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# *lagC*-Null and *gbf*-Null Cells Define Key Steps in the Morphogenesis of *Dictyostelium* Mounds

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**The transition to multicellularity is a key feature of the *Dictyostelium* life cycle, and two genes, *gbf* and *lagC*, are known to play pivotal roles in regulating this developmental switch. *lagC*-null and *gbf*-null cells fail to induce cell-type-specific genes ordinarily expressed during multicellular development. The null mutants also share a similar morphological phenotype: mutant cells repeatedly aggregate to form a loose mound, disperse, and reform a mound, rather than proceeding to form a tip. To characterize defects in morphogenesis in these mutants, we examined cell motion in the mutant mounds. In analogy with the failed transition in gene expression, we found that *lagC*-null and *gbf*-null mounds failed to make a morphogenetic transition from random to rotational motion normally observed in the parent strain. One reason for this was the inability of the mutant mounds to establish a single, dominant signaling-wave center. This defect of *lagC*-null or *gbf*-null cells could be overcome by the addition of adenosine, which alters cAMP signaling, but then even in the presence of apparently normal signaling waves, cell motility was still aberrant. This motility defect, as well as the signaling-wave defect, could be overcome in *lagC*-null cells by overexpression of GBF, suggesting that *lagC* is dispensable if GBF protein levels are high enough. This set of morphogenetic defects that we have observed helps define key steps in mound morphogenesis. These include the establishment of a dominant signaling-wave center and the capacity of cells to move directionally within the cell mass in response to guidance cues.** 

## INTRODUCTION

During *Dictyostelium* development, solitary amoebae aggregate to form a hemispherical mass of cells called a mound. The mound then undergoes dramatic morphogenesis, elongating to form a slug that ultimately differentiates into a fruiting body composed of two cell types, stalk cells and spore cells. A defining aspect of this life cycle is the transition from independent amoebae to a multicellular organism. One protein known to play a key role in this transition is the transcription factor, GBF. GBF (G-box-binding factor) is required for the induction of postaggregative genes at the mound stage and prestalk and prespore genes that are characteristically expressed at the multicellular stage (Hjorth *et al.*, 1990; Schnitzler *et al.*, 1994; Schnitzler *et al.*, 1995).

*gbf* null cells exhibit a striking phenotype: the mutant cells aggregate normally and form mounds, but the mounds do not proceed further in development and instead disperse yielding a lawn of solitary amoebae which eventually reaggregate forming new mounds which again disperse, and so on (Schnitzler *et al.*, 1994). A second gene, *lagC* (loose aggregate mutant C), has also been identified, which, when knocked out, exhibits a very similar morphological phenotype of mound dispersal (Dynes *et al.*, 1994). LagC's sequence suggests that it is a transmembrane protein with a large extracellular domain. Like *gbf*, *lagC* is also required for expression of a number of postaggregative genes and subsequent prestalk and prespore cell differentiation. Analyses suggest that it functions nonautonomously as a signaling molecule.

Gene-expression studies in both *gbf*-null and *lagC*-null lines have demonstrated the importance of these genes in regulating the developmental switch that ultimately leads to cell-type differentiation (reviewed in Firtel, 1995). Virtually nothing however is known about the role of these genes

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in morphogenesis, except that they are clearly vital given the mound-dispersal phenotype observed when either gene is knocked out. To investigate their morphogenetic role, we analyzed cell movement and cell signaling in *lagC*-null and *gbf*-null mounds. We have found, in analogy with the failed transition in gene expression, that cells from both mutants also fail to undergo a morphogenetic transition, namely the transition from random to rotational motion normally observed in mounds of the parent strain. We have also found that both mutants suffer signaling defects, specifically an inability to establish a dominant signaling center within a mound. The signaling defects in the mutants can be overcome by the addition of adenosine, which is known to suppress cAMP signaling. However, despite proper signals, directed motion is still absent. In a *lagC*-null mutant, both proper signaling and directed motion can be restored by overexpression of GBF. These sets of mutant phenotypes help define several steps in mound morphogenesis: first the establishment of a dominant signaling center and second the capacity to move directionally in response to guidance signals. Our analysis also suggests that these morphogenetic requirements can be satisfied in the absence of *lagC* if enough GBF is expressed, supporting the possibility that a key function for *lagC* may be upregulation of *gbf*.

## MATERIALS AND METHODS

### *Cell Growth, Development, and Fluorescent Labeling*

Cells were grown on plates in HL5 medium (Spudich, 1982) at 21°C. The parent strain was KAx-3. *gbf*-null cells were those described by Schnitzler *et al.* (1994). *lagC*-nulls were those described by Dynes *et al.* (1994). Methods for GBF overexpression, previously described (Schnitzler *et al.*, 1994), were used to create a *gbf*-overexpressor/*lagC*-null strain.

To induce multicellular development, cells were collected by centrifugation, washed several times in phosphate buffer (McDonough *et al.*, 1980), and then allowed to settle on a dialysis membrane laid on top of a 2% agar plate. As mounds began to form, the membranes were transferred to a coverslip that was placed in a humidified chamber optimized for microscope imaging. In some cases, dialysis membranes were transferred immediately to this chamber, and aggregation was allowed to proceed there.

To fluorescently label cells, an aliquot of cells obtained after collection and washing was incubated for 20 min in 0.1 mM CMF (5-chloromethylfluorescein diacetate, Molecular Probes, Eugene, OR). These cells were then washed several times in phosphate buffer and allowed to sit for 10–15 min in phosphate buffer to release excess dye. The labeled cells were centrifuged once more and then recombined with unlabeled cells, typically in a ratio of about 1:20 labeled:unlabeled cells. This cell mixture was then allowed to settle on a dialysis membrane as described above.

### *Time-Lapse 3D Fluorescence Microscopy*

Imaging set-up and collection were as described by Doolittle *et al.* (1995). In brief, images were collected with a cooled, scientific-grade CCD camera attached to a custom-modified Olympus inverted microscope equipped with a computer-controlled microstepping motor to control focus. An Olympus 20×/0.8 N.A. oil objective or an Olympus 10×/0.3 N.A. dry objective was used. $x$, $y$, and $z$ resolution was 1.3 $\mu$m for the 20× objective or 2.6 $\mu$m for the 10× objective. Images were typically either 256 × 256 or 128 × 128 pixels, and 16, 32, or 64 focal planes were collected depending on the size of the mound under study. Depending on the brightness of the sample, exposure time varied from 30 to 500 ms with a selected neutral-density filter (50–95%) in the light path of either a 100-W mercury arc lamp or 150-W xenon arc lamp. Intervals between 3D images also varied from 30 s to 2 min depending on the experiment. 3D images were typically processed to reduce out-of-focus light by several well-characterized restoration methods (Conchello *et al.*, 1994; Preza *et al.*, 1992; McNally, 1994). 3D cell tracking was done with custom software modified from that described by Awasthi *et al.* (1994).

### *Visualization of Signaling Waves*

The microscope was set up for bright-field microscopy, but the Nomarski prism and analyzer were pushed in partially to create a shadow on one side of the aggregate. Either the 10× or 20× objective described above was used. 2D images were acquired at 3- to 10-s intervals, and then time-lapse movies were generated in order to see the wave patterns. To make these wave patterns visible in a single image for display in a figure, we subtracted images from the time-lapse movie that were separated by 3–10 s. These difference images were then enhanced by applying a median filter and then displayed by adjusting contrast so that the final image most closely represented the wave pattern visible in the movie. The actual movies for all of the data reported are available at our web site: http://tyrone.wustl.edu or on CD-ROM by request.

### *Adenosine Treatment*

Cells were collected and washed as described above, except that 5 mM adenosine (Sigma, St. Louis, MO) was added to the phosphate buffer used for washing. In addition, 5 mM adenosine was added to the 2% agar on which the dialysis membrane containing developing cells was laid. Identical protocols were used to test the effects of cAMP on wave patterns in the mutant mounds.

### *Chimeric Mounds*

Cells of the two strains to be used were harvested, washed, and then mixed in the proportions of 10%:90%. When added to a predominantly parent strain mound, *lagC*-null cells were labeled with the fluorescent dye CMF. When added to a predominantly *lagC*-null mound, parent strain cells were used expressing a blue variant of GFP under the control of an actin 15 promoter. In some of these mounds, *lagC*-null cells were also labeled, again with CMF.

## RESULTS

### *lagC-Null and gbf-Null Cells Exhibit Disorganized Motion in the Mound*

Both *lagC*-null cells and *gbf*-null cells aggregate to form loose mounds, but the mounds then disperse (Schnitzler *et al.*, 1994; Dynes *et al.*, 1994). In an attempt to understand

why development arrests in these mutants, we used 3D time-lapse microscopy to examine cell motion in mounds of the mutant strains. In normal mounds from the parent strain, KAx-3, cells sometimes moved randomly in the loose mound (Fig. 1a), but always exhibited highly organized, rotational motion in the tight mound (Fig. 1b). In stark contrast, we found that *lagC*-null or *gbf*-null cells always moved randomly (Figs. 1c and 1d), never exhibiting rotational motion. Thus, cells in these mutant mounds appeared unable to make the transition from random to directed, rotational motion. (The actual movies for all of the data reported here are available at our web site: http://tyrone.wustl.edu or on CD-ROM by request.)

### *Signaling Is Deranged in the lagC-Null and gbf-Null Mounds*

To test if this lack of rotational motion in *lagC*-null or *gbf*-null mounds might be caused by improper signaling, we examined ''dark-field'' wave patterns in the mound (see Materials and Methods and also Siegert and Weijer, 1995). These dark-field waves are thought to reflect an underlying cAMP wave that is thought to play a role in organizing cell motion (Siegert and Weijer, 1995). In mounds of the parent strain, KAx-3, we observed dark-field waves that formed multiarmed spirals rotating either clockwise or counterclockwise (Figs. 2a–2c) about a center point (61/66 mounds). Cells normally moved perpendicular to the spiral wave fronts, thus yielding circular motion. Similar results have been obtained by Rietdorf *et al.* (1996) in Ax-3 strains.

We found that dark-field wave patterns were profoundly disorganized in mounds of *lagC*-null or *gbf*-null cells. Unlike the parent strain, the mutant mounds never exhibited a single, dominant wave center. Rather, multiple centers appeared both at different times and in different places in the mutant mounds (Figs. 2d–2i). Each of these multiple centers produced either a spiral or concentric wave pattern, with both pattern types often present in the same mound. The 12 *gbf*-null mounds examined had an average of 5–6 centers per mound; the 23 *lagC*-null mounds examined had an average of 8–12 centers per mound; the 66 parent mounds examined had an average of only a single center per mound. These observations suggest that in the mutant mounds, wave centers were unable to suppress other competing centers, with this defect even more pronounced in *lagC*-null vs *gbf*-null mounds.

In both mutants, multiple centers pulsed asynchronously and were fleeting. New centers continually emerged at new locations in the mound, and old centers disappeared. *lagC*-null centers lasted an average of 0.5–1 min, *gbf*-null centers lasted an average of 2 min, and centers in the parent normally persisted for 2–3 h. These observations suggest that waves in the mutant mounds were temporally unstable, with waves in *lagC*-null mounds even more unstable than *gbf*-null mounds.

This wave phenotype of ''percolating'' multiple centers observed in both mutants was in marked contrast to the single, persistent organizing center routinely observed in normal mounds. In rare cases (5/66), two centers were observed in mounds of the parent strain, but these two centers tended to pulse in synchrony with one center being extinguished after 10–15 min. In contrast, the multiple centers that were routinely present in *lagC*-null or *gbf*-null mounds pulsed asynchronously, and the percolation of competing centers persisted for 2–3 h. Taken together, these observations of wave patterns suggest that the *lagC*-null and *gbf*-null mutants suffer defects in the cell–cell signaling required to establish and maintain a dominant organizing center for dark-field waves in the mound.

### *Adenosine Rescues the Aberrant Dark-Field Wave Patterns*

The lack of a single, persistent wave center in the mutant cell lines suggested a failure of wave-center dominance that would normally prevent secondary wave centers from initiating. Competition among wave centers also occurs during *Dictyostelium* aggregation, and there studies have shown that 5 mM adenosine could inhibit wave-center initiation and increase aggregate territory size (Newell and Ross, 1982; Newell, 1982). In slugs, 5 mM adenosine has been shown to increase tip dominance, that is to increase the territory size over which new tips are inhibited from forming (Schaap and Wang, 1986). The slug tip is also believed to be an initiation center of signaling waves (Siegert and Weijer, 1992; Steinbock *et al.,* 1993), suggesting again that adenosine can increase the territory size of these waves. Although the specific mechanism by which adenosine increases wave-center dominance is not known, adenosine is known to noncompetitively inhibit cAMP binding to cAMP receptors (Newell, 1982; Van Haastert, 1983) and also to slightly inhibit the cAMP signaling response (Theibert and Devreotes, 1984).

To test whether the disorganized wave patterns in the *lagC*-null and *gbf*-null mutants reflected a problem with wave-center dominance, we allowed mutant cells to aggregate in the presence of 5 mM adenosine. For both mutant strains, adenosine treatment yielded significant improvements in the wave patterns. In many *gbf*-null mounds (6/10) treated with 5 mM adenosine, only a single center formed instead of the five to six unstable, competing centers observed in untreated *gbf*-null mounds. In each case where a single center formed in an adenosine-treated *gbf*-null mound, this single center produced concentric waves (Figs. 3a–3c) that persisted for as long as they were followed (at least 25 min) compared to the 2-min average center lifetime of untreated *gbf*-null mounds. Although some *gbf*-null mounds showed dramatic improvements in the wave pattern phenotype following adenosine treatment, our results were variable, as other adenosine-treated *gbf*-null mounds

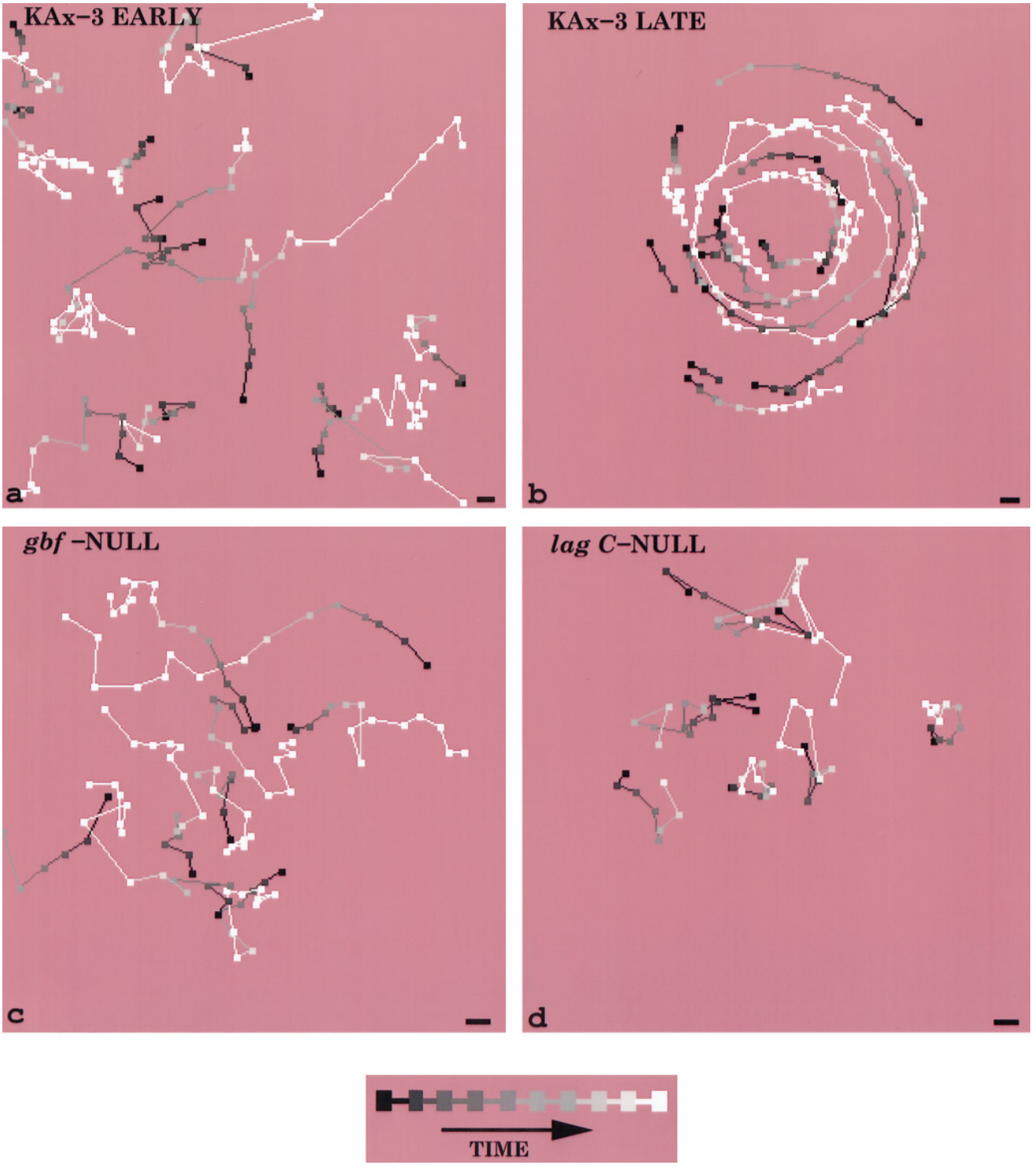


**FIG. 1.** Projected view of 3D cell trajectories from normal (a,b) and mutant mounds (c,d). Cell positions are indicated by small squares and connected at successive time points by lines. The flow of time is indicated by the transition from black to gray to white. The cell tracks represent three-dimensional data viewed looking down from the top of the mound. (a) Trajectories from an early, loose mound of the parent strain, KAx-3. Cells move randomly at this early developmental stage. (b) Same mound as in a, but 60 min later at which time a tight mound has formed. At this later developmental stage, cells from the parent strain exhibit vigorous rotational motion. (c) Random cell motion in a mound of *gbf*-null cells. Rotational motion is never observed in these mutant mounds. (d) Random cell motion in a mound of *lagC*-null cells. Rotational motion is likewise never observed in this mutant strain. Interval between time points is 3 min in a, 3 min in b, 4 min in c, and 5 min in d. Scale bars = 20 $\mu$m. (Movies for this and subsequent figures are available at our web site: http://tyrone.wustl.edu or on CD-ROM by request.)

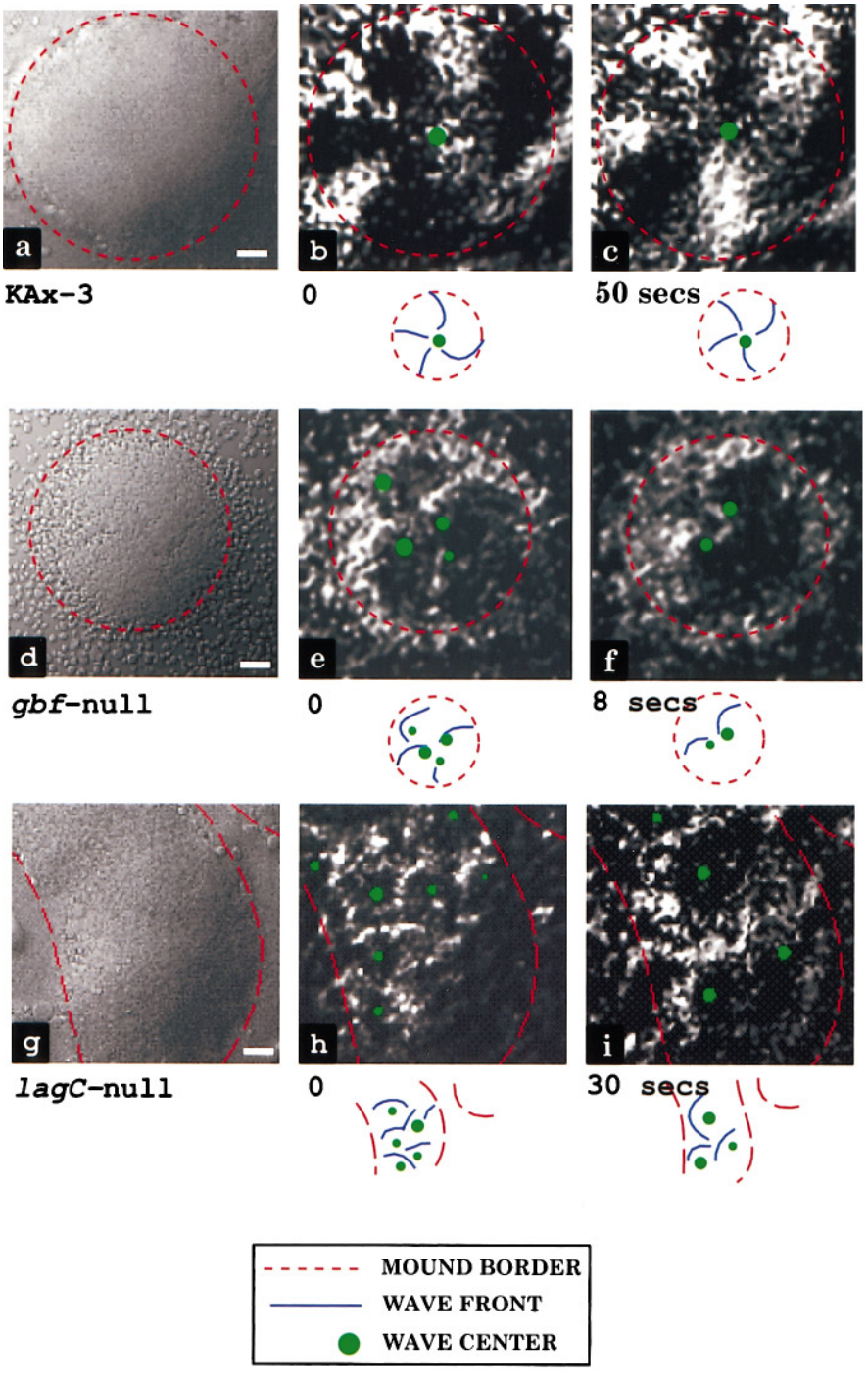


**FIG. 2.** Dark-field images of mounds and their corresponding signaling wave patterns obtained for the parent strain (a–c), for *gbf*-null cells (d–f), and for *lagC*-null cells (g–i). The wave pattern images are obtained by subtracting one dark-field image from its predecessor, and then the subtraction image is filtered using custom software (see Materials and Methods) to enhance the wave pattern. The subtraction procedure amplifies features which have changed from one image to the next, thus highlighting the changing wave profile. Dashed red lines indicate the mound boundary, and green dots indicate the wave centers as determined from time-lapse movies. Schematics of the wave patterns are shown below the wave images. (a) Dark-field image of a mound from the parent strain, KAx-3. (b,c) Subtraction images obtained by computing the difference between two consecutive dark-field images. Here the signaling wave pattern takes the form of a four-armed spiral in this KAx-3 mound. The wave is shown at two time points and rotates clockwise. Note that in this normal mound there is a single center (green dot) about which the spiral arms pivot. (d) Dark-field image of a *gbf*-null mound. (e,f) Signaling wave patterns in this *gbf*-null mound. These subtraction images reveal 2–3 wave centers (green dots) which are temporally and spatially unstable. (g) Dark-field image of a *lagC*-null mound. (h,i) Signaling wave patterns in this *lagC*-null mound. These subtraction images reveal the presence of multiple, unstable centers (green dots). Scale bars = 20 $\mu$m.

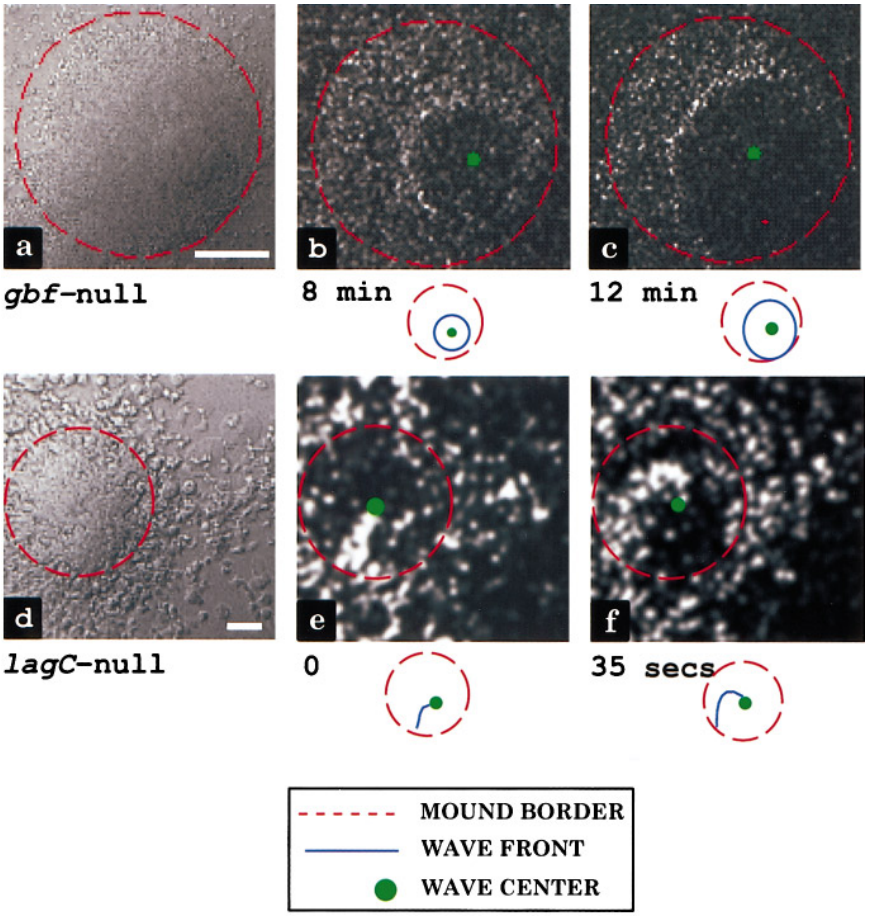


**FIG. 3.** Adenosine rescue of the aberrant wave patterns in *gbf*-null (a–c) and *lagC*-null (d–f) mounds. Procedure and labeling conventions as in Fig. 2. (a) Dark-field image of a *gbf*-null mound in the presence of 5 mM adenosine. (b,c) Signaling waves at two time points from this mutant mound. The subtraction images reveal a single center (green dot) from which a circular wave expands. Compare with Figs. 2e and 2f. (d) Dark-field image of a *lagC*-null mound in the presence of 5 mM adenosine. (e,f) Signaling waves at two time points from this mutant mound. Note again the presence of a single center about which a single-armed spiral pivots. Compare with Figs. 2h and 2i. Scale bars = 40 $\mu$m.

(4/10) exhibited absolutely no apparent improvement in aberrant wave patterns.

In contrast, for all of the *lagC*-null mounds (over 40 examined), 5 mM adenosine completely rescued the wave pattern phenotype: competing centers were absent; the one center present persisted for as long as the mound was examined (at least 30 min), and this center always produced apparently normal concentric waves or one-armed spiral waves (Figs. 3d–3f). This stable, single-wave center was in marked contrast to what was observed in untreated *lagC*-null cells. As a control, cells of the parent strain were also exposed to 5 mM adenosine. This had no effect on the wave patterns in these mounds (data not shown), although larger mounds tended to form, consistent with adenosine's known effect on territory size. As an additional control, *lagC*-null mounds were exposed to 10 mM cAMP to see if it could elicit a comparable rescue in the wave pattern phenotype. It did not. Rather, in the presence of cAMP, no waves were visible in the *lagC*-null mounds.

### *Cell Movement Is Still Aberrant in Adenosine-Treated lagC-Null Mounds*

Although adenosine rescued the wave pattern defect in *lagC*-null and *gbf*-null mounds, these mounds showed only a marginal improvement in development. Since the effects of adenosine on *gbf*-null mounds were variable, we focused attention on *lagC*-null mounds, which reliably produced normal wave patterns during adenosine treatment. Untreated *lagC*-null mounds ordinarily formed loose mounds and then dispersed, but in the presence of adenosine the mutant mounds tended to progress slightly further to the tight mound stage before dispersing. To obtain insights as

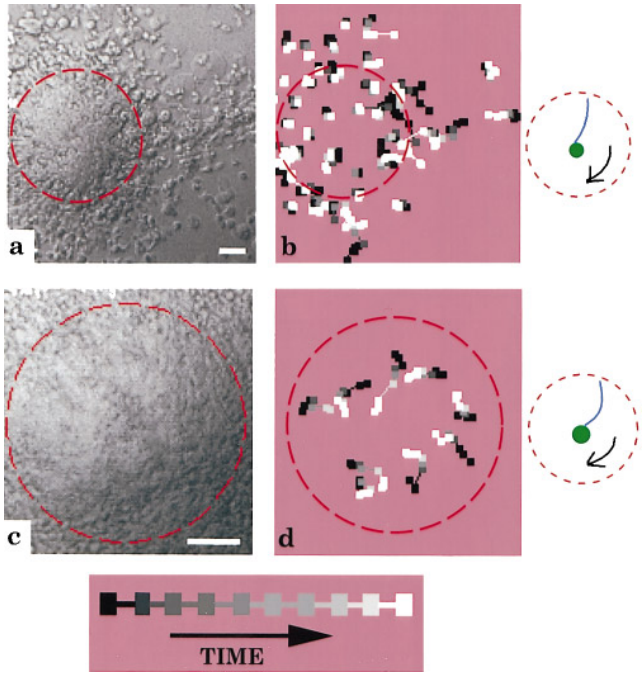


**FIG. 4.** Motion of *lagC*-null cells (b) and parent cells (d) in *lagC*-null mounds (a,c) treated with 5 mM adenosine. Dashed lines indicate the mound border, and cell trajectories are indicated as detailed in Fig. 1. (a) Dark-field image of a *lagC*-null mound treated with adenosine. (b) Projected 3D trajectories of fluorescently tagged *lagC*-null cells from the mound shown in a. Cells within the mound move poorly and show no hint of rotation, even though a single-armed spiral was observed in this mound, as in all mounds treated with 5 mM adenosine. See for example Figs. 3e and 3f. A schematic of the spiral wave in this mound is shown to the right of b. The wave movie is available at http://tyrone.wustl.edu or on CD-ROM by request. Despite the absence of significant directional motion within the mound, note that some cells outside of the mound move directionally inward. (c) Dark-field image of a chimeric mound: 90% *lagC*-null cells + 10% parent cells (d) Projected 3D trajectories of labeled parent cells in this mound. These cells show some hints of directed rotational motion. Once again a spiral wave was observed in this mound and is shown schematically to the right of d. The wave movie is available as described above. Scale bars = 40 $\mu$m.

to why *lagC*-null mounds exposed to adenosine failed to proceed even further through development despite an apparent improvement in wave signaling, we examined cell motion in adenosine-treated *lagC*-null mounds. We found that in the presence of adenosine, *lagC*-null cells moved apparently normally in streams (Figs. 4a and 4b), but that motion became constrained by the time tight mounds had formed (over 40 mounds examined). Within these tight mounds, the *lagC*-null cells moved feebly, despite normal wave patterns. Some cells moved small distances, but many cells remained almost stationary (Figs. 4a and 4b). In the parent strain, control experiments demonstrated that adenosine treatment had no effect on directional cell motion within mounds: in the presence of 5 mM adenosine, parent cells continued to rotate counter to the multiarmed spiral wavefronts.

In a further attempt to determine if the wave signal were functional in the *lagC*-null mounds treated with adenosine, we formed chimeric mounds containing 10% wild-type cells and then examined the motion of the wild-type cells. We found that these normal cells moved much greater distances and more directionally than the mutant cells (Figs. 4c and 4d), suggesting that appropriate signals for directional movement were present in these mounds (8 chimeric mounds examined) and that the *lagC*-null cells were simply unable to move in response to these signals. Control studies showed that the *lagC*-null cells continued to move feebly in these chimeric mounds and that the wave patterns in these chimeric mounds were one-armed spirals. Both of these features were characteristic of mounds composed only of *lagC*-null cells, demonstrating that the addition of 10% parental cells had not changed the basic signaling properties of the mound.

To test further the hypothesis that the *lagC*-null cells were unable to move in response to guidance signals in the tight mound, we mixed a low percentage of the *lagC*-null cells (10%) with wild-type cells (90%) and found that the

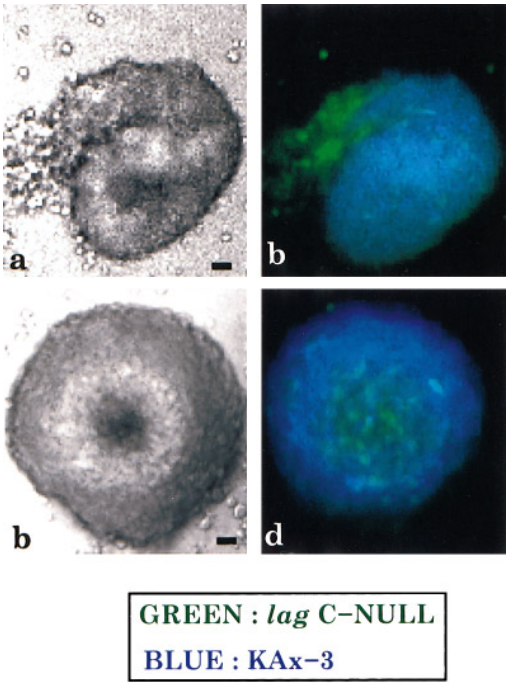


**FIG. 5.** Distribution of *lagC*-null (green) and parent cells (blue) in tight mounds undergoing rotation. The chimeric mounds are composed of 90% parent cells and 10% *lagC*-null cells. In these mixed mounds (bright-field images in a and c), the mutant cells became relegated to either the outer periphery (b) or the inner core (d) of the mound. Scale bars = 20 $\mu$m.

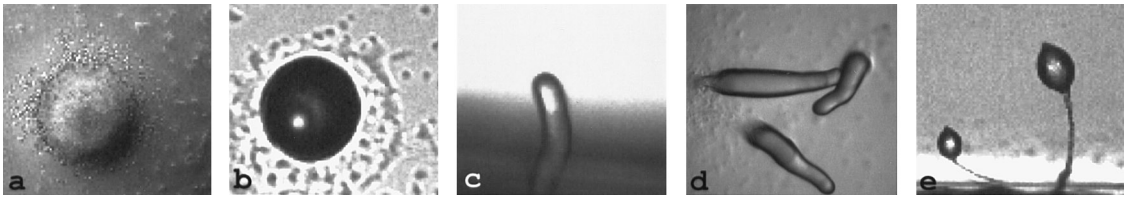


**FIG. 6.** Development is normal in the *lagC*-null/*gbf* overexpressors. (a) Mound (11 h). (b) Tipped mound (12.5 h). (c.) First finger (13 h). (d) Migrating slug (13.5 h). (e) Fruiting body (19 h).

mutant cells were always segregated to the mound periphery or mound center (Figs. 5b and 5d) (8 chimeric mounds examined). This segregation typically occurred during the period of rotational motion. Some mutant cells were observed to rotate with the parent cells, but most other mutant cells did not rotate and instead drifted to the inner core or outer edge of the mound, despite the fact that neighboring wild-type cells were rotating vigorously. Eventually all mutant cells suffered this fate, suggesting that they could not sustain rotational motion and/or that they segregated based on differential-adhesive preferences.

### *GBF Overexpression in lagC-Null Rescues Defects in Both Signaling and Directional Motion*

One possible role for *lagC* function is to enhance GBF expression and its downstream functions (Firtel, 1995). Consistent with this, we found that *lagC*-null cells overexpressing GBF appeared to undergo normal development (Fig. 6), in particular forming comparable structures at comparable rates in side-by-side wild-type controls. This suggests that *lagC* is dispensable as long as GBF levels are high enough.

To determine whether the morphogenetic defects of *lagC*-nulls were completely rescued by GBF overexpression, we examined signaling waves and cell motion in mounds of this strain. We found that a single, dominant center was consistently observed in these mounds (Figs. 7a and 7c) (41 mounds examined), demonstrating that GBF overexpression rescued the multiple-center phenotype of the *lagC*-nulls. However, some differences in wave patterns were observed compared both to adenosine-treated *lagC*-nulls and to the parental strain. Whereas adenosine treatment consistently yielded single-armed spirals in the *lagC*-nulls, GBF overexpression yielded either single-armed spirals in 10 mounds (Fig. 7a) or concentric waves in 21 mounds (Fig. 7c). The parental strain typically yielded multiarmed spirals. Concentric waves, however, are quite common in closely related *Dictyostelium* laboratory strains (Siegert and Weijer, 1995), so their appearance in the *lagC*-null/*gbf* overexpressor probably does not reflect a radical shift in signaling.

Cell motion in the *lagC*-null/*gbf* overexpressors appeared completely normal. In mounds producing spiral waves, cell motion was rotational and counter to the spiral wavefronts (Fig. 7b). In mounds producing concentric waves, cell motion was largely random with some radially inward and outward motion (Fig. 7d). The latter motion is very similar to the motion of other *Dictyostelium* laboratory strains, which ordinarily produce concentric wavefronts (Siegert and Weijer, 1995; Kellerman and McNally, unpublished observations). Thus, GBF overexpression in a *lagC*-null background appears to restore essentially normal signaling and cell motion.

## DISCUSSION

Previous work (reviewed in Firtel, 1995) has demonstrated that there is a developmental switch in *Dictyostelium* that regulates the conversion between expression of early genes required for aggregation to late genes required for multicellularity. In parallel, examination of cell motion during *Dictyostelium* mound formation has provided evidence for a morphogenetic switch that governs a transition from random to organized motion in the mound (Rietdorf *et al.*, 1996; Kellerman and McNally, unpublished observations). Our studies here show that two genes, *gbf* and *lagC*, are required for this morphogenetic transition in the KAx-3 strain. Cells lacking either of these genes form mounds in which motion is only random and a transition to rotation never occurs. These same two genes play key roles in regulating the transition in gene expression patterns that accompanies the transition to multicellularity. Thus, the failed transition to late gene expression appears to have a morphogenetic counterpart, the failed transition to rotational motion. Our data suggest that this block in morphogenesis reflects failures in both cell–cell signaling and cell motility that may be mediated by the expression of new genes ordinarily regulated by *gbf* and *lagC* (see below).

In our analysis of cell motion in mounds of *lagC*-null or *gbf*-null cells, we observed only random motion. Our examination of signaling waves in these mounds suggested that this random motion was caused at least in part by disrupted signaling. Ordinarily, in the parental strain KAx-3, dark-field waves in the form of rotating multiarmed spirals are observed. Several lines of evidence suggest that these dark-field waves reflect an underlying cAMP signaling wave in mounds (Siegert and Weijer, 1995, 1997). We found that these dark-field waves are profoundly altered in

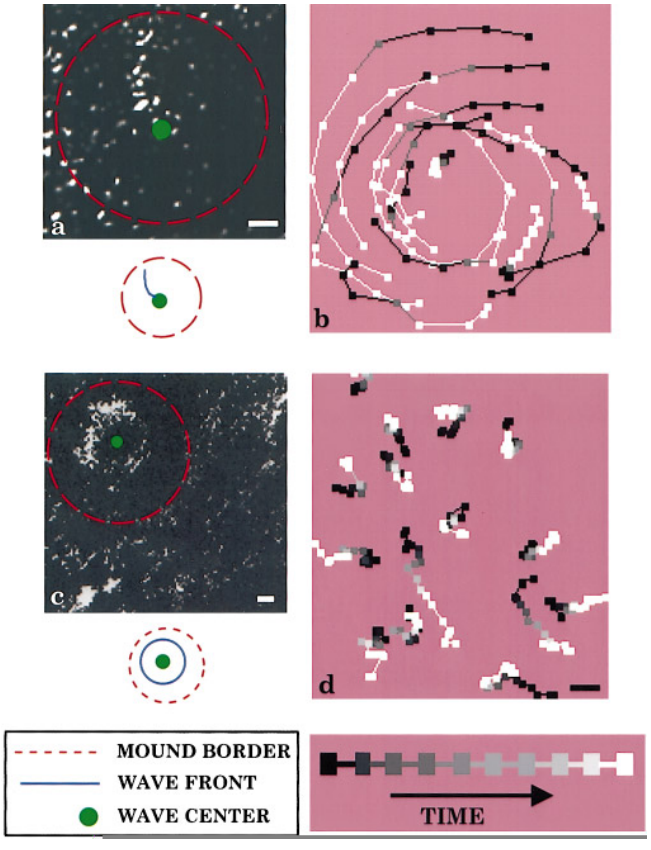


**FIG. 7.** Wave patterns and 3D cell motion in mounds of *lagC*-null/*gbf* overexpressors. (a) A single, dominant center exhibiting a one-armed spiral wave in a *lagC*-null/*gbf* overexpressor mound. The image was produced by subtraction of two dark-field images obtained 3 s apart. (b) Cell trajectories from the mound in a. Cells respond appropriately to the spiral wave and exhibit rotational motion. Time points are 3 s apart. (c) A single, dominant center exhibiting a concentric wave in a *lagC*-null/*gbf* overexpressor mound. The image was produced by subtraction of two dark-field images obtained 10 s apart. (d) Cell trajectories from the mound in c. Cell motion is mostly random with some radial motion, characteristic of normal mounds which exhibit such concentric wave patterns. Time points are 10 s apart. Scale bars = 20 $\mu$m.

*lagC*-null or *gbf*-null mounds, raising the possibility that cAMP signaling is disrupted. In these mutant mounds, we never observed a single, dominant wave center, but rather found multiple centers that transiently appeared and disappeared. These observations may help explain earlier studies in which mounds composed of mixtures of *lagC*-null and wild-type cells yielded a multiple-tip phenotype (Dynes *et al.*, 1994). We now think the multiple tips most likely arise from competing centers present in the same mound that were perhaps stabilized by the presence of normal cells. In any event, the multiple-center defect in dark-field waves that we observed in *lagC*-null or *gbf*-null cells suggests a defect in some mechanism of cell–cell communication that is used ordinarily to establish a single, dominant center and suppress other competing centers within the same mound.

To test more directly this dominant-center hypothesis, we exposed mutant mounds to 5 mM adenosine. Comparable adenosine treatment has been shown to inhibit center formation during aggregation (Newell and Ross, 1982) and also to suppress secondary-tip formation in slugs (Schaap and Wang, 1986). We reasoned that if the multiple-center phenotype in *lagC*-null or *gbf*-null mounds was due to a failure of the mutants to suppress competing centers, adenosine treatment might rescue the phenotype. We found in fact that this was the case. In the presence of adenosine, *lagC*-null mounds always exhibited a single, dominant center, and *gbf*-null mounds often exhibited a comparable rescue of this aspect of the phenotype. We conclude that the mutant mounds do suffer from an inability to suppress competing centers and that this presumably reflects a signaling defect that can be compensated for by the presence of adenosine.

It is not understood exactly how adenosine mediates its effect on center inhibition observed now in our study and also at identical concentrations in previous studies of aggregation and slug-tip formation (Newell and Ross, 1982; Schaap and Wang, 1986). Two classes of binding sites have been identified in *Dictyostelium*. Binding of adenosine to the $\beta$ class of receptors ($K_d$ = 350 $\mu$m) noncompetitively inhibits cAMP binding to cAMP receptors (Newell, 1982; Van Haastert, 1983). In addition, it has been shown that concentrations of adenosine in the 5–10 mM range slightly inhibit cAMP signaling, which could alter excitability and lead to center inhibition, as suggested by Theibert and Devreotes (1984). It seems likely that adenosine may be having a similar effect in our studies and so may be compensating for a center-inhibition function in mounds normally provided either directly or indirectly by *lagC* and *gbf*.

Even though the effect of adenosine on the dominance of wave centers was striking, adenosine treatment did not fully rescue the wave patterns. In the presence of adenosine, *gbf*-null mounds formed concentric waves (or no dominant wave center at all), and *lagC*-null mounds typically formed one-armed spiral waves, while mounds of the parent strain typically formed multiarmed spiral waves. Adenosine treatment also did not yield significant improvements in cell motility or continued development, consistent with an underlying defect in gene expression required for complete development in the mutant cells. Neither *gbf*-null or *lagC*-null mounds formed fruiting bodies in the presence of adenosine: adenosine-treated *lagC*-null mounds appeared to proceed a little further in development to form a tight mound instead of a loose mound, but then dispersed as in the non-adenosine-treated mounds. Cell motion in the mutant mounds was still random in early mounds but then became more constrained in tight mounds, even in the presence of stable wave patterns induced by adenosine.

To define more clearly these other defects, we examined other aspects of the *lagC*-null phenotype in the presence of

adenosine. We selected this mutant for further analysis because its wave-dominance defect had exhibited complete rescue by adenosine. Our data suggest that adenosine-treated *lagC*-null mounds produce functional signaling waves, since low numbers of normal cells added to these mutant mounds appeared to rotate counter to the waves. In contrast, in these same chimeric mounds, *lagC*-null cells showed no hint of rotational motion, suggesting that the *lagC*-null cells could not respond appropriately. It should be noted that in these chimeric mounds, the rotational motion of the parent cells was not as vigorous as in a normal mound, even though it was significantly better than the motion of *lagC*-null cells in the same mound. The reduced vigor of the parental cells seeded into the adenosine-treated *lagC*-null mounds may reflect the difficulty of moving through a cell mass in which most of the cells are nearly stationary. Alternatively, it could reflect difficulties of wild-type cells in gaining traction on *lagC*-null cells, if these cells suffer from an adhesive defect.

Why do the *lagC*-null cells fail to respond properly to passage of a wave in an adenosine-treated mound, when parent cells in the same mound can respond? *lagC*-null cells appear to sense the underlying wave signal, since dark-field waves were visible in *lagC*-null mounds with or without adenosine treatment. The presence of dark-field waves implies that the mutant cells were capable of a cell-shape change, or ''cringe,'' which ordinarily accompanies passage of the underlying signal (Alcantara and Monk, 1974) and thereby makes a wave pattern visible by dark-field microscopy. Thus, the *lagC*-null cells apparently detect an underlying signal, but cannot move in response to it.

The defects in motion in *lagC*-null cells were especially pronounced at the tight mound stage which is only attained in the presence of adenosine, suggesting that *lagC* is required for directed motion within this cell mass. Mutant cells in streams and at the mound periphery moved reasonably well, but, once inside the tight mound, these cells were unable to move vigorously or directionally. When seeded into mounds composed of predominantly normal cells, some *lagC*-null cells appeared capable of limited rotational motion. Inevitably however, the mutant cells drifted to the inner core or outer edge of these chimeric mounds. This result suggests that the *lagC*-null cells suffer from motility defects that handicap them in these chimeric mounds and/or that these mutant cells have different adhesive properties from normal cells, which lead to their segregation in the mixed mound.

We did find that the apparent motility defects of the *lagC*-null cells could be overcome by overexpression of GBF. In the *lagC*-null cells, this GBF overexpression rescued defects in both signaling-wave patterns and cell motility and also led to essentially normal fruiting-body formation. Thus, the motile and/or adhesive defects still present in adenosine-treated *lagC*-null mounds are bypassed in untreated *lagC*-null mounds by GBF overexpression. Figure 8 shows one model consistent with these data and earlier gene expression studies, all of which suggest

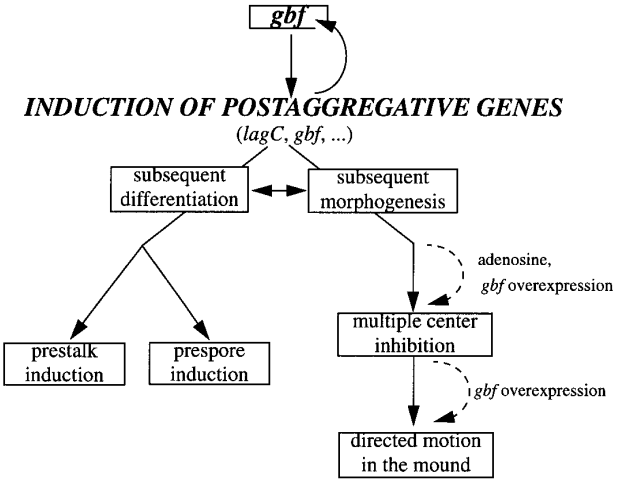


**FIG. 8.** A model for *gbf* and *lagC* pathways based on earlier models for gene expression (Firtel, 1995), but now incorporating roles for morphogenesis. *gbf* is required for induction of a host of postaggregative genes, including *lagC*. *gbf* expression may be enhanced by autoactivation and/or *lagC* expression (curved upward arrow). As has been shown in earlier studies, genes downstream of *gbf* and *lagC* are then required for cell-type differentiation (left pathway) and morphogenesis (right pathway). The bidirectional arrow indicates that there are likely to be interactions between these two pathways. The current study illuminates roles for *lagC* and *gbf* in the morphogenetic pathway. Both genes are required to establish a single, dominant center for wave signaling within the mound, presumably by assisting in multiple-center inhibition. This requirement for multiple-center inhibition can be completely bypassed in a *lagC*-null, but only partially overcome in a *gbf*-null by adding 5 mM adenosine (curved dashed arrow). In the *lagC*-nulls, GBF overexpression yields a comparable phenotype to adenosine treatment in that single, dominant wave centers emerge. However, in *lagC*-null mounds treated with adenosine, directed cell motion in mounds is still absent, indicating a second requirement for *lagC* in mound morphogenesis. The motile requirement for *lagC* can also be overcome by GBF overexpression, raising the possibility that a principal function for *lagC* in mound morphogenesis is enhancement of GBF expression.

that *lagC*'s principal function is to enhance GBF expression and/or the pathway leading to GBF-mediated gene expression. According to this model, *lagC* would not play a direct role in either the establishment of a dominant signaling center or the capacity for directed cell motility in the mound. Instead, *lagC* may enhance GBF expression or the signaling pathway required for GBF function (Schnitzler *et al.*, 1995). This in turn could induce genes that provide both the proper signaling machinery to establish a single, dominant wave center and the motile and/or adhesive machinery to move directionally within the densely packed cell mass that is the mound. Adenosine treatment of the *lagC*-null mounds may substitute for the mechanism ordinarily required to produce a single, dominant wave center, but it

cannot replace the requirements for new motile or adhesive components.

In sum, our mutant analysis has characterized several significant defects in the morphogenesis of *lagC*-null and *gbf*-null cells. These data enable us now to incorporate a morphogenetic pathway for *gbf* and *lagC* function into previous models (Firtel, 1995; Schnitzler *et al.*, 1995) that were based solely on gene expression data (Fig. 8). Establishment of a dominant wave center within a mound is one critical step in morphogenesis mediated by both *lagC* and *gbf*. Our data show that this step can be bypassed in either null mutant by addition of adenosine. However, our data also argue that proper signaling waves are not sufficient for correct morphogenesis and that both *lagC* and *gbf* are required additionally for directed cell motility within mounds. This step can be overcome in a *lagC*-null by overexpression of GBF. This latter result suggests that new genes are required for proper motion within the mound quite apart from those genes required to set up the guidance signals for motion. These genes associated with directed motion are likely to code for cytoskeletal and adhesive proteins that may well be required to meet the new challenges for movement within a densely packed cell mass.

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