A Cell Number-counting Factor Regulates Group Size in *Dictyostelium* by Differentially Modulating cAMP-induced cAMP and cGMP Pulse Sizes*

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A secreted counting factor (CF), regulates the size of *Dictyostelium discoideum* fruiting bodies in part by regulating cell-cell adhesion. Aggregation and the expression of adhesion molecules are mediated by delayed pulses of cAMP. Cells also respond to cAMP with a short cGMP pulse. We find that CF slowly down-regulates the cAMP-induced cGMP pulse by inhibiting guanylyl cyclase activity. A 1-min exposure of cells to purified CF increases the cAMP-induced cAMP pulse. CF does not affect the cAMP receptor or its interaction with its associated G proteins or the translocation of the cytosolic regulator of adenyl cyclase to the membrane in response to cAMP. Pulsing streaming wild-type cells with a high concentration of cAMP results in the formation of small groups, whereas reducing cAMP pulse size with exogenous cAMP phosphodiesterase during stream formation causes cells to form large groups. Altering the extracellular cAMP pulse size does not phenocopy the effects of CF on the cAMP-induced cGMP pulse size or cell-cell adhesion, indicating that CF does not regulate cGMP pulses and adhesion via CF’s effects on cAMP pulses. The results suggest that regulating cell-cell adhesion, the cGMP pulse size, or the cAMP pulse size can control group size and that CF regulates all three of these independently.

Controlling the size of a group of cells is critical not only for the development of multicellular organisms but also for the maintenance of their normal functions (1). Defects in cell number regulation can cause severe problems such as cancer; however, little is known about how cell number and group size are regulated in multicellular organisms.

The unicellular eukaryote *Dictyostelium discoideum* is an excellent model system for the study of cell number regulation. *Dictyostelium* are haploid amebae that live on soil surfaces and eat bacteria (2, 3). When *Dictyostelium* outgrows its food supply, the starving cells signal other cells by secreting an array of factors such as the glycoprotein conditioned medium (CM) factor (2, 4–7). After more and more cells in a population have starved, the extracellular conditioned medium factor reaches a threshold. This permits the cells to aggregate using delayed pulses of cAMP as a chemoattractant. When a pulse of cAMP arrives at a starved *Dictyostelium* cell that is in the presence of high levels of conditioned medium factor, a burst of cAMP is released by the cell to relay the signal. The cell moves toward the source of cAMP, and the expression of specific classes of genes is altered. The incoming cAMP pulse is sensed by the serpentine receptor cAR1. cAR1 activates Gβγ, which subsequently activates cytosolic factors such as phosphoinositide 3-kinase, Akt/protein kinase B, and CRAC thereby transiently activating adenyl cyclase, while Go2 activates guanylyl cyclase to generate a rapid cGMP pulse, with a peak at ~10 s and a return to baseline at ~30 s (8–21). The aggregating cells form streams that flow toward the aggregation center. If the cell density is high, the streams break into groups (22). This process forms a number of groups of cells, with each group developing into a fruiting body containing a mass of spore cells held on top of a cellular stalk (2, 3).

A normal *Dictyostelium* aggregate and fruiting body contains up to 10⁵ cells (23, 24). Numerous mutants give rise to groups with significantly larger or smaller numbers of cells (25–30). For example, streamer F cells, which have a defect in the cGMP-specific phosphodiesterase (cGMP PDE) and have abnormally large cAMP-induced cGMP pulses, form streams that do not break up and thus form abnormally large aggregates (28, 31, 32). Mutants with a disruption of *Ddmek1* have a decreased guanylyl cyclase activity, a small cAMP-stimulated cGMP pulse, and form small groups and fruiting bodies, although this appears to be caused by the formation of small aggregation territories rather than excessive breakup of streams in normal sized aggregation territories (33). Cells with abnormally high levels of extracellular cAMP PDE, which significantly represses the extracellular cAMP pulses, form small fruiting bodies (29, 30). Two transformants, *smlA* and *countin*, have normal growth, differentiation, and aggregation territory sizes but form smaller and larger aggregates and fruiting bodies, respectively (24, 34, 35). The significant feature of these two mutants is that their cell number regulation depends on the concentration and function of a secreted cell number-regulating factor, a 450-kDa protein complex called counting factor (CF) (24, 34). *smlA*, a CF gain-of-function mutant, overexpresses CF, resulting in streams breaking up into many small groups that form small fruiting bodies. Knocking out *contin*, a 40-kDa component of CF which is essential for its activity, has the opposite effect of knocking out *smlA*: the *contin* knockout cells form unbroken streams and thus huge groups and fruiting bodies (24).

CF modulates group size at least in part by inhibiting cell-
cell adhesion (36). High levels of CF repress cell-cell adhesion, resulting in aggregation streams dissipating and breaking up into small groups. Low levels of CF increase cell-cell adhesion, causing streams to remain intact and form large groups. In this report, we show that CF regulates group size by regulating both the cAMP-induced cGMP and cGMP pulses via a diverging pathway that acts downstream of the activation of G proteins by cAR1. Altering just the cGMP pulse size can regulate group size (28, 31–33), and we found that altering just the cAMP pulse size during stream formation can also regulate group size by breaking up the aggregation stream. Changing the size of the extracellular cAMP pulses does not phenocopy the effects of CF on the size of the cAMP-induced intracellular cGMP pulses and cell-cell adhesion, suggesting that CF does not regulate cGMP pulses and adhesion via its effects on cAMP pulses. *Dictyostelium* thus appears to regulate group size using several different mechanisms simultaneously.

### EXPERIMENTAL PROCEDURES

#### Cell Culture—Cell culture, development of cells, and preparation of CM from starving cells were performed as described by Jain et al. (37).

Unless noted otherwise, *smlA-* designates the *smlA* antisense transfectant in the Ax4 background, and *countin* designates the *countin* knockout in the Ax4 background (24, 34). Filtered assays were performed as described by Breck et al. (34) with 80 l of cells at 4 × 10^6 cells/ml in PBM (20 mM KH2PO4, 10 mM CaCl2, 1 mM MgCl2, pH 6.1) placed on a Millipore filter. After 2 h, the filter pads were transferred to new plates and placed on filter papers soaked with either PBM or PDE. Submerged culture was also performed as described by Breck et al. (34) with 150 l of cells at 1 × 10^6 cells/ml in PBM placed in the well of a Falcon 24-well plate and mixed with 300 l of PBM. When indicated, 0.9 l of buffer or 15 l of PBM was added to a well (final concentration, 30 nM). Preimmune and immune antisera against countin were applied at a 1:300 dilution (24) to starving cells after 2 h of starvation.

CRAC-GFP cells were a kind gift from Dr. William Loomis. The translocation of CRAC-GFP induced by cAMP stimulation was studied using a Zeiss LSM410 laser confocal microscope following Parent et al. (20). cAMP and cGMP production, cAMP Binding, GTP Binding, and GTPase Assays—The cGMP production in response to a 1 l extracellular cAMP pulse was measured following Kesbeke et al. (38), and the concentration of cGMP was determined using a [3H]cGMP assay kit (Amersham Pharmacia Biotech). To study the effect of cAMP pulses on cAMP-induced cGMP pulses, Ax4 cells were treated with beef heart cAMP PDE (0.01 unit/ml, Sigma), PBM, or cAMP pulses (final concentration, 30 nM administered every 6 min for 5 h starting 1 h after starvation). The cAMP-induced production of cAMP was measured in the absence of dithiothreitol following Van Haastert (39). Cells were starved for 6 h, stimulated with 10 l 2′-deoxy-cAMP (a functional cAMP analog that does not interfere with the subsequent cAMP assay), and lysed at 0, 1, 2, 3, 5, and 8 min. cAMP concentrations were determined using a [3H]cAMP assay kit (Amersham Pharmacia Biotech). cAMP binding to cells was performed following Kesbeke et al. (38) with the exception that cells were starved for 6 h. The effect of cAMP on the binding of [3H]GTP to membranes was measured following Snaar-Jagalska and Van Haastert (40) with the exception that instead of spinning membranes through silicone oil, membranes were collected by centrifugation for 2 min at 14,000 × g. cAMP-induced high affinity GTPase activity was measured following Brazill et al. (41), adding the creatine phosphokinase to the mixture immediately before use.

Guanylyl Cyclase and cGMP-specific PDE Assays—Guanylyl cyclase activity was measured following Snaar-Jagalska and Van Haastert (42) with the exception that cells were starved in PBM at 1 × 10^6 cells/ml for 6 h, collected by centrifugation, washed twice, and resuspended in 40 ml HEPES/NaOH, pH 7.0, at 1.5 × 10^6 cells/ml. After neutralization, the formation of cGMP by guanylyl cyclase was determined using a [3H]GMP assay kit.

cGMP-specific PDE was assayed following Van Haastert et al. (43). Cells were starved in PBM at 1 × 10^6 cells/ml for 6 h, collected by centrifugation, washed twice, resuspended in PB (3 mM Na2HPO4, 7 mM KH2PO4, pH 6.5) at 5 × 10^6 cells/ml, and lysed through a 5-μm filter. The assay mixture was incubated for 20 min at 22 °C to allow hydrolysis of cGMP to GMP by the cGMP PDE. The reaction was terminated by boiling for 2 min. To convert GMP to guanosine, 100 μl of 1 mg/ml snake venom (Ophiophagus hannah, Sigma) was added followed by incubation at 22 °C for 30 min. 1 ml of anion exchanger (1 part AG X-8 Bioreal + 2 parts ethanol) was added, and the mixture was shaken gently for 15 min. The anion exchanger was pelleted, and 500 l of supernatant was used to measure the guanosine radioactivity.

Adhesion Assay—Cell-cell adhesion was measured following Roisin-Bouffay et al. (36). Ax4 cells were starved at 5 × 10^6 cells/ml in PBM buffer on a shaker. At 2, 4, and 6 h after starvation, 500 l of cells was mixed with 500 l of PBM and vortexed for 20 s. 500 l of the diluted cell sample was then added to a new Eppendorf tube and rotated on a Labquake rotator for 1.5 min. Adhesion was measured by counting both the total number of cells and the number of single cells with a hemacytometer.

### RESULTS

**CF Reduces the cAMP-induced cGMP Pulse Size**—One of the first observed responses of aggregating *Dictyostelium* cells to a cAMP pulse is a rapid cGMP pulse, with a peak at ~10 s and a return to base line at ~30 s (44, 45). Alterations of cGMP pulse size affect group size in *Dictyostelium*, with large cGMP pulses causing the formation of large groups (28, 31, 33, 46). To determine if CF regulates group size by regulating the cAMP-induced cGMP pulse, we examined the cAMP-induced cGMP pulse in Ax4 parental, *smlA*−, and *countin*− cells. As observed previously, stimulating Ax4 cells with cAMP generated a cGMP pulse with a peak at ~10 s and a return to basal levels at ~30–40 s. With the same stimulus, *countin*− cells generated a much larger cGMP pulse, whereas *smlA*− cells responded with
a very small cGMP pulse (Fig. 1A). smlA knockouts in a DH1 background also had a very small cGMP pulse (data not shown). Thus, we hypothesized that CF is capable of inhibiting the cAMP-induced cGMP pulse.

To verify our hypothesis, we measured the cAMP-induced cGMP pulse in Ax4 cells in the presence or absence of anti-countin antibodies. We found previously that adding anti-countin antibodies to developing cells essentially eliminated CF in the medium (24). Compared with the control cells treated with preimmune serum, cells exposed to anti-countin antibodies for 4 h had a significantly larger cAMP-induced cGMP pulse (Fig. 1B). This suggested that extracellular countin regulates the cAMP-induced cGMP pulse.

Regulation of the cGMP pulse by CF could be caused either by a regulation of the guanylyl cyclase or the cGMP PDE. Compared with wild-type cells, countin− cells had a higher GTPγS-stimulated guanylyl cyclase activity, whereas smlA− cells had a lower GTPγS-stimulated guanylyl cyclase activity (Fig. 2A). Both smlA− and countin− cells had higher cGMP PDE activities than parental cells (Fig. 2B). To determine if the cGMP PDE is in the CF signal transduction pathway, we starved Streamer F cells (which have a defective cGMP PDE) and control cells on pads soaked with either wild-type CM, which contains a normal amount of CF, or smlA− CM, which contains abnormally high levels of CF. Compared with wild-type CM, smlA− CM induced a 1.5-fold increase in the number of groups formed by wild-type cells and a 1.6 ± 0.3 -fold increase in the number of groups formed by Streamer F cells. This indicated that Streamer F cells respond to CF. Together, the data suggest that cells developing in the presence of abnormally high or low levels of CF both have higher levels of cGMP PDE activity, but the presence of the cGMP PDE is not necessary for the CF effect on group size; the levels of the GTPγS-stimulated guanylyl cyclase activity roughly match the observed effects on the size of the cGMP pulse.

**Cell-counting Factor Signal Transduction**

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**FIG. 2.** Guanylyl cyclase activities and cGMP-specific PDE activities in smlA−, wild-type (WT), and countin− cells. Panel A, guanylyl cyclase activities were measured in triplicate for smlA−, wild-type, and countin− cells 6 h after starvation. Panel B, cGMP PDE activities were measured in wild-type, smlA−, and countin− cells. Cells were starved for 6 h, and the cGMP PDE activities were measured in triplicate followed by normalizing the average of each triplicate to the average PDE activity of the wild-type cells. Both panels show the means ± S.E. for three separate experiments.

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**FIG. 3.** CF affects the cAMP-induced cAMP pulse. Panel A, wild-type (WT), smlA−, and countin− cells were starved for 6 h and treated with 2′-deoxy-cAMP. The levels of cAMP were measured in triplicate. Data shown are the means ± S.E. for three separate experiments. Panel B, short term effect of CF on cAMP signal relay. countin− cells were starved for 6 h followed by exposure to purified CF for 1 min before 2′-deoxy-cAMP treatment. The amount of cAMP produced in 3 min was measured in duplicate followed by normalizing the average of each duplicate to the average amount of cAMP produced by the control cells. Data shown are the means ± S.E. for four separate experiments.

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**CF Increases the cAMP-induced cAMP Pulse Size**—Starving Dictyostelium cells secrete cAMP and use it to coordinate development. A number of Dictyostelium mutants with abnormal aggregate sizes have defects in their cAMP signal transduction pathway (29, 30, 47). To determine whether CF regulates the cAMP pulse, the cAMP-induced cAMP signal relay was measured in wild-type, smlA−, and countin− cells. The smlA− cells had a much stronger cAMP signal relay than the parental wild-type Ax4 cells, whereas the cAMP signal relay in countin− cells was much weaker (Fig. 3A). When countin− cells were exposed for 1 min to purified CF, there was a 40% increase in
the size of the cAMP-induced cAMP pulse (Fig. 3B). countin<sup>−</sup> cells were also treated for 5 min with either wild-type or smlA<sup>−</sup> CM. These also increased the cAMP signal relay significantly, whereas exposure of the countin<sup>−</sup> cells to countin<sup>−</sup> CM (which contains no functional CF) had no effect (data not shown). Similar 5-min treatments of countin<sup>−</sup> cells with wild-type or smlA<sup>−</sup> CM had no significant effect on the cAMP-stimulated GMP pulse size. These data suggest that the presence of CF potentiates the size of the cAMP-induced cAMP pulse and that CF affects cAMP-stimulated cAMP accumulation within 1 min but requires several hours to affect the cAMP pulse. Further studies demonstrated that CF regulates cAMP-induced cAMP pulsing by controlling GTP<sub>γ</sub>S-stimulated adenyl cyclase activity.<sup>2</sup>

**Table I**

| Presence or Absence of 10<sup>−6</sup> M cAMP | GTP<sub>γ</sub>S-stimulated GTP binding | cAMP-stimulated GTP binding |
|---------------------------------------------|---------------------------------------|-----------------------------|
| Presence of 10<sup>−6</sup> M cAMP          | 654 ± 161                             | 674 ± 163                   |
| Absence of 10<sup>−6</sup> M cAMP           | 632 ± 175                             | 705 ± 175                   |
| Mean S.E.                                    | 358 ± 135                             | 301 ± 115                   |

**Table II**

| Presence or Absence of 10<sup>−6</sup> M cAMP | GTP<sub>γ</sub>S-stimulated GTP binding | cAMP-stimulated GTP binding |
|---------------------------------------------|---------------------------------------|-----------------------------|
| Presence of 10<sup>−6</sup> M cAMP          | 654 ± 161                             | 674 ± 163                   |
| Absence of 10<sup>−6</sup> M cAMP           | 632 ± 175                             | 705 ± 175                   |
| Mean S.E.                                    | 358 ± 135                             | 301 ± 115                   |

As with other G protein-coupled receptors, GTP<sub>γ</sub>S inhibits the binding of cAMP to cAR1 (39, 50). The addition of GTP<sub>γ</sub>S inhibited cAMP binding, but there was no significant difference between the different cell types or cells exposed to the immune or preimmune antibodies (Table II). Another feature that cAR1 shares with G protein-coupled receptors is that binding of ligands causes membranes to bind GTP with high affinity (48, 49). We found that the basal and cAMP-induced high affinity binding and the normalized ratios of these two were indistinguishable in Ax4, smlA<sup>−</sup>, countin<sup>−</sup> and Ax4 treated with preimmune, or Ax4 treated with immune anti-countin antibodies. As with all known G proteins, the bound GTP is eventually hydrolyzed to GDP (40). We found previously that a different secreted factor, CM factor, regulates signaling through cAR1 by regulating the lifetime of the GDP bound to Go2, the Gα protein that interacts with cAR1 (41). To determine if CF similarly regulates Go2-GTP hydrolysis, we measured the cAMP-induced high affinity GTPase of membranes. There was no statistically significant difference in the basal or cAMP-induced GTPase activities of Ax2, smlA<sup>−</sup>, or countin<sup>−</sup> membranes (Table II and data not shown). Cells exposed to immune or preimmune anti-countin antibodies also had similar GTPase activities, although these were different from those of cells not

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exposed to any antibody (Table II).

Taken together, the data suggest that the increased CF levels in smlA <sup>−</sup> cells or the decreased CF activity in countin <sup>−</sup> cells (or cells exposed to anti-countin antibodies), does not affect cAMP binding, the GTPγS inhibition of cAMP binding, cAMP-induced GTP binding, or the subsequent GTP hydrolysis. This then indicates that CF does not affect cAR1, its associated Go, or their interactions.

We also studied the cAMP-induced CRAC subcellular relocation, which is essential for cAMP-induced adenyl cyclase activation (51, 52). Wild-type cells expressing GFP-tagged CRAC were starved in PBM buffer for 6 h, and the cAMP-induced CRAC subcellular relocation was examined. CF did not affect the subcellular relocation process of CRAC (data not shown). Together, the data suggest that CF regulates cAMP signal transduction utilizing a mechanism that is apparently downstream from the cAR1-G<sub>γ</sub>γ-CRAC pathway.

**Changing the Size of cAMP Pulses Causes Wild-type Cells to Mimic smlA <sup>−</sup> and countin <sup>−</sup> Cells**—Exposure of cells to CF increases the size of the cAMP-stimulated cAMP pulse and decreases group size. To determine if the cAMP pulse size seen by streaming cells affects group size, wild-type cells were starved on filter pads until just before stream formation and were then treated with beef heart cyclic nucleotide PDE to decrease the intensity of cAMP pulses. 0.01 unit/ml PDE-treated wild-type cells formed larger aggregates and fruiting bodies compared with the control group (Fig. 4, A–C). However, when the PDE concentration was higher than 0.1 unit/ml, and the cAMP pulses were extremely small, wild-type cells formed a large number of smaller aggregates and fruiting bodies. We also investigated the effect of large cAMP pulses by applying high levels of cAMP pulsing (to mimic that of smlA <sup>−</sup> cells) to both wild-type and countin <sup>−</sup> cells in submerged culture (because exogenous pulsing is not possible with filter pad assays). Pulsing cells with buffer had no effect, whereas large (30 nM or 1 μM) cAMP pulses added every 6 min starting at 6 h after starvation (when streams were forming) caused aggregation streams to break up into abnormally small groups (Fig. 4, E and G). Together, the data suggest that after streams have formed, small cAMP pulses lead to reduced stream breakup and large groups, whereas large pulses cause streams to break into small groups.

Compared with wild-type or countin <sup>−</sup> cells, smlA <sup>−</sup> cells have large cAMP pulses and small cGMP pulses (Figs. 1 and 3). We found that reducing the magnitude of cAMP pulses in smlA <sup>−</sup> cells by exposure to PDE from either the beginning of starvation or the beginning of stream formation did not increase the group size (data not shown). This suggests that in smlA <sup>−</sup> cells, the high level of CF overrides the effect of decreasing the cAMP pulse size.

**Changing the Size of cAMP Pulses Affects the cAMP-induced cGMP Pulse Size and Cell-Cell Adhesion**—Mimicking the effect of CF on cAMP pulse size was capable of reproducing the smlA <sup>−</sup> and countin <sup>−</sup> phenotypes. We thus examined whether mimicking the effect of CF on cAMP pulse size was able to reproduce its effect on the size of cGMP pulses and cell-cell adhesion. Ax4 cells were treated with beef heart PDE or 30 nM cAMP (final concentration) or PBM pulsing (every 6 min for 5 h started 1 h after starvation), and their cAMP-stimulated cGMP pulses were measured. Exposing cells to large cAMP pulses (to mimic CF gain of function) significantly enlarged the size of the cAMP-stimulated cGMP response, although it came down quickly to the basal level within 30–60 s (Fig. 5). Nevertheless, decreasing the cAMP pulse size seen by streaming cells using beef heart PDE (to mimic CF loss of function) also increased the cAMP-stimulated cGMP pulse size, which did not return to the basal level even after 60 s (Fig. 5). The data suggest that the effect of CF on cGMP pulse size is not dependent on its regulation of the cAMP-stimulated cAMP pulse. Although CF can regulate cGMP pulses indirectly through the cAMP signal.
transduction pathway, CF inhibition of the cAMP-stimulated cGMP pulse overrides its indirect effect.

To determine if CF regulates cell-cell adhesion via cAMP-stimulated cAMP pulses, cell-cell adhesion under the above conditions was also examined. PDE-, cAMP-, and PBMTreated cells were collected at 2, 4, 6 h after starvation, and their cell-cell adhesion was measured. We found that increasing the cAMP pulse size increased cell-cell adhesion, whereas decreasing the pulse size decreased cell-cell adhesion (data not shown). Thus, as with the cGMP pulses, the observed inhibition of cell-cell adhesion by CF (36) suggests that CF inhibits adhesion via a pathway that is independent of the CF-potentiated cAMP pulses.

**DISCUSSION**

*Dictyostelium* cells use relayed pulses of cAMP both for aggregation and to regulate the expression of a variety of genes during early development (16, 17, 53). Under our laboratory conditions, aggregating wild-type cells form streams that break into groups of $\sim 2 \times 10^4$ cells. In this report, we find that the breakup into groups of a specific size may be caused in part by the secreted CF altering cAMP signal transduction. We found that compared with wild-type cells, smlA$^-$ cells (which oversecrete CF) have a smaller cAMP-induced cGMP pulse and a larger cAMP-induced cAMP pulse, whereas countin$^+$ cells (which do not secrete functional CF) had a relatively large cGMP pulse and a small cAMP pulse. The results suggest that CF is capable of increasing cAMP pulse magnitude and decreasing the cGMP pulse magnitude in *Dictyostelium* cells. The effect can be phenocopied: low levels of CF prevent stream breakup and cause the formation of large groups, and either large and prolonged cGMP pulses (28, 31, 32) or small cAMP pulses (33) or large cAMP pulses (Fig. 4) have this same effect.

CF does not appear to affect the amount of cAMP receptors or their measurable interactions with G proteins (Tables I and II). This suggests that CF is not regulating group size by regulating the cAMP receptor or its activation of G proteins. It thus appears that CF regulates cAMP signal transduction at a step downstream of the cAMP receptor and G protein activation. Our data indicate that long-term exposure of cells to CF decreases the cAMP-induced cGMP pulse size. Treating Ax4 cells with purified CF for 1 min (Fig. 1B) or treating countin$^+$ cells with wild-type or smlA$^-$ CM for 5 min potentiated the cAMP-induced cAMP pulse size (data not shown). The same treatment failed to affect the cAMP-induced cGMP pulse. This suggests that CF regulates cAMP pulses and cGMP pulses in opposite directions and with different time scales. We also found that altering the size of extracellular cAMP pulses does not phenotype the effect of CF on the size of the cAMP-induced cGMP pulse, indicating that CF regulates cAMP and cGMP signal transduction independently.

Knocking out key components of the cAMP and cGMP signal transduction pathways such as the cAMP receptor, Go2, Gβ, adenyl cyclase, or guanylyl cyclase totally eliminates aggregation and multicellular development (2, 3, 12, 15, 51, 54–58). Because smlA$^-$ and countin$^+$ cells show normal aggregation, high or low levels of CF do not appear to abolish cAMP or cGMP production completely.

Some *Dictyostelium* mutants form small fruiting bodies apparently caused by having small cAMP pulses. Kesbeke and Van Haastert (47) found that in the mutant HB3 the secretion of cAMP is reduced significantly, which causes these cells to form very small fruiting bodies. Small fruiting bodies are also generated by abnormally high levels of extracellular cAMP PDE and thus small cAMP pulses (29, 30, this report). Altering cAMP pulses from the beginning of development affects the size of aggregation territories (59, 60). In this case small cAMP pulses promote large aggregation territories, whereas large cAMP pulses cause small aggregation territories. We found previously that smlA$^-$ and countin$^+$ cells form normal sized aggregation territories (24, 34). Developing cells do not secrete detectable levels of CF until roughly 4 h after starvation, when aggregation territories have already been established and streams are forming. Thus, the key difference may be that CF affects cAMP pulses just before the stream formation, not from the beginning of development, and so does not affect the size of the aggregation territory.

We found that changing the size of cAMP pulses is sufficient to regulate group size by affecting stream breakup (Fig. 4). Increasing the size of cAMP pulses, which mimics the situation of having high levels of CF, caused the premature breakup of streams formed by wild-type cells; reducing the size of cAMP pulse resulted in wild-type cells forming countin$^+$-like aggregation streams. Unlike what we expected, reducing the intensity of cAMP pulses in smlA$^-$ cells did not rescue the small group phenotype. Neither exposure to PDE from the beginning of starvation nor from the beginning of stream formation could increase the size of groups formed by smlA$^-$ cells. This suggests that although the regulation of cAMP pulse size by CF itself is sufficient to affect group size in *Dictyostelium*, lowering the cAMP pulse size cannot override other effects of a high level of CF, such as a decreased cGMP pulse size and cell-cell adhesion.

Together, these data indicate that the cell number counting factor CF regulates several different signal transduction pathways (such as those regulating cAMP and cGMP pulses) in *Dictyostelium*. Although regulating only the cAMP or cGMP pulses or cell-cell adhesion is sufficient to control group size, CF appears to utilize all of these mechanisms simultaneously. Higher eukaryotes have many G protein-mediated signal transduction pathways similar to the *Dictyostelium* cAMP signal transduction pathway. CF represents a simple mechanism to sense structure size, and an exciting possibility is that having a secreted signal regulating a G protein-mediated chemotactic signal transduction pathway may be a general way to regulate group or cell size.

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