in b. Power in
the other Fourier frequencies is required for accurate determination
of peak shapes. (Inset) Bright-field photomicrograph of the whorl
during rotation. (Bar = 40 μm.) The equatorial distribution of
anti-Pgl101-specific antigen was recorded by repeatedly photographing
stained whorls rotated in thin capillaries (see Materials and
Methods).

FIG. 3. False-color images of anti-Pgl101 immunofluorescence.
(a–c) Late whorls with well-established tips. (d–j) Equatorial anti-
Pgl101 staining on whorls prior to tip formation. (k) Hoechst nuclear
staining pattern around the equatorial surface of a whorl prior to tip
formation. Numbers in the upper right corner of each panel are used
to identify the whorls throughout this report. The circumference of
each whorl is listed in Table 1.
cells, small random differences exist. The initial distribution is transformed into a periodic one by autocatalysis and lateral inhibition. To compare model spatial patterns to the Pg101 patterns discussed here, we modeled the whirl equator as a ring of cells and simulated tip formation using a set of reaction–diffusion equations. Although other models, and other formulations of reaction–diffusion theory, could have been used to illustrate the general character of our results, we use this particular version because it is computationally simple, and because we have used it in another study to examine the relationship between the regularity of radial patterning and the variance in the initial morphogen concentration at \( t = 0 \). It is also worth pointing out here that for an organism the size of P. pallidum, it is realistic to imagine that diffusing chemicals can operate over the required distances in the required time \((11)\).

Fig. 5a illustrates morphogen concentration over time for one such simulation. This example displays a wide variety of intermediate periodic patterns. There are three morphogen peaks at equilibrium (Fig. 5b; \( t = 1600 \)). The initial morphogen profile (Fig. 5b; \( t = 0 \)) was chosen to be statistically uniform. As expected, Fourier transformation of this profile resulted in a power spectrum with no favored frequency. The first obvious periodic pattern has seven morphogen peaks (Fig. 5b; \( t = 80 \)). Each of these peaks is present, though less evident, in the initial morphogen profile. At this early stage, the periodicities detected by Fourier analysis are often related to the initial morphogen distribution rather than the equilibrium pattern. Over time several morphogen peaks are suppressed, and Fourier analysis reveals a consistent selection for lower frequencies. This often leads to a time when there is clearly an emerging pattern, yet no single Fourier frequency in the power spectrum is significant (Fig. 5b; \( t = 400 \)). This stage is inevitably followed by growth of a lower frequency, which persists to equilibrium (Fig. 5b; \( t = 1600 \)). There is significant agreement between this model and the experimental results. First, the power spectra for whirls 9 and 10 reveal no evidence for a radial prepattern, and thus we can conclude that the very early origins of the tip pattern lie in an almost constant distribution of antigen about the equator. This was Turing’s postulate \((3)\). Second, during the evolution of the radial prepattern, both in simulation and experiment, nonequilibrium frequencies grow and then disappear, as evidenced by the shift in the power spectra with time from higher to lower frequencies (Figs. 4 and 5). And finally, it is characteristic of our results that the regularity of

**Table 1. Summary of spectral analysis**

<table>
<thead>
<tr>
<th>Whorl</th>
<th>Circumference, ( \mu m )</th>
<th>Tips (patches)*</th>
<th>Significant frequencies†</th>
<th>Spacing, ( \mu m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>182</td>
<td>4 (4)</td>
<td>4, 5 (0.543)</td>
<td>41.7</td>
</tr>
<tr>
<td>2</td>
<td>197</td>
<td>5 (5)</td>
<td>5 (0.580)</td>
<td>39.4</td>
</tr>
<tr>
<td>3</td>
<td>123</td>
<td>3 (3)</td>
<td>2, 3 (0.631)</td>
<td>53.4</td>
</tr>
<tr>
<td>4</td>
<td>183</td>
<td>— (5)</td>
<td>4, 6 (0.663)</td>
<td>40.3</td>
</tr>
<tr>
<td>5</td>
<td>211</td>
<td>— (5)</td>
<td>5 (0.412)</td>
<td>42.2</td>
</tr>
<tr>
<td>6</td>
<td>222</td>
<td>— (6)</td>
<td>6 (0.217)</td>
<td>37.0</td>
</tr>
<tr>
<td>7</td>
<td>129</td>
<td>— (3)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>137</td>
<td>— (3)</td>
<td>6 (0.317)</td>
<td>29.0</td>
</tr>
<tr>
<td>9</td>
<td>146</td>
<td>— (–)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>201</td>
<td>— (–)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>117</td>
<td>— (–)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

A summary of the power spectra for anti-Pg101-stained whirls. Whorls are numbered according to Fig. 3.

*The number of tips, or antigen patches (in parentheses), is given.
†The significant Fourier frequencies were determined using the methods described by Shimshoni (9). The number in parentheses is the fraction of the total power contained in the significant frequencies. When two significant frequencies are present, the spacing is a weighted average of their periods. The average spacing of tips in whorls 1–3 is 44.8 \( \mu m \) (SD = 7.5). The mean spacing of antigen patches for whorls 4–6 is 39.8 \( \mu m \) (SD = 2.6). Using a two-way \( t \) test, these mean values are not significantly different \((P > 0.5)\).**

**DISCUSSION**

The Fourier transformation of Pg101 density profiles was particularly valuable during the initial stages of prepattern formation when periodicities were not obvious (Fig. 3 e and \( f \)) or absent (Fig. 3 g–j). A modified version \((9)\) of Fisher’s test \((10)\) was used to determine which frequencies in the power spectrum were significantly more powerful than the random expectation. By this test we conclude that tip-specific antigens are initially expressed in a random fashion (Fig. 4 e and \( f \)). The subsequent transformation of this initial pattern into a periodic prepattern is not entirely synchronous (Fig. 3 g–j) but, in general, occurs uniformly about the circumference. We emphasize that the prepattern is established before tips or their visible precursors can be seen.

The transformation of the initial distribution of Pg101 into a periodic prepattern is strikingly similar to the pattern-forming process observed in models first formulated by Turing \((3)\), Gierer and Meinhardt \((4)\), and Oster et al. \((5)\). These models postulate that within a homogenous field of
the final pattern—the extent to which the radial spacing of tips, arms, and patches of Pg101 deviate from perfect spacing, as judged by nearest-neighbor analysis—exhibits the same degree of order for both the model and the experiments (ref. 1; and results not shown). In sum, the final distribution of tips on whorls, the angle between arms within whorls, the partitioning of whorl cells into arms, and the distribution of tip-specific antigens throughout the prepatterning stages are accurately described by generic autocatalysis lateral inhibition models. We note that many different formulations of reaction–diffusion systems (13, 14), the chemotactic model of Keller and Segel (15), and the mecanochemical models of Oster et al. (5) and Odell and Bonner (16) might be expected to produce broadly similar results.

This study does not attempt to answer the question of whether the spatio-temporal signals required for tip formation are derived from diffusing morphogens, as predicted in a reaction–diffusion model, or from the cell–cell and cell–substrate interactions predicted by mecanochemical models. Our observations that a radial prepatterning is established before we see tips or bulges on the whorl mass suggests that the prepatterning is established by a reaction–diffusion-like mechanism, since in most mechanical models visible changes are expected to accompany symmetry breaking. It is clear, however, that the patterning of tips on whorls cannot be accurately described by models based on the activities of a few cells (17, 18), growth of crystalline-like precursors (19), or cell-lineage processes. These models all predict that the spatio-temporal signals for pattern formation arise locally, in contrast to the global Pg101 patterns we observe.

Patterns with similar origins have been described for the early expression of two Drosophila segmentation genes (20–22). Transcripts from both the fushi tarazu and hairy genes are initially expressed in an apparently random manner, over a broad region of the Drosophila blastoderm. Over time this diffuse pattern evolves into the seven abdominal bands with an additional area of expression in the head region for hairy. The techniques described in this report can be used to analyze the origins of these patterns and to quantify the spatial characteristics of the evolving prepatterning.

To determine in detail the patterning mechanism for tip formation of P. pallidum whorls, several fundamental questions must still be addressed. What contributions do cellular motion and antigen turnover make in establishing the periodic prepatterning? What functions do these antigens have, and what other tip-specific antigens exist? Is the rapid appearance of these antigens the result of new expression or local modification of existing cell products? We are pursuing answers to these questions through both a detailed morphological and molecular analysis of the early patterning stages in hope of determining the character of the initial symmetry breaking event.

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