

A cell-counting factor regulating structure size in *Dictyostelium*

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Developing *Dictyostelium* cells form large aggregation streams that break up into groups of 0.2×10^5 to 1×10^5 cells. Each group then becomes a fruiting body. *smlA* cells oversecrete an unknown factor that causes aggregation streams to break up into groups of $\sim 5 \times 10^3$ cells and thus form very small fruiting bodies. We have purified the counting factor and find that it behaves as a complex of polypeptides with an effective molecular mass of 450 kD. One of the polypeptides is a 40-kD hydrophilic protein we have named *countin*. In transformants with a disrupted *countin* gene, there is no detectable secretion of counting factor, and the aggregation streams do not break up, resulting in huge (up to 2×10^5 cell) fruiting bodies.

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Very little is known about how a group of cells or a tissue senses the number of cells in it or its absolute size. An excellent system to study processes such as cell counting and size determination is the simple eukaryote *Dictyostelium discoideum*. *Dictyostelium* normally exists as a single cell that eats bacteria on soil surfaces and increases in number by fission. When a cell starves, it signals that it is starving by slowly secreting a cell-density sensing factor, the glycoprotein CMF (Mehdy et al. 1985; Gomer et al. 1991; Jain et al. 1992; Jain and Gomer 1994; Yuen and Gomer 1994; Yuen et al. 1995). As more and more cells in a 1 to 10 mm diameter area of soil starve, the concentration of CMF increases. When there is a high density of starving cells and thus a high concentration of CMF, the cells aggregate by use of relayed pulses of cAMP as a chemoattractant (for review, see Robertson and Grutsch 1981). Starving cells also secrete a phosphodiesterase and a phosphodiesterase inhibitor; the phosphodiesterase causes the levels of cAMP to return to a baseline level in the interval between pulses (Riedel and Gerisch 1971; Dicou and Brachet 1979; Kessin et al. 1979; Tsang and Coukell 1979; Franke and Kessin 1981; Orlov et al. 1981; Faure et al. 1988, 1989; Hall et al. 1993; Wu et al. 1995) and also steepens the cAMP gradient sensed by the cell (Nanjundiah and Malchow 1976). The aggregate forms a migrating slug, which, in turn, forms a fruiting body containing a mass of spore cells supported by a column of stalk cells (for review, see Loomis 1975, 1993; Devreotes 1989; Schaap 1991; Firtel 1995).

The function of the fruiting body is to hold the spore

mass as high off the ground as possible, for optimal spore dispersal. Thus, there is a strong selective pressure to have a large number of cells in the fruiting body. However, there is a limit to the strength of both the stalk and the attachment of the spore mass to the top of the stalk, so that if there are too many cells in a fruiting body, the fruiting body will fall over or the spore mass will slide down the stalk. Because spores on the ground will probably not get dispersed, there is a strong selective pressure to have an upper limit on the number of cells in a fruiting body. Therefore, in a field of starving *Dictyostelium*, the slugs are usually of approximately the same size. Depending on the plating density, slugs contain 2×10^4 to 1×10^5 cells (Bonner and Hoffman 1963).

There are many *Dictyostelium* mutants with an abnormal aggregate size (Sussman and Sussman 1953; Hohl and Raper 1964; Gerisch 1968). Once aggregate size is determined, later events may alter fruiting body size and number. Overexpression of the gp80 adhesion protein causes aggregation streams to break up and form small fruiting bodies (Faix et al. 1992). In Streamer F cells, which lack the cGMP-specific phosphodiesterase, the aggregation streams do not break up (Newell and Liu 1992). A mutant described by Sussman and Sussman (1953), *bushy*, makes aggregates of normal size, but then the slug breaks up into many tiny fruiting bodies. There also exist species of *Dictyostelium* that form large aggregates, which then break up into a bouquet of small fruiting bodies (for review, see Schaap 1986). Overexpression of a modified *ras* gene or disruption of a MEK also cause this phenotype (Reymond et al. 1986; Ma et al. 1997).

Hohl and Raper (1964) examined several small-aggregate mutant strains of *D. discoideum* and found that the phenotypes were due to disruption of either of two dif-

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ferent mechanisms. The first mechanism is aggregation, and mutants with defective aggregation could be rescued by crowding the cells together so that aggregation became unnecessary. Mutants with a defect in the extracellular phosphodiesterase fall into this class (Riedel et al. 1973; Faure et al. 1988). The second mechanism is a cell number or mass sensor, which in *Dictyostelium* and other systems has been hypothesized to regulate aggregate size and cause the aggregate to break up if it exceeds a critical size (Spratt and Haas 1961). Hohl and Raper (1964) also found mutants of this type, as some mutant cells, when starved at very high cell densities, still formed small aggregates and fruiting bodies.

One possible mechanism that would allow individual cells to sense the number of cells in an aggregate or group could theoretically be mediated by a signal that is simultaneously secreted and sensed by cells, and that can diffuse into or be degraded by the surrounding environment (Clarke and Gomer 1995; Gomer 1999). With a small number of cells in the group, the signal concentration in the vicinity of the cells will be low, and as the number of cells increases, the signal concentration increases.

To isolate genes involved in aspects of *Dictyostelium* morphogenesis such as size determination, we developed shotgun antisense, in which *Dictyostelium* cells are transformed with an antisense vector containing a library of cDNAs (Spann et al. 1996). The transformed cells were cloned out and allowed to form plaques on a lawn of bacteria, and clones with unusual fruiting body morphologies were picked. A transformant that develops at a normal speed, but which forms very small fruiting bodies, was designated *smlAas* for small aggregates, with the *as* suffix designating antisense. A 275-bp antisense cDNA fragment was purified from *smlAas* cells by PCR. The isolated antisense cDNA showed no significant sequence similarity to any known gene. The antisense cDNA was religated into the antisense vector, and the resulting construct, when transformed into wild-type cells, recapitulated the original *smlAas* phenotype (Spann et al. 1996). These transformants were called *smlAasr*. The 275-bp antisense cDNA fragment was also used to screen a cDNA library, and a 1.2-kb cDNA was isolated and used to make a gene disruption construct. Cells transformed with this construct, designated *smlA* cells, had a disruption of the *smlA* gene as determined by Southern blots, had no detectable *smlA* mRNA on Northern blots, and had a small-fruiting-body phenotype (Spann et al. 1996).

smlA mRNA is expressed in vegetative and early developing cells (Brock et al. 1996). The sequence of the cDNA and the derived amino acid sequence of the SmlA protein show no significant similarity to any known sequence. There are no obvious motifs in the protein or large regions of hydrophobicity or charge. Immunofluorescence and staining of Western blots of cell fractions indicated that SmlA is a 35-kD cytosolic protein present in all vegetative and developing cells and is absent from *smlA* cells. The absence of SmlA did not affect the growth, motility, differentiation, or developmental speed of cells. Mixing 5% *smlA* cells with wild-type cells

caused the wild-type cells to form smaller fruiting bodies and aggregates. Although there was no detectable SmlA protein secreted from cells, conditioned starvation medium (CM) from *smlA* cells also caused Ax-4 cells to form many small aggregates (Brock et al. 1996). Fractionation indicated that the component in the *smlA* CM that affects aggregate size is a protein with molecular weight >100 kD, but is not CMF, phosphodiesterase, or the phosphodiesterase inhibitor. The data thus suggest that there exists a secreted factor that regulates aggregate size, and that the secretion or processing of this factor is regulated by SmlA. In this report, we characterize the factor oversecreted by *smlA* cells, and find that it is a complex of polypeptides we call counting factor. Counting factor appears to be an example of a signal that is part of a general mechanism that measures the approximate number of cells in a group.

Results

We showed previously that the *smlA* transformant forms small aggregates and fruiting bodies due to the oversecretion of an unknown factor that is retained in a 100-kD cutoff centrifugal microfilter (Brock et al. 1996). This activity appeared to allow aggregation to begin normally, but then caused aggregation streams to break up (Brock et al. 1996). To characterize the factor, we used conventional column chromatography to fractionate starvation buffer in which either *smlA* or wild-type Ax4 cells had been starved (CM). After ion exchange chromatography of concentrated CM from both cell types, a bioassay of the various fractions showed that in the fractionated *smlA* CM, a peak that eluted at ~0.56 M NaCl, caused aggregation streams to break up. The fractions from the 0.56 M peak were pooled and further fractionated by hydroxyapatite chromatography. As a control, the same fractions from the Ax4 CM were also pooled and run on a hydroxyapatite column. In a bioassay, the Ax4 hydroxyapatite column fractions all formed approximately the same number of aggregates. In the *smlA* fractions, an activity eluting at 0.3 M phosphate allowed streams to form normally, but then caused them to break up into small subaggregates.

When the hydroxyapatite peak was fractionated by electrophoresis on a nondenaturing polyacrylamide gel (exposure to SDS destroyed the activity of counting factor), a 30-fold dilution of the material eluting out of two adjacent slices (2 and 3) of the *smlA* hydroxyapatite fractions gel showed a strong peak of bioassay activity that caused an increase in aggregate number. Similarly eluted and diluted material from the Ax-4 hydroxyapatite fractions gel slices had little effect on cell aggregation in the bioassay. We observed that this activity increased aggregate number by causing aggregation streams to break up (Fig. 1A,B). At higher concentrations, the material eluting out of gel slices 2 and 3 from the Ax4 prep also caused aggregate streams to break up. As shown in Figure 1C, there are several different proteins present in the nondenaturing gel peak. Comparing the material purified from the *smlA* cells and from the Ax4 cells, we observed the

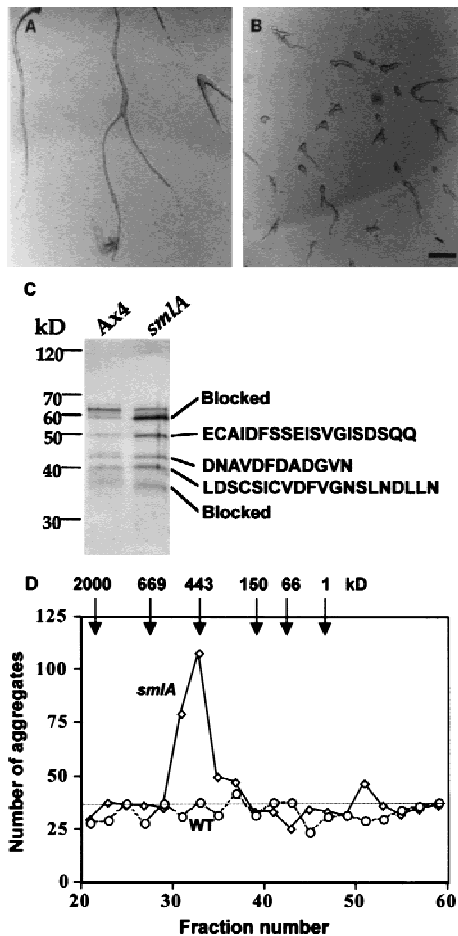


Figure 1. Purification of the counting factor. (A,B) Bioassay of the purified counting factor activity. Fractions 18–23 from the hydroxyapatite column were pooled, concentrated, desalted, and run on a 10% nondenaturing polyacrylamide gel. Then, 0.5-cm slices were excised, electroeluted, and concentrated. Ax4 cells were starved in submerged culture for 15 hr in the presence of (A) the material eluted from slice 3 of the nondenaturing gel of Ax4 CM, (B) material eluted from the same slice from *smlA* CM. Slice 2 from the *smlA* preparation had a similar number of small aggregates as seen for slice 3 from *smlA*; all other slices from the *smlA* prep and all the slices from the Ax4 prep had normal aggregation streams as shown for the Ax4 slice 2. Bar in B, 200 μ m. (C) Silver-stained SDS-polyacrylamide gel of the combined material eluted from gel slices 2 and 3. In other experiments, the polypeptide composition of the material from the two slices was seen to be identical. The amino-terminal amino acid sequences of the proteins are shown at right. The sequence we obtained for the 43-kD band was a mixture of two sequences; the sequence of the more abundant polypeptide is shown. (D) Gel-filtration chromatography of the active fractions from the hydroxyapatite column. (Solid line) Number of aggregates formed in the presence of fractionated *smlA*; (broken line) wild-type (WT) control; (dotted line) number of aggregates formed in PBM.

same proteins, with a roughly fourfold greater amount in the *smlA* material. This suggests that *smlA* cells over-secrete a factor normally secreted by Ax4 cells.

We tried a variety of columns to further fractionate the

polypeptides from the nondenaturing gel purification, but were unsuccessful. When the partially purified counting factor from the hydroxyapatite chromatography was fractionated on a gel filtration column, a single peak of activity was found, with an approximate molecular mass of 450 kD (Fig. 1D). An SDS-polyacrylamide gel of this peak showed essentially the same pattern of bands as seen in a gel of the material purified by elution from a nondenaturing gel. The observation that a factor with an effective molecular mass of 450 kD consists of polypeptides with molecular masses below 80 kD suggests that some of these polypeptides form a complex.

We attempted to sequence the amino termini of each of the proteins from the nondenaturing gel peak, and obtained sequences for the 50-, 43-, and 40-kD polypeptides (Fig. 1C). The 60- and 36-kD proteins appeared to have blocked amino termini. Iijima et al. (1995) described a 450-kD complex of proteins secreted by *Dicystostelium* cells. Although the protein profiles of counting factor and the Iijima factor are mostly different, both factors contain a roughly 50-kD polypeptide. The sequence of the 50-kD protein had only 3 of 14 amino acids identical with a 49-kD protein present in the Iijima factor, suggesting that the two proteins are different, and the difference in protein profiles suggests that the two complexes are different.

To further characterize the polypeptides eluted from the nondenaturing gel, we made degenerate oligonucleotides to isolate cDNA clones. As of yet, we were unable to isolate clones for the 50- and 43-kD proteins. For the 40-kD protein, we isolated a 920-nucleotide cDNA with the first AUG being followed by a 738-nucleotide ORF (Fig. 2). The ORF encodes a protein with a predicted molecular mass of 26.7 kD. The amino acid sequence of the originally sequenced peptide was present in the predicted amino acid sequence. The predicted sequence shows no significant similarity to any known protein. The predicted protein, which we have named countin, is hydrophilic, has a potential signal sequence upstream of where we observe the amino terminus of the secreted protein, and has two potential glycosylation sites (Fig. 2). To examine when during development the *countin* gene is expressed, a Northern blot of RNA from cells at different stages of development was probed with the *countin* cDNA. The *countin* mRNA is ~1.2 kb, and is expressed in vegetative cells and throughout development (Fig. 3H and data not shown). To determine whether *countin* is present in all or just some cells, as well as its subcellular localization, cells were stained by immunofluorescence with anti-countin antibodies. As shown in Figure 3A, staining of vegetative wild-type cells shows countin present in all cells, associated with what appear to be vesicles. *smlA* cells show a weaker staining with a distribution roughly equivalent to wild-type cells (Fig. 3C).

Next, we wanted to see the effect of disrupting expression of *countin*. A gene disruption construct made with the cDNA failed to give homologous recombination as determined by PCR of DNA from transformant clones, so the cDNA was used to probe Southern blots to map

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1  GTA AAT TGT TTT TTG TTA AAT TAA TAA TAA AAT TAA ATT AAT TTT AAT TTT TTT TTT 60
61  TTT TAT GAA AAA ATA AAA CCA TGG GCC ATT GTT TTT TTT TTT TTT TTT TTT AAA ATA ATG 120
121 GAA AAT TAA AAC AAT GCC CAA TAG GTT TTT TTT TTT TTT TTT TTT TTT TTT GGT GCC CAT TAA 180
181 AAG ATT TTT TTT TTT CAA TTT AAA AAC AAG TAA AAA AAA ATT TTT TAA AAA AAT CTA 240
241 TAC CCA AAG GGT TTC TAA ATG GTC ATG ATG ATT TCA AAA AAA AAA AAA AAA AAG GGT 300
301 ATY TTC ATT GAT TTT TTT TTA TTT TAT TTT TAT TTT TTT ATT TTT TTT TTT AAT AAT TAT 360
361 TTT TTT TTT TAA AAA AAA GGT TAA AAA AGT TTT TTT TTT TTT TTT TTA TTC ACT YTT CTA 420
421 AAA TCC AGA ATT GGT AGA TAT ATA TAT ATA ATA AAA ATG AAT AAA TTA TTT TCA TTA ATT 480
      M N K L F S L I
481  TTA GCT TTA TTC CTT GTC AAC TCC GGT GTT GTC TCT TCA CTC GAC TCA TGT AGT ATT TGT 540
      L A L F L V N S A V V S S L D S C S I C
541  GTT GAT TTT GTT GGT AAC TCA CTC AAT GAT TTT TTA AAT ATT ATC CTT agt aag taa att 600
      V D F V G N S L N D L L N I I L
601  att att ttt tta ttt ttt att ttt ttg ttt taa ata ttt acc aat ttt att ttt tta ttt 660
      KpnI
661  ttt att tta taa aaa gac TCT GGT GTT ATT GGT ACC TGT GCT GAT CTT TGC TCA GCT GTT 720
      S G V I I G T C C G D L C S A V
721  CCA GGT GGT CAA ATT GTT GAT ACC GTT TGC GAC TTA TTA TCC CAT TAT GTT GGC GTT GAT 780
      P G G Q I V D T V C D L L C D Y V G D
781  GAA TTT ATT AAA CTC ATT TCA CAT GTT GAC CCA GAT CCA AIT TAC ATT TGT GAA AAG ATC 840
      E F I K L I S D V D P O P I Y I C E K I
841  AGT GTT TGC AAA ACC AAC GAT AAC GCA AAT GCT TCA TTA GAT TTA GTT CAA AAT AAC CCA 900
      S V C K T O L A F N I I D P G N A F V G D
901  CAA AAC GGT ACT GTC GGT GGA ACA TTC ACC CTC TCA ATT GCT TAC ACT GTT ACC AAC ACC 960
      Q N G T V I G G T F T L S I A Y T V T N T
961  ATC GCC ACT GGT CAA TTA GCT TTC AAT ATC ATC GAT CCA ACC GGT AAT GCT TTT GGT GAT 1020
      I A T G Q L A F N I I D P G N A F V G D
1021 GCC GTT TTA TTA GTT CAA CAA TCA CCA GAA CAA TAC ACC CAA CAA TTC CAA TTC CAA GCT 1080
      A V L L V Q Q S P E Q Y T Q Q F Q F Q A
1081 ACC CCA TCA CAA CAA CAA TCA TTC CCA AAT GGT TTA TAC ACT GTC CAA GCT CTC GTT TGT 1140
      T P S E Q E S F P N G Y T V D A L V C
1141 GAA GGT TCA TGT GGT TCA CCA CAT CCA AAC ACT TAC ACT CTT GCC AAC GGT ACC ACC ACT 1200
      E G S C G S P H P N T Y T L A N G T T I
1201 TTC ACC ATC TCT GGT GGT GAT TCT TCA TCA ATG TCT GGT GCT GGT TCT TTC TCT GGT TCT 1260
      F T I S C A D S S S H S G A G S F S G S
1261 TCC CAA TCA ACC CAA ACT GGT GCC GCC TCT GGT TCA GGT TCA GGT TTT GCT TTA TTT TAA 1320
      S Q S T Q T G A A S G S G S C F A L F *
1321 AAA AAT ACT TAT TAA AAT AAA TAT TTA ATT TTA TAA TTT TTT ATT TAA TTC TAT AAA TAA 1380
1381 CTT GGA TTT TTT TAT TTT TTA TTT TTA AAA TTA AAA TTT TTT TTT TTA AAA CCA ATA TTC 1440
1441 CCT CTC GTT TTT TCA AAA ATA AAA AAA AAA AAA AAA TTA AAA TTA AAC GAT TTA GAT 1500
1501 CCC CAA CTC TCT ATA TCT AAA AAA TGT TTA AAA AAT TAA AAA AAA CTT ATT CCA TAA AAA 1560
1561 AAA AAA ATT AAA AAG AAA ATC ATA TTT TTA CCA AAT CTA TAA AAA CCG GAT TTA TAA ATC 1620
1621 TGT TTT TTT TTT AAA CAA CCA ACC ACC CAA CTT GTT ATT TAA AAA CAA AAT 1671

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Figure 2. Sequence of the *countin* gene. The sequence starts with genomic DNA; the 5' end of the cDNA is indicated by an arrow. The first AUG is at nucleotide 457. A broken underline indicates a potential signal sequence. The amino-terminal amino acid sequence obtained from the purified 40-kD protein is marked with a heavy underline. Two potential amino-linked glycosylation sites (Alexander 1997) are marked with boxes around the amino acid sequence. There is an intron between nucleotides 552 and 681. A light underline marks the sequence of the synthetic peptide used to immunize rabbits for antibody production. The *KpnI* sites used for the insertion of a blasticidin resistance cassette to make a gene disruption transformant are indicated by lines over the DNA sequence. (*) Start of an AAUAAA poly(A) addition signal. An open arrow marks where the cDNA sequence had 21 A's and then terminated. The remainder of the sequence is genomic DNA. The sequence is available as AF140780 in Genbank.

the genomic DNA, and a 3-kb genomic fragment was then isolated. This was used to make a gene disruption construct to replace 0.5 kb of the *countin* coding region with a blasticidin resistance cassette. When transformed into Ax2 or Ax4 cells, this disrupted the *countin* gene as determined by PCR (data not shown). Northern blots of parental and transformed cells indicated that the *countin*

cells lack the *countin* mRNA (Fig. 3H). Immunofluorescence showed that *countin* cells do not show the staining observed in wild-type cells (Fig. 3B), and Western blots indicated that a 40-kD band is stained with anti-*countin* antibodies in concentrated CM from wild-type but not *countin* cells (Fig. 3G). The above results suggest that *countin* mRNA and protein are absent from the *countin* cells.

The *countin* cells grew in shaking culture and on bacterial lawns approximately as well as the parental cells. At low cell density, the differentiation of *countin* cells into CP2-positive and SP70-positive was indistinguishable from wild-type cells (data not shown). When the *countin* cells were starved on either agar or filter pads, their development was slightly delayed with respect to that of the parental cells. On both agar and filter pads, the *countin* and parental cells formed aggregation territories of approximately the same size. However, the aggregation streams of the parental cells broke up into subgroups, whereas the streams of the *countin* cells continued to flow in toward the aggregation center (Fig. 4A,B). Occasionally, the large aggregates formed by the *countin* cells would then separate into several smaller groups. The *countin* cells were able to form fruiting bodies, but

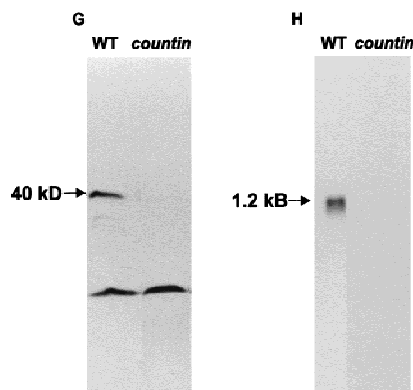
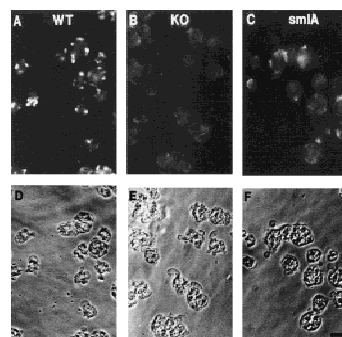


Figure 3. *Countin* is present in all cells and is absent from *countin* transformants. (A-F) Cells stained with anticountin antibody. (A,B,C) Immunofluorescence staining of cells; (D,E,F) corresponding phase images. A and D are Ax4 cells, B and E are *countin* knockout cells, and C and F are *smIA* cells. Bar in F, 10 μ m. (G) Western blot of protein from wild-type (WT) and *countin* CM stained with anti-*countin* antibodies. (H) Northern blot of total RNA from vegetative cells probed with the *countin* cDNA.

these were considerably larger than those of the parental cells (Fig. 4C), and as a consequence often fell over (Fig. 4D,E). Disruption of *countin* in Ax4 or *smlA* cells also resulted in larger aggregates (data not shown).

To determine whether the phenotype of the *countin* cells was due to the lack of a secreted factor, the CM from *countin* cells was assayed for counting factor activity. When *countin* cells were starved at low density in the presence of CM from *countin* cells starved at high cell density, they formed no more aggregates than when they were starved in buffer alone; but when they were starved in CM from wild-type or *smlA* cells, they formed more aggregates (Fig. 5). Similar results were obtained starving Ax4 cells in the various conditioned media. When CM from *smlA* cells was treated for 1 hr at room temperature with a 1:500 dilution of anti-countin antibodies, the number of aggregates decreased by ~50%. No effect was seen using *countin* CM or preimmune sera. When *countin* cells were mixed with 10% Ax4 cells and starved on either agar or filter pads, they formed Ax4-size fruiting bodies, and when mixed with *smlA* cells, they formed small fruiting bodies. A similar effect was ob-

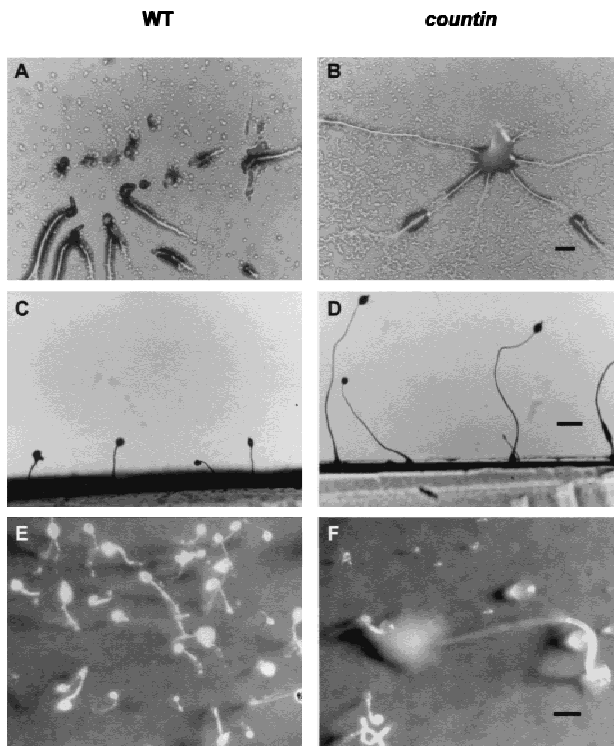


Figure 4. The development of countin cells. (A,B) Aggregation of wild-type (WT) and *countin* cells. Cells were starved at 10^7 cells/cm² on PBM agar. (A) Wild-type cells showing aggregation streams breaking up. (B) *countin* cells with unbroken streams forming a large aggregate. Bar, 200 μ m. (C,D) Side views of wild-type and *countin* fruiting bodies. (C) Wild-type fruiting bodies are normal size. (D) Fruiting bodies formed by starved *countin* cells are much taller than wild-type. Bar, 500 μ m. (E,F) The effect of forming large fruiting bodies. Cells were starved at 10^7 cells/cm² on filter pads moistened with PBM. (E) A field of wild-type fruiting bodies depicting normal size. (F) A large, toppled *countin* fruiting body. Bar, 500 μ m.

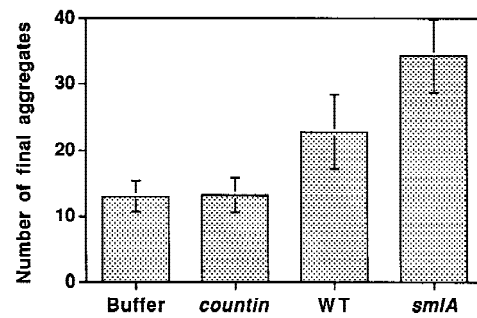


Figure 5. Assay of conditioned medium for counting factor activity. *countin* cells were starved in the presence of either PBM alone (Buffer), or buffer conditioned by a high density of starved *countin*, Ax4 (WT), or *smlA* cells. The number of aggregates was counted after 20 hr. Bars, S.E.M.

served when *countin* cells were starved on filter pads soaked with Ax4 or *smlA* CM. These results suggest that *countin* cells do not secrete a factor that causes aggregate streams to break up, but can sense and respond to a such a factor secreted by Ax4 and *smlA* cells.

Discussion

Very little is known about how groups of cells are able to count themselves to regulate tissue size. An evenly spread culture of starved *Dictyostelium* cells breaks up into groups of $\sim 2 \times 10^4$ cells. Previously, we hypothesized that individual cells would be able to sense the number of cells in a group by secreting and sensing a diffusible factor. We found a transformant (*smlA*) that appears to oversecrete such a factor, and apparently as a result of the high levels of the cell-counting factor, gives cells a false sense of the nearby presence of a large number of cells. A prediction of this hypothesis is that in the absence of the cell-counting factor, cells will be unable to sense whether they have formed an excessively large group, and thus will tend to form large aggregates. To test this prediction, we made transformants with a defective factor by purifying the factor, isolating the gene encoding a putative component of the factor, and then using this to make a gene disruption construct. To our delight, transformants lacking this component formed large aggregates, verifying the hypothesis.

The nature of the counting factor is rather unusual, in that it appears to be a complex of polypeptides (Fig. 1B,C). The counting factor would thus appear to be a multimer of countin, or a complex of countin and other proteins. If the counting factor is a multimer of different polypeptides, countin could either interact with a cell-surface receptor by itself, furnish part of the binding domain for a receptor, activate one receptor while another polypeptide in the counting factor complex activates a different receptor, or hold other proteins in a configuration necessary for them to interact with receptors. Like the cell-density signal CMF, countin has a polypeptide backbone that is smaller than the observed protein molecular mass, suggesting that like CMF and other se-

creted *Dictyostelium* proteins, countin has a considerable amount of glycosylation (Yuen et al. 1991; Alexander 1997). The ability of the *countin* transformants, as well as the *smlA* cells, which oversecrete countin, to form fruiting bodies indicates that countin is not involved with the differentiation of cells.

We were surprised to see a secreted signal with such a large molecular mass. Diffusion calculations by a secreted signal with a molecular mass of 10 kD, or even 1 kD, indicated that signals of these sizes could be used to sense the number of cells in a group (Fig. 6). Using an effective diffusion distance of $x = \sqrt{4Dt}$, in which D is the diffusion coefficient and t is the amount of time the material has been diffusing, we find that for a 450-kD signal diffusing through agar or moist dirt for 5 hr, the effective distance is ~ 1 mm. For a 10-kD signal, the effective distance is roughly twice this. These distances are typical for the spacing between fruiting bodies, but much greater than the width of an aggregation stream or the diameter of the aggregated group of cells forming a fruiting body. Thus, one possible reason that the counting factor is so large is that this results in a relatively small propagation distance, allowing cells within a group to communicate with each other, but reducing the amount of signal a group of cells would see from neighboring groups.

Low-power microscopy of cells in the presence and absence of countin suggests that when *Dictyostelium* cells are starved at high density they form a converging aggregation stream pattern over a relatively very large area, and that countin is involved in the aggregation streams breaking up into chains of discrete groups of cells, each of which will go on to form a fruiting body. Disruption of the MAP kinase kinase, MEK1, causes a highly broken up stream phenotype very similar to that seen in *smlA* (Ma et al. 1997), suggesting the possibility that MEK1 is involved in either counting factor secretion or signal transduction. Because cells lacking countin

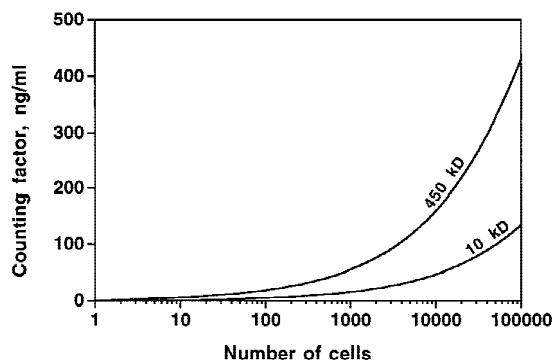


Figure 6. The theoretical concentration of counting factor at the center of a group of cells as a function of the number of cells in the group. The calculations were done for close-packed disks of cells on an agar or moist dirt surface, starting with one cell, then one cell with a ring of 6 cells around it (a total of 7 cells), up to a total of 200 concentric rings. The cells in the calculation were secreting counting factor continuously for 5 hr. The graph shows the concentrations for a 450-kD factor as well as a 10-kD factor, both being secreted from cells at 3×10^{-10} ng/sec.

have streams that do not break up, but form aggregates that occasionally break up, one possibility is that there exists more than one size determination mechanisms in *Dictyostelium*.

There is ~ 2 μ g of protein in the active fractions eluted from the nondenaturing gel peak from the prep of *smlA* CM (Table 1). The 40-kD polypeptide is $\sim 10\%$ of this protein (Fig. 1B), so from 150 ml of 5×10^6 cells/ml secreting for 20 hr we purified ~ 0.2 μ g of the 40-kD protein. Assuming that there is no internalization or degradation of the 40-kD protein, that the secretion is uniform with time, and that the yield is 13% (Table 1), there are ~ 250 molecules of the 40-kD protein secreted per *smlA* cell per minute.

Previously, we used calculations to show that a secreted factor that diffuses away from a group of cells can be used as part of a mechanism to sense the number of cells in a group (Yuen and Gomer 1994; Clarke and Gomer 1995). We used 450 kD as the molecular mass of counting factor to calculate a diffusion coefficient of 3.4×10^{-7} cm^2/sec . Assuming that countin is secreted at an even rate by all starving cells, from the purification table we determined the approximate secretion rate of countin to be 3×10^{-10} ng/sec. This allows us to calculate the concentration of countin as a function of the number of cells secreting it. With the cells closely spaced, and the cells in a flat disk on a surface with the bulk diffusion properties of moist dirt or agar, the theoretical concentration of countin increases with the number of cells in the group (Fig. 6). At the lower number of cells, the concentration approaches the concentration for a single cell; previously, we saw that this concentration quickly reaches and stays at a fixed value (Yuen and Gomer 1994). We also calculated the concentration of a 10-kD signal, also being secreted at 3×10^{-10} ng/sec (Fig. 6, bottom curve). The concentration of this signal also increases with the number of cells, but is lower overall, due to the dissipation of this faster-diffusing signal. Similar results were obtained for different geometries of cells, such as an elongated stream. These calculations were done for groups of cells that had been secreting the factor continuously for 5 hr. Qualitatively similar results (signal concentration increasing with the number of cells in the group) were obtained for shorter and longer secretion times (results not shown).

From the purification (Table 1), 2 μ g of counting factor was purified from 150 ml of *smlA* CM with a 13% purification efficiency, giving an estimated concentration of counting factor in *smlA* CM to be ~ 100 ng/ml. The diffusion calculations indicate that this concentration is reached when there are $\sim 3 \times 10^3$ cells in a group (Fig. 6). If we assume that the secretion of counting factor from *smlA* cells is fourfold greater than the secretion from wild-type cells (Fig. 1C), then the counting factor concentration would theoretically reach ~ 100 ng/ml when there are $\sim 10^5$ Ax4 cells in a group. Thus, there is a very crude agreement between the theoretical effect of countin and the observed number of cells in aggregates.

Our current working hypothesis is that to sense the number of cells in a group, the cells secrete a factor that

Table 1. Purification of the counting factor

Material	Volume (ml)	Differentiation activity			Protein			Specific activity (U/μg)
		U/ml	units	%	μg/ml	μg	%	
Whole CM	150	1	150	100	14	2100	100	0.07
Ion exchange fractions	8	5	40	26	60	480	23	0.08
Hydroxyapatite fractions	5	5	25	16	30	150	7	0.16
Gel elution purification	0.66	30	20	13	3	2	0.1	10

The U/ml activity is defined as the lowest fold dilution at which the material produces a significant (1.5×) increase in the number of aggregates for Ax4 starved in submerged culture.

diffuses into the surrounding environment. The environment thus becomes a concentration sink for the material. The cells measure at their plasma membranes the extracellular concentration of the factor, and this increases, although not linearly, with the number of cells in the group. Such a mechanism would work with the surrounding environment being either a tissue completely surrounding the group, or the surface of a permeable material that the cells sit on, such as moist dirt or agar. In addition to passive diffusion being a sink for the secreted factor, one can envision a surrounding layer of cells or material that absorbs, adsorbs, or enzymatically breaks down the signal, or removes it by a process such as endocytosis. In addition, the essential polarity of the mechanism could also be reversed, with the surrounding material or tissue supplying the signal, and the cells competing for the signal in any of the ways described above (Raff et al. 1998). Then, in the vicinity of the cells, the concentration of the signal would decrease as the number of cells increase. In several ways, a secreted signal can thus form the basis of a simple and elegant cell-counting mechanism.

Materials and methods

Cell culture

Dictyostelium discoideum Ax4 wild-type and *smlA* knockout cells (Brock et al. 1996) were grown as described previously (Jain and Gomer 1994), with the exception that for the HL5 growth medium the peptone was a mix of 7.15 grams of bacterial peptone (Oxoid Limited, Basingstoke, Hampshire, UK) and 7.15 grams of BBL Thiotone E peptone (Becton Dickinson, Cockeysville, MD) per liter; the HL5 was supplemented, after autoclaving, with 20 μg/liter biotin, 5 μg/liter vitamin B12, 200 μg/liter folic acid, 400 μg/liter lipoic acid, 500 μg/liter riboflavin, and 600 μg/liter thiamine. A mixture of 0.3 grams/liter streptomycin sulfate and 0.1 grams/liter ampicillin was used for the antibiotics. CM was made by harvesting mid-log phase cells by centrifugation at 500g for 5 min, resuspending cells in PBM [20 mM KH₂PO₄, 1 mM MgCl₂, 0.01 mM CaCl₂ (pH 6.1) with KOH], recentrifuging, resuspending to 5 × 10⁶/ml, and shaking at 110 RPM. After 20 hr, the cells were removed by centrifugation as above, and the supernatant was clarified by centrifugation at 12,000g for 10 min. Cell differentiation at low cell density was assayed following Wood et al. (1996).

Gel electrophoresis

Nondenaturing protein separating gel stock was 7.5 ml of 40% acrylamide (Fisher, Fairlawn, NJ), 2 ml of 2% bis-acrylamide

(Fisher), 11.2 ml of 1 M Tris/HCl at pH 8.7, and 9.3 ml of water. Gels were polymerized with 15 μl of 20% ammonium persulfate and 1.5 μl TEMED/4 ml of gel stock, and overlaid with isobutanol. Nondenaturing stacking gel stock was 1.25 ml of 40% acrylamide, 0.65 ml of 2% bis-acrylamide, 1.25 ml of 1 M Tris/HCl (pH 6.8) and 6.85 ml of water. Stack stock (1 ml) was polymerized with 5 μl of 20% ammonium persulfate and 1.5 μl of TEMED. Gels were cast and run in a Mini-Protean II apparatus with 0.75-mm spacers and 15-tooth combs (Bio-Rad, Hercules, CA).

Protein purification and sequencing

CM (150 ml) from Ax-4 and *smlA* cells was concentrated with Centricon Plus-80 30,000 NMWL centrifugal filter devices (Millipore, Bedford, MA) to 1–1.5 ml. This and all subsequent concentration steps were done at 4°C. The chromatographic steps were done with an Econo system (Bio-Rad, Hercules, CA) at 4°C collecting 1-ml samples with all flow rates 1 ml/min. The concentrated CM was loaded on a 5-ml Econo-Pac High Q column (Bio-Rad), after washing the column with 5 ml of PBM, the bound material was eluted with a 35-ml gradient of 0–800 mM NaCl in PBM. To prevent proteolysis, a Boehringer complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN) was dissolved in 1 ml of PBM to make a 50× cocktail, and 20 μl was added to each collected fraction. The peak activity fractions from the High Q column were pooled (typically a total of 6 ml) and diluted with PBM to 15 ml. This was then concentrated with an Ultrafree-15 10-kD cutoff centrifugal concentrator (Millipore) to 1 ml, and loaded on a 5-ml Econo-Pac CHT-II hydroxyapatite cartridge (Bio-Rad). After washing the column with 5 ml of PBM, the bound material was eluted with a 35-ml gradient of 0–800 mM potassium phosphate at pH 6.1 in PBM. Protease inhibitor was added to the fractions as described above. The peak activity fractions from the hydroxyapatite column were concentrated as described above to 1 ml. This material was then concentrated to 50 μl with a Microcon 30-kD cutoff centrifugal concentrator (Millipore). A total of 12.5 μl of 5× nondenaturing sample buffer (3.5 ml of 1 M Tris/HCl at pH 6.8, 3.6 ml of glycerol, 1.2 mg of bromphenol blue, with water added to 10 ml) was added, and the mixture was clarified by centrifugation at 14,000g for 2 min at room temperature. The supernatant was loaded on a 10% polyacrylamide nondenaturing gel. The gel was run at 50 V for 15 min, and then at 100 V for ~1 hr, until the dye front was 1 cm from the bottom of the gel. The gel was cut into 5 mm slices. Each slice from one lane was crushed in 200 μl of PBM with a small pestle in an eppendorf tube, and 400 μl of PBM was used to rinse the pestle. After adding 12 μl of 50× protease inhibitor, the crushed gels were rotated end over end at 4°C overnight. The eluted protein was then clarified by centrifugation at 14,000g for 5 min and stored at –80°C. Alternatively, protein was electroeluted out of gel slices with a model 422 electroeluter with 12- to 15-kD cutoff membranes following

the manufacturer's directions (Bio-Rad). To obtain protein for amino acid sequencing, the material from several preps was pooled and concentrated as described above. This was loaded in a well of a 20% SDS-polyacrylamide gel. The top chamber contained 40 μ l of thioglycolic acid (Sigma, St. Louis, MO) in 200 ml of running buffer. After electrophoresis, the gel was blotted to Problot PVDV (Applied Biosystems, Foster City, CA) in Laemmli running buffer containing 20% methanol for 5 hr at 35 V in a water-cooled transblot apparatus (Bio-Rad). The blot was stained with amido black following Jain et al. (1992) and the bands were excised. Amino-terminal amino acid sequencing of the protein in the bands was done at the Baylor College of Medicine core facility.

Gel filtration

Size fractionation of conditioned medium or partially purified counting factor was done at 4°C with a 1.5 \times 47.5-cm column of A 1.5-m resin (Bio-Rad) equilibrated in PBM and collecting 1.4-ml fractions. The column was calibrated with Bromphenol Blue and a 29- to 700-kD gel filtration chromatography standards kit (Sigma).

Assay for counting factor activity

To avoid interference from protease inhibitors and salt from the column fractions, 300 μ l of every sample for the counting factor bioassay (except for whole CM and sieving gel fractions) was concentrated with a Microcon 30-kD cutoff centrifugal concentrator as described above and resuspended in 300 μ l of PBM. The concentration and resuspension was then repeated two more times. For the bioassay, Ax4 cells growing in shaking culture at 1×10^6 to 2×10^6 cells/ml were harvested by centrifugation, washed in PBM, and resuspended in PBM to 1×10^6 cells/ml. A total of 150 μ l of these cells were mixed with 10–300 μ l of column fraction or eluted material and 40 μ l of Ax4 conditioned medium. PBM was added to a final volume of 450 μ l. This was placed in the well of a 24 well plate (type 3047, Falcon, Lincoln Park, NJ). The plates were examined 15–24 hr later with an inverted microscope, and the number of aggregates was counted.

cDNA and genomic DNA isolation

A degenerate oligonucleotide encoding the amino acid sequence ICVDFVG from the 40-kD protein, with a *Mlu*I site at the 5' end, GCACGCGTAT(TCA)TG(TC)GT(AGTC)GA(TC)TT(TC)-GTAGG and a primer ACCTCTATACTTTAACGTCAAGGAG binding to the λ YES plasmid were used for PCR following Compton (1990) with a *Dictyostelium* cDNA library (a gift from Dr. Gino de Hostos, UCSF) as the template. The products from the PCR reaction were cloned into a TA-cloning vector (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Several PCR products were obtained and were sequenced at the University of Texas Houston core facility. One cloned PCR product had a sequence corresponding to the primer, and then encoded a protein with the same initial sequence as the rest of the sequenced protein. An oligonucleotide with a portion of the sequence of the PCR product was then labeled with 32 P and used to screen the λ yes library, and a larger cDNA clone was obtained. This clone and the original PCR product were sequenced by primer walking.

To isolate genomic *countin* DNA, Southern blots of *Dictyostelium* genomic DNA digested with a variety of restriction enzymes were probed with the *countin* cDNA following Wood et al. (1996). After generating a restriction map, we chose to

isolate a 3.0 kb *Hind*III–*Sac*I fragment that had the *countin*-coding region near its center. A genomic sublibrary was made by digesting genomic DNA with *Sac*I and *Hind*III, and gel purifying the ~3-kb DNA. This was cloned into pBluescript, and this library was screened with the *countin* cDNA as a probe following Wood et al. (1996).

Gene disruption

The 3.0-kb fragment of *countin* genomic DNA in pBluescript was excised with *Hind*III and *Sac*I and ligated into the same sites in pUC 19. A 0.5-kb *Kpn*I fragment in the approximate middle of the coding region in the genomic DNA was then replaced with the 1.4-kb *Kpn*I fragment from pBSR479 containing a *Dictyostelium* blasticidin resistance cassette. The pBSR479 plasmid was a gift from Dr. Frantisek Puta (Charles University, Prague, Czech Republic), and is a modification of the blasticidin resistance cassette developed by Sutoh (1993). This construct was then digested with *Eco*R1 (this cuts inside the genomic DNA, on the 3' side of the *countin* gene, and in pUC 19, outside the genomic DNA) to yield a 3.3-kb linear construct with 0.6 kb of the *countin* 5' sequence, the blasticidin resistance cassette, and 1.3 kb of *countin* 3' sequence. This was used to transform both Ax2 and Ax4 cells following Shaulsky et al. (1996).

Antibody preparation, immunofluorescence, and Western blots

The synthetic peptide CGSPHPNTYTLANGTT was conjugated to keyhole limpet hemocyanin and this was then used to immunize a rabbit at Biosynthesis Inc. (Lewisville, TX). Antibodies were purified from sera with a 25%–50% ammonium sulfate cut followed by dialysis against 10 mM sodium phosphate at pH 7.4. After dialysis, the antibodies were clarified by centrifugation at 12,000g for 5 min, and then sodium azide and NaCl were added to 10 mM and 120 mM, respectively. For immunofluorescence, cells were fixed with ethanol and stained following Gomer (1987). With stained cells mounted in glycerol, we observed that the antibody came off the cells slowly, so slides were observed immediately after mounting.

Diffusion calculations

The diffusion of a factor such as *countin* was calculated following Yuen and Gomer (1994). The calculations were done for point sources on a thick layer of moist dirt secreting a 450-kD molecule at 3×10^{-10} ng/sec continuously for 5 hr. We used an effective diffusion coefficient of 1.7×10^{-7} cm²/sec (Moore 1962). As a comparison, we also did the calculations for a 10-kD signal, with a diffusion coefficient of 1.2×10^{-6} cm²/sec. The cells were assumed to be in a circular disk in a closely packed array. The concentration at the center of the disk was calculated for one cell at the center, then for one cell plus a contribution from a ring of surrounding cells, etc.

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