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REVIEW

Combining experiments and modelling to understand size regulation in *Dictyostelium discoideum*

Wonhee Jang[1,*] and Richard H. Gomer[2]

[1]*Department of Life Science, Dongguk University, 3-26 Pil-dong, Chung-gu, Seoul 100-715, Korea*
[2]*Department of Biochemistry and Cell Biology, Rice University, MS-140, 6100 South Main Street, Houston, TX 77005-1892, USA*

Little is known about how the sizes of specific organs and tissues are regulated. To try to understand these mechanisms, we have been using a combination of modelling and experiments to study the simple system *Dictyostelium discoideum*, which forms approximately 20 000 cell groups. We found that cells secrete a factor, and as the number of cells increases, the concentration of the factor increases. Diffusion calculations indicated that this lets cells sense the local cell density. Computer simulations predicted, and experiments then showed, that this factor decreases cell–cell adhesion and increases random cell motility. In a group, adhesion forces keep cells together, while random motility forces cause cells to pull apart and separate from each other. As the group size increases above a threshold, the factor concentration goes above a threshold and the cells switch from an adhered state to a separated state. This causes excessively large groups to break apart and/or dissipate, creating an upper limit to group size. In this review, we focus on how computer simulations made testable predictions that led the way to understanding the size regulation mechanism mediated by this factor.

Keywords: biological clocks; *Dictyostelium*; size regulation

1. SIZE REGULATION

The regulation of size is a major issue in developmental and evolutionary biology (Bonner 2006). Generally, there are two different ways to affect the size of an animal, organ or appendage. One is by controlling the number and size of the cells it contains and the other is by controlling the amount of extracellular matrix and fluid (Conlon & Raff 1999). Recent studies show that size regulation in higher eukaryotes, including *Drosophila*, mice, cows and *Arabidopsis*, primarily depends on the control of cell growth and division (McPherron & Lee 1997; McPherron *et al.* 1997; Bohni *et al.* 1999; Montagne *et al.* 1999; Metcalf *et al.* 2000; Chae *et al.* 2002; Garofalo 2002). In most cases, an increase or reduction in body or organ size is achieved by modifying the levels of growth hormones, growth factors, their receptors or by interfering with their signal transduction pathways (McPherron & Lee 1997; McPherron *et al.* 1997; Bohni *et al.* 1999; Montagne *et al.* 1999; Metcalf *et al.* 2000; Chae *et al.* 2002; Garofalo 2002). In an extreme case, a mutation in a member of the transforming growth factor-β superfamily (myostatin) results in a 100–200% increase of muscle mass in mice (McPherron *et al.* 1997). In mice and *Drosophila*, mutants with increased size usually contain more cells, not necessarily bigger cells; mutants with a reduction in size contain fewer cells, with or without decreases in cell size (Fero *et al.* 1996; Kiyokawa *et al.* 1996; Nakayama *et al.* 1996; Bohni *et al.* 1999; Montagne *et al.* 1999; Garofalo 2002). The numerous factors autonomously regulating the size of an organism make it extremely difficult to study size determination mechanisms in complex higher eukaryotes. Therefore, a relatively simple organism such as *Dictyostelium discoideum* is useful as a model system for this type of study.

2. *DICTYOSTELIUM DISCOIDEUM* AS A MODEL SYSTEM FOR SIZE REGULATION

The social amoeba *D. discoideum* provides an excellent model system to study size regulation. *Dictyostelium* is a haploid unicellular eukaryote that grows on soil and

*Author for correspondence (wany@dongguk.edu).

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feeds on bacteria (for review, see Loomis 1975; Kessin 2001). Upon starvation, a developmental programme is initiated that results in the formation of a multicellular structure that contains, for the laboratory strains that we use, approximately 20 000 cells.

Dictyostelium cells use a variety of signals to coordinate development. Even in a small (approx. 1 cm^2) area of soil, some cells will starve before nearby cells starve. It appears to be advantageous for the cells to wait and form a multicellular structure when most of the cells in the area have starved, and to coordinate this the cells signal that they are starving by secreting a glycoprotein called conditioned medium factor (CMF; Gomer *et al.* 1991; Jain *et al.* 1992; Jain & Gomer 1994; Yuen & Gomer 1994; Yuen *et al.* 1995; Deery & Gomer 1999; Deery *et al.* 2002). As more and more cells starve, the local CMF concentration passes a threshold level. This allows cells to aggregate using relayed pulses of secreted cAMP as a chemoattractant (for review see Parent & Devreotes 1999; Jin & Hereld 2006; Willard & Devreotes 2006; Van Haastert & Veltman 2007). First, some cells start emitting cAMP pulses, and when a pulse of cAMP reaches a starving cell, it triggers the cell to relay the signal by emitting a pulse of cAMP (Roos *et al.* 1975; Shaffer 1975). The cAMP waves generated by the cells act as a chemoattractant, and the cells move towards the cAMP signal, forming dendritic aggregation streams (figure 1*a–d*; for review, see Varnum-Finney *et al.* 1987; Kessin 2001). Starving cells also secrete phosphodiesterase (PDE), which degrades the extracellular cAMP to bring its concentration back to a basal level in the interval between pulses. The cAMP pulses also regulate the expression of many genes specifically expressed during early development (for review, see Verkerke-van Wijk & Schaap 1997). A rapid cGMP pulse is also generated in response to a cAMP pulse (Mato *et al.* 1977; Wurster *et al.* 1977).

After the formation of a hemispherical mound of approximately 2×10^4 cells, the aggregate forms a migrating slug that, if necessary, can crawl towards the soil surface. The slug forms an approximately 1–2 mm tall fruiting body consisting of a spore mass held up off the soil by a thin column of stalk cells (figure 1*f*). The spores can then be dispersed by the wind, and when a spore lands in a moist environment it cracks open, an amoeba crawls out and hopefully finds bacteria and can start dividing to form a new colony of cells. The purpose of fruiting bodies is thus to disperse spores. When a fruiting body is too small, the spore mass will be too close to the ground for optimal spore dispersal (figure 1*e*). On the other hand, if a spore mass is too big, it will slide down the stalk or the fruiting body will fall over (figure 1*g*). Therefore, the formation of a spore mass and stalk of the proper size is of utmost importance (for review, see Loomis 1975; Devreotes 1989; Schaap 1991; Firtel 1995). Even though multicellularity in *Dictyostelium* results from aggregation of a large number of cells rather than division and growth as in metazoans, size regulation mechanisms in all multicellular organisms must address similar conceptual problems: tissue size must be measured, compared with a critical value and adjusted properly.

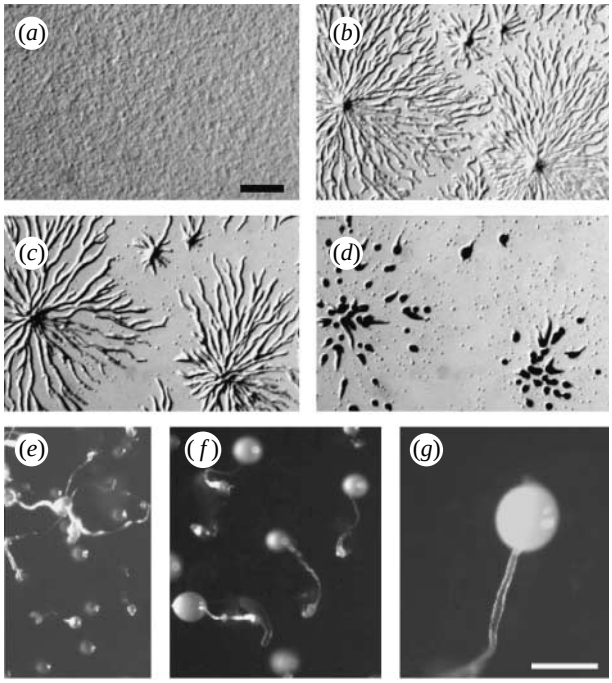

Figure 1. Aggregation and fruiting bodies of *D. discoideum* cells. For (*a–d*), the cells were spread on a non-nutrient agar plate, and video microscopy was used to observe the starvation-induced development. For (*e*,*f*), the cells were grown on an agar plate that was spread with bacteria. After consuming all the bacteria, the starving *Dictyostelium* cells aggregated and formed fruiting bodies, and the fruiting bodies were photographed from above. Bars on (*a*,*g*) are 1 mm. (*a*) There is an even field of cells approximately 30 min after starvation. (*b*) At 5 hours, the field has broken into aggregation territories, and in each territory cells in dendritic aggregation streams are moving towards a common centre. (*c*) At 6 hours after starvation, the streams are well organized. (*d*) At 7 hours, the streams have broken into groups. Each group will then go on to form a fruiting body. (*e*) Fruiting bodies of *smlA*$^-$ cells. (*f*) Fruiting bodies of Ax2 cells. (*g*) Fruiting bodies of *countin*$^-$ cells.

3. GENERAL MECHANISMS OF FRUITING BODY SIZE REGULATION

There seem to be three mechanisms that determine the size of the *D. discoideum* fruiting body. In a large lawn of starving cells, the lawn will break up into aggregation territories where all the cells in a territory are streaming in towards a common centre. The first mechanism regulates the size of the aggregation territory by regulating the range of the relayed cAMP signals (Thadani *et al.* 1977; Faure *et al.* 1988). Changes in aggregation territory size can be observed when the cAMP pulse size is altered. One of the ways to alter the cAMP pulse size is by altering the PDE level. There are two different types of cAMP-specific PDEs in *D. discoideum*: secreted and membrane-bound forms. High levels of secreted PDE decrease cAMP pulse size, which decreases the signal relay, and the cells then form small aggregation territories and aggregates (Riedel *et al.* 1973; Thadani *et al.* 1977; Faure *et al.* 1988). Mutants with a defect in a secreted PDE inhibitor also have small cAMP pulses and form small aggregates (Adames *et al.* 1994).

The second mechanism that regulates fruiting body size can be observed when cells are forming aggregation streams. If the cell density is high, as aggregation streams flow into an aggregation centre, the streams break into groups, with each group then forming a fruiting body (Shaffer 1957). The stream breakup prevents the formation of excessively large fruiting bodies. Two mutants, *smlA*$^-$ and *countin*$^-$ (*ctnA*$^-$), provide an insight into how stream breakup is regulated in *D. discoideum* (for review, see Brown & Firtel 2000). Although the aggregation territories formed by these cell lines are essentially the same, a major difference between the parental strain Ax2, *smlA*$^-$ and *countin*$^-$ cells is observed in stream breakup (Brock & Gomer 1999; Roisin-Bouffay *et al.* 2000). We found a mutant, which we named *smlA* because it formed very *small* fruiting bodies (Brock *et al.* 1996; figure 1*e*). We found that a factor secreted by *smlA*$^-$ cells could cause parental Ax2 cells to form small fruiting bodies. We purified the factor and found that it was a complex of proteins (Brock & Gomer 1999). The protein complex, named 'counting factor' (CF), is secreted by parental Ax2 cells at a low to moderate concentration. When the *countin* (*ctnA*) gene (encoding one of the components of the CF complex) is disrupted, CF appears to lose bioactivity, and aggregation streams seldom break, and coalesce into huge fruiting bodies (Brock & Gomer 1999). The size of the spore mass of the *countin*$^-$ fruiting body is so large that it often slides down or falls over (figure 1*g*). The development of this mutant is otherwise normal.

The third mechanism acts after groups are formed. Even after CF-mediated stream breakup, if the groups are above a threshold size, they will break into smaller subgroups (Hohl & Raper 1964; Kopachik 1982). This phenomenon is occasionally observed in *countin*$^-$ cells, which suggests that the group breakup mechanism does not require countin and by inference CF. Together, the data suggest the existence of three distinct fruiting body size regulation mechanisms in *D. discoideum*.

4. DIFFUSION CALCULATIONS VERIFIED THAT CF CAN BE USED TO SENSE CELL NUMBER

Our working model of how CF regulates stream breakup is that as the number of cells in a stream increases, the concentration of CF will increase, and if there are too many cells in a stream, the associated high levels of CF will signal this fact to the cells. We had observed that cells appear to secrete CF continuously, and a puzzling aspect of this model was the idea that CF concentrations would simply increase with time, so that for a given group or stream size the CF concentration would be different at different times. To help understand this, we did diffusion calculations for cells in a group or stream secreting CF. To our surprise, the diffusion calculations predicted that the CF concentration in the vicinity of cells quickly reaches a steady state, with the amount added by secretion being balanced by the amount diffusing away from the group or stream (Brock & Gomer 1999). In addition, the calculated concentration of CF that is reached when a stream contains more than 2×10^4 cells matched the observed concentration of CF that causes stream breakup.

5. COMPUTER SIMULATIONS LED THE WAY IN UNDERSTANDING HOW CF REGULATES GROUP SIZE

In the phenomenon of stream breakup, a stream of closely packed cells appears to relatively quickly break across the stream, forming a cell-free gap running across the stream. We were baffled by this and could not conceive of a mechanism that could cause this. To investigate what properties of individual cells could determine whether a stream breaks up, and the size of the groups it breaks up into, we wrote a simple computer simulation of cells moving in a stream (figure 2*a*). The stream was approximately 10 cells wide and several hundred cells long. The individual cells were modelled as points on a grid and could move onto an adjacent grid point if the point was unoccupied. We could then change a variety of cell properties to see if this could cause the model stream to break. The key insight for us came when we started playing with the model and examined the effect of motility and adhesion forces. In the simulation, if the ratio of the random cell motility force F_M to the cell–cell adhesion force F_A was low, the stream stayed intact. If, however, this ratio was high, the random motility of the cells overcame the cell–cell adhesion and as a result cells would begin to pull away from the stream for a while before returning to the stream by chemotaxis. This effectively caused the stream to dissipate (figure 2*b*). If the F_M/F_A ratio then decreased, the dispersed cells would condense (figure 2*b*). If the stream was sufficiently dissipated, the cells in the simulation did not condense back into an even stream but rather condensed into groups (Roisin-Bouffay *et al.* 2000). Altering the timing or extent of the changes in the F_M/F_A ratio strongly affected the size of the groups that a stream breaks up into. In the simulations, a F_M/F_A ratio that simply increased with time caused streams to break into groups, but thick streams broke into large groups while thin streams broke into smaller groups. If, however, in the simulations a secreted diffusible factor slightly decreased cell–cell adhesion and/or increased cell motility, thick and thin streams broke into groups of the same size. Our simulation generated groups using a mechanism that is different from the classic Turing mechanism or models that involve different cell types separating from one another (Turing 1952; Oster *et al.* 1983). The key testable prediction from our simulations was that CF might regulate cell–cell adhesion and/or cell motility.

The computer simulations suggested that motility and cell–cell adhesion forces play major roles in the breakup of aggregation streams during development, when all the conditions are the same. The cell–cell adhesion of *D. discoideum* cells increases gradually over time during development. Using the observed increase in cell–cell adhesion during development and the observed probability distribution of the random cell motility, we found that if the initial adhesion force is greater than the motility force, aggregation streams do not fragment and thus form large fruiting bodies. We also found that the timing of the increase of cell–cell adhesion significantly affects group size, and that

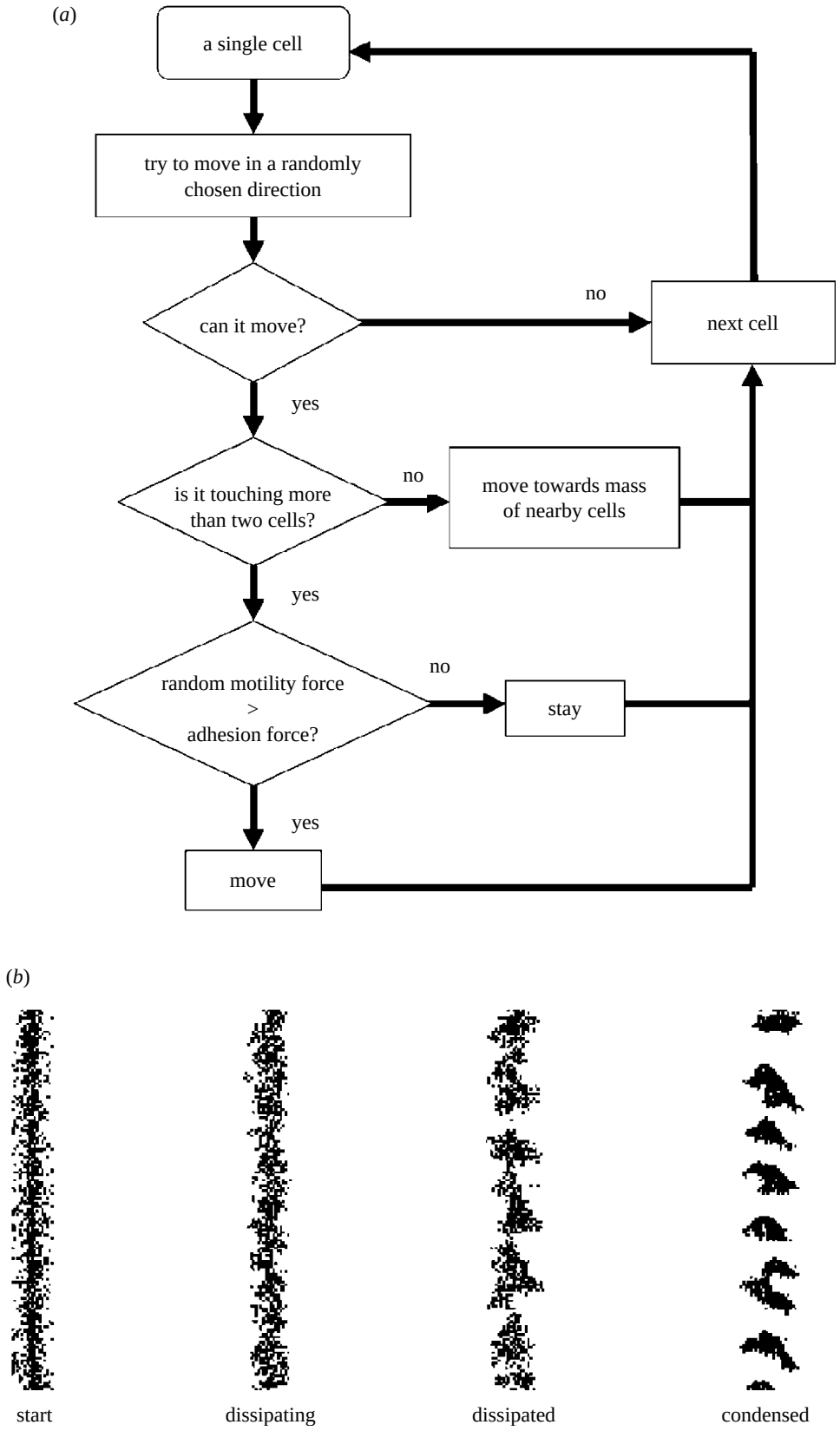

Figure 2. General outline of computer simulation algorithm, and computer simulation results showing stream breakup. (*a*) Simulation algorithm for one cell. (*b*) The first column (start) shows a model of cells in a stream, with each cell represented as a dot. With respect to figure 1, this represents a stream at approximately 5.5 hours after starvation. The second column (dissipating) shows the result of allowing cells to have a random motility that often is higher than the cell–cell adhesion force, allowing the stream to lose some cohesiveness; this represents a stream at approximately 6 hours. The third column (dissipated) shows a model stream at approximately 6.5 hours, with relatively high motility forces and relatively low adhesion forces allowing the cells in the stream to further disperse, with weak points (areas of few cells) appearing in the dispersed stream. At this point in the model, the adhesion force was increased and the random motility force was decreased. The last column (condensed) shows the effect of this change: the dispersed cells have coalesced into groups.

changing the patterns of cell motility by shifting the cell motility distribution affects group size (Dallon *et al.* 2006). More sophisticated models where the cells were not on a grid, and were aggregating using relayed pulses of cAMP, also showed that varying the motility force to adhesion force ratio can cause streams to break up or remain intact (Dallon *et al.* 2006).

The computer simulations showed that when the ratio of motility force to adhesion force is simply a function of time, thick (approx. 20 cells wide) streams break into large groups and thin (approx. 5 cells wide) streams break into small groups (Roisin-Bouffay *et al.* 2000). However, in this model, having the concentration of a diffusible factor secreted by cells regulate cell–cell adhesion and/or cell motility successfully caused both thick and thin streams to break up into similar-sized groups. This result suggested that *D. discoideum* cells secrete CF to modulate cell–cell adhesion and motility for a more precise regulation of group size. In addition, the simulations predicted that the ratio of cell–cell adhesion and cell motility determines the group size, not cell–cell adhesion or cell motility *per se*. Finally, a virtual mutant created in simulations which changes the direction of cell motility predicted that *D. discoideum* cells can regulate group size not only by modulating cell–cell adhesion and cell motility, but also by changing the direction of cell motility (Dallon *et al.* 2006).

6. EXPERIMENTS SUGGESTED BY THE COMPUTER SIMULATIONS VERIFIED THE PREDICTIONS OF THE MODEL

In higher eukaryotes, adhesion is required to connect cells together and plays an essential role in overall tissue organization and proper physiological function of the tissue and organ (Gumbiner 1996). In addition, regulating adhesion is thought to be an important suppressor of epithelial tumour cell invasiveness and metastasis (Takeichi 1993; Bergelson & Hemler 1995). In order to form a multicellular structure, proper cell–cell adhesion is required in *D. discoideum*. Two major types of adhesions can be observed during the development of *D. discoideum*: EDTA-sensitive and EDTA-resistant adhesion. Early in development, aggregation of *D. discoideum* can be prevented by adding EDTA. Later (8–12 hours), the cells will agglutinate even in the presence of EDTA. EDTA-sensitive adhesion is mediated by glycoprotein24 (gp24) and EDTA-resistant adhesion by glycoprotein80 (gp80). The gp80 expression is induced by cAMP pulses and cell–cell contact (for review, see Loomis 1975).

The computer simulations predicted that CF might regulate stream breakup. To our surprise and delight, we found that CF indeed regulates stream breakup and group size by regulating adhesion (Roisin-Bouffay *et al.* 2000). A high adhesion observed in *countin*$^-$ cells is due to the high expression level of gp24 early in the development. The gp24 expression in *smlA*$^-$ cells is delayed and, moreover, the level of expression is significantly lower. Exogenous administration of CF also reduces cell–cell adhesion significantly, showing that the decreased level of adhesion is, indeed, caused by CF. The *smlA*$^-$ cells also show a delayed pattern in gp80 expression. The gp80 expression level is not significantly altered in *countin*$^-$ compared to Ax2, but blocking gp80 adhesion by adding anti-gp80 antibodies to cells results in the formation of smaller groups (Siu 1990). We found that adding anti-gp24 antibodies to streaming cells also causes the formation of smaller groups. These results show that cell–cell adhesion is regulated by CF, and can regulate group size in *D. discoideum*.

The computer simulations also predicted that another way to regulate stream breakup is to regulate the random motility of cells (Tang *et al.* 2002). *Dictyostelium discoideum* cells depend on myosin and actin for motility (Noegel & Schleicher 2000). *Dictyostelium discoideum* cells move by extending a pseudopod, and actin is present in the protruding pseudopod (Cox *et al.* 1992; Fukui *et al.* 1999). Normally, myosin II is uniformly distributed throughout the cytoplasm and also forms a cortical ring of polymerized myosin. Upon cAMP stimulation, it depolymerizes at the front of the cell and localizes in the posterior end of migrating cells and in the tip of retracting pseudopods (Ogihara *et al.* 1988; Nachmias *et al.* 1989; Moores *et al.* 1996; Clow & McNally 1999). We found that CF decreases group size by increasing the amount of polymerized actin (F-actin) and decreasing the amount of assembled myosin II, which in turn increases the motility of cells (Tang *et al.* 2002).

7. CF SIGNAL TRANSDUCTION

We also found that cAMP and cGMP signalling pathways are implicated in the CF-mediated size regulation mechanism (Tang *et al.* 2001). cAMP is known to control motility and regulate the expression of adhesion proteins (Souza *et al.* 1994; Tang *et al.* 2001). cAMP signals are used as chemoattractants, morphogens, hormones and as intracellular messengers. In *Dictyostelium*, the synthesis of cGMP is triggered by cAMP stimulation (Mato *et al.* 1977; Wurster *et al.* 1977). cGMP regulates a wide variety of processes in *D. discoideum* as a secondary messenger, such as cell motility and cell–cell adhesion (for review, see Verkerke-van Wijk & Schaap 1997; Bosgraaf & van Haastert 2002; Bosgraaf & van Haastert 2006). We found that CF upregulates the cAMP-induced cAMP signal while at the same time downregulating the cAMP-induced cGMP signal (Tang *et al.* 2001; Brock *et al.* 2002). In addition, we observed saturable cell surface binding of recombinant countin and CF50, suggesting that there exist cell surface receptors for CF (Gao *et al.* 2002; Brock *et al.* 2003*a*). It thus appears that after binding to its receptor, CF regulates intracellular signalling processes that subsequently regulate key components of the cAMP and cGMP signal transduction pathways.

During *Dictyostelium* aggregation, each pulse of cAMP causes a transient translocation of Akt/protein kinase B (Akt/PKB) to the leading edge of the plasma membrane and a concomitant activation of the kinase activity, which in turn stimulates motility by phosphorylating and activating a kinase (PAKa) that

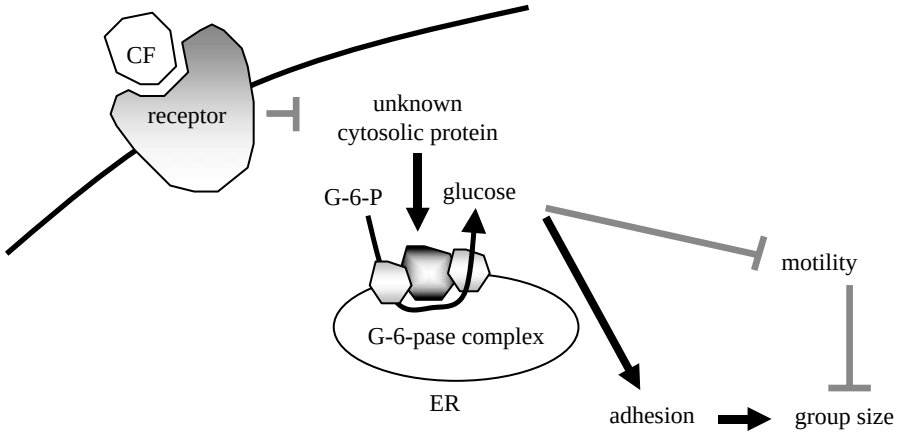

Figure 3. Our current model showing how CF may regulate glucose-6-phosphatase, glucose, cell–cell adhesion, cell motility and group size. Our working hypothesis is that CF binds to its surface receptor and the signal inhibits an unknown cytosolic protein. In the absence of CF, this protein increases glucose-6-phosphatase activity by decreasing the $K(m)$ to produce more glucose inside cells. High intracellular glucose inhibits random cell motility and increases cell–cell adhesion to increase group size.

regulates myosin II assembly (Chung & Firtel 1999; Chung *et al.* 2001; de la Roche & Cote 2001). The *countin*$^{-}$ cells, as well as Ax2 cells starved in the presence of anti-countin antibodies, showed a decreased level of cAMP-stimulated Akt/PKB membrane translocation and kinase activity compared with control cells (Gao *et al.* 2004). A one-minute exposure of cells to recombinant countin increased Akt/PKB translocation to membranes and Akt/PKB activity without affecting the total level of Akt/PKB. Together, the data indicated that CF potentiates cAMP-stimulated activation and translocation of Akt/PKB, which then activates PAKa to regulate myosin II heavy chain phosphorylation and assembly, thereby increasing the motility of *D. discoideum* cells.

Garrod & Ashworth (1972) showed that *Dictyostelium* cells grown with a high concentration (86 mM) of glucose formed larger fruiting bodies than control cells. To our surprise, we found that transformants lacking bioactive CF and Ax2 cells with extracellular CF depleted by antibodies have high glucose levels, while transformants oversecreting CF have low glucose levels (Jang *et al.* 2002). The addition of glucose does not change CF secretion, and there is not a significant amount of glucose secreted into conditioned media. Adding as little as 1 mM exogenous glucose negates the effect of high levels of extracellular CF on group size and mimics the effect of depleting CF on glucose levels, cell–cell adhesion, cAMP pulse size, actin polymerization, myosin assembly and motility (Jang *et al.* 2002; Brock *et al.* 2003*b*; Gao *et al.* 2007). The addition of beef heart cAMP-specific PDE to cells, or pulsing cells with cAMP, did not have any effect on internal glucose levels, suggesting that glucose is upstream of cAMP with respect to group size. CF represses internal glucose levels by increasing the $K(m)$ of glucose-6-phosphatase, an enzyme that is associated with microsomes (Jang *et al.* 2002; Jang & Gomer 2005, 2006).

8. CF AND ITS COMPONENTS

CF is an approximately 450 kDa protein complex that contains prominent bands at approximately 40, 45, 50 and 60 kDa (Brock & Gomer 1999). Countin, CF45, CF50, and CF60, the 40, 45, 50, and 60 kDa components, respectively, regulate group size by breaking up aggregation streams. Disrupting the genes encoding countin, CF45 and CF50 causes cells to secrete almost no detectable CF activity (Brock *et al.* 2002, 2003*b*). The streams formed by *countin*$^{-}$, *cf45-1*$^{-}$ or *cf50*$^{-}$ cells do not break up thereby forming huge but few aggregates. Consistent with our model that breakup is determined by the balance of cell–cell adhesion and cell motility, each component of the CF complex modulates cell–cell adhesion and cell motility in different ways to regulate group size. Our current working model on how CF regulates group size is illustrated in figure 3.

9. COUNTIN AND ITS HOMOLOGUES

Countin is the most well-studied component of the CF complex. The *countin* gene encodes a 40 kDa protein (Brock & Gomer 1999). Countin has some similarity to amoebapores, polypeptides that form pores in target cell membranes. Recombinant countin has some amoebapore activity at low pH, but no amoebapore activity in the extracellular buffer where it affects group size (Gao *et al.* 2002). Transformants with a disruption of the *countin* gene cause a decrease in the cAMP-induced cAMP pulse, and a decrease in cAMP-stimulated Akt/PKB membrane translocation and kinase activity, which in turn decrease cell motility (Tang *et al.* 2002; Brock *et al.* 2003*a*; Gao *et al.* 2004). The *countin*$^{-}$ cells also have increased cell–cell adhesion and myosin polymerization. These cause a net effect of increasing group size in *countin*$^{-}$ cells. The direct addition of recombinant countin to developing Ax2 cells causes an increase in F-actin polymerization and myosin phosphorylation and a decrease in myosin polymerization (Gao *et al.* 2004). We also found that the effect of countin is partly mediated by repressing internal glucose levels (Jang *et al.* 2002).

Dictyostelium discoideum has two proteins, countin2 (CtnB) and countin3 (CtnC), which have sequence similarity to countin (Okuwa *et al.* 2001, 2002; Katayama *et al.* 2003). The countin2 shares 40% identity and countin3 has 49% identity to countin. Both countin2 and countin3 seem to be secreted proteins

and have peak expression during aggregation and mid-developmental stages, respectively. The *countin2*$^-$ cells have low levels of gp24 and gp80, and thus have low cell–cell adhesion compared with parental Ax2, whereas *countin*$^-$ cells have higher adhesion due to the increased expression of gp24 (Okuwa *et al.* 2002). Like *countin2*$^-$ cells, *countin3*$^-$ cells also have a decreased cell–cell adhesion compared with the parental Ax2 strain, but it is not yet known whether these cells have altered patterns of adhesion proteins (Katayama *et al.* 2003). The loss of countin2 or countin3 results in decreased group size. The increased group size of *countin*$^-$ cells was restored close to Ax2 levels when *countin*$^-$ cells were starved with an equal number of *countin2*$^-$ cells. Together, the data suggest that countin2 and countin3 also play a role in group size regulation by antagonizing countin function, and that both countin2 and countin3 may serve to fine-tune the process of group size regulation.

10. CF50

CF50 is the 50 kDa component of the semi-purified CF complex (Brock *et al.* 2002). CF50 shows some similarity to *Entamoeba histolytica* lysozyme II precursor, but has a very low lysozyme activity. The *cf50* mRNA and CF50 protein are present in both vegetative growth and development, with an increase in the early development stage. Like *countin*$^-$ cells, disruption of the *cf50* gene causes large groups to form. However, the developing structures formed by *cf50*$^-$ cells are more aberrant than those formed by *countin*$^-$ cells. Disruption of the *cf50* gene also causes some degradation of secreted countin in the conditioned media, suggesting the role of CF50 may be to protect countin. Like *countin*$^-$ cells, the *cf50*$^-$ cells also have increased cell–cell adhesion and internal glucose levels and decreased cell motility (Chung *et al.* 2001; Brock *et al.* 2002, 2003*a*). Disrupting CF50 affects the differentiation of cells into spores and stalk cells, whereas disrupting countin does not affect this process. While the addition of either recombinant countin or recombinant CF50 to Ax2 cells decreased cAMP-induced cGMP pulses, they had an opposing effect on cAMP-induced cAMP pulses (Brock *et al.* 2003*b*). The addition of recombinant countin potentiates the cAMP-induced cAMP pulse, whereas the addition of recombinant CF50 decreases cAMP pulses. Although countin and CF50 both appear to negatively regulate group size, it seems that countin and CF50 have overlapping but different functions during development.

11. CF45-1

Another component of the CF complex, CF45-1, shares some similarity to *E. histolytica* lysozyme, although recombinant CF45-1 has no detectable lysozyme activity (Brock *et al.* 2003*b*). CF45-1 shares approximately 67% identity with CF50. Transformants with a disrupted *cf45-1* gene form huge groups when cells are grown with bacteria on agar plates. Unlike other transformants with disrupted components of CF complex (*countin*$^-$, *cf50*$^-$), *cf45-1*$^-$ cells sometimes form smaller groups than Ax2 cells when cells are starved on filter pads. At this time, we do not have an explanation for this behaviour. The *countin*$^-$ cells and *cf50*$^-$ cells accumulate a large amount of extracellular CF45-1 during development. Recombinant CF45-1 reduces group size when added to Ax2 or *cf45-1*$^-$ cells, but has a very slight effect on *cf50*$^-$ or *countin*$^-$ cells at the concentration effective on Ax2 or *cf45-1*$^-$ cells. This indicates that countin and CF50 are necessary for CF45-1 to affect group size. Like *countin*$^-$ and *cf50*$^-$ cells, *cf45-1*$^-$ cells also have increased internal glucose levels, slightly increased cell–cell adhesion and significantly decreased cell motility.

12. CF60

CF60 is the fourth component of CF (Brock *et al.* 2006). CF60 has some similarity to acid phosphatases, but recombinant CF60 has a very little or no phosphatase activity. When the *cf60* gene was disrupted, no viable transformants were obtained, suggesting that CF60 may have a crucial role in early development. Therefore, attempts were made to knock down *cf60* expression using an antisense construct. The introduction of the *cf60* antisense vector diminished CF60 levels very slightly, whereas the same vector with different antisense inserts (*countin* or *smlA*) successfully decreased the levels of countin or SmlA, respectively (Brock *et al.* 1996; Brock & Gomer 1999). Transformants oversecreting CF60 formed large numbers of abnormally small groups, suggesting that CF60 plays a role in aggregation stream breakup and group size. Adding recombinant CF60 to Ax2, *cf45-1*$^-$ and *countin*$^-$ cells decreased group size, while adding recombinant CF60 to *cf50*$^-$ cells caused no significant effect on group size, indicating that the effect of CF60 on group size requires the presence of CF50.

13. HOW DID CF EVOLVE?

Two components of the CF complex (CF45-1 and CF50) have similarities to lysozyme, but they have minimal lysozyme activities. CF60 has similarity to various types of acid phosphatase but the enzyme activity is not readily detectable. Countin has some amoebapore activity, but this activity is not detectable at a normal extracellular pH. The CF complex is thus made up of polypeptides with similarities to proteins that could be toxic to other cells. This then suggests the possibility that the CF complex may have evolved from a secreted defensive mechanism, and that at some point the cells were able to use the fact that sensing the concentration of the complex allowed them to sense the density and thus number of cells in a stream.

There exists a factor in mammals which acts analogously to CF. Leptin molecules are expressed and secreted by white adipose tissues in proportion to their triglyceride stores and are detected by the hypothalamus and/or choroid plexus in the brain (for review, see Khosla *et al.* 1996). When there is more than a threshold value of leptin, the brain sends a signal to reduce food intake and thus fat storage. Mutations in the signal domain of the leptin receptor result in 'leptin resistance' and obesity in mice and humans. Even

though there is no sequence homology between CF and leptin, it seems as if their function is quite analogous. Both proteins function as secreted signal molecules to detect size, with their levels increasing or decreasing in accordance with body size. In addition, exogenous administration of the proteins seems to affect body size. Together, the data strongly suggest that there may exist a CF-like mechanism in mammals. Combining diffusion calculations, computer simulations and experiments will hopefully provide an insight into how higher eukaryotes regulate tissue and organ size.

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