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The different components of a multisubunit cell number-counting factor have both unique and overlapping functions

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SUMMARY

Dictyostelium **aggregation streams break up into groups of 10^3 to 2×10^4 cells. The cells sense the number of cells in a stream or group by the level of a secreted counting factor (CF). CF is a complex of at least 5 polypeptides. When the gene encoding countin (one of the CF polypeptides) was disrupted, the cells could not sense each other's presence, resulting in non-breaking streams that coalesced into abnormally large groups. To understand the function of the components of CF, we have isolated cDNA sequences encoding a second component of CF, CF50. CF50 is 30% identical to lysozyme (but has very little lysozyme activity) and contains distinctive serine-glycine motifs. Transformants with a disrupted *cf50* gene, like *countin*$^-$ cells, form abnormally large groups. Addition of recombinant CF50 protein to developing *cf50*$^-$ cells rescues their phenotype by decreasing group size. Abnormalities seen in aggregating *countin*$^-$ cells (such as high cell-cell adhesion and low motility) are also observed in the *cf50*$^-$ cells. Western blot analysis of conditioned medium sieve column fractions showed that the CF50 protein is present in the same fraction as the 450 kDa CF complex. In the absence of CF50, secreted countin is degraded, suggesting that one function of CF50 may be to protect countin from degradation. However, unlike *countin*$^-$ cells, *cf50*$^-$ cells differentiate into an abnormally high percentage of cells expressing SP70 (a marker expressed in a subset of prespore cells), and this difference can be rescued by exposing cells to recombinant CF50. These observations indicate that unlike other known multisubunit factors, CF contains subunits with both overlapping and unique properties.**

Key words: Counting factor, Cell population size, *Dictyostelium discoideum*

INTRODUCTION

Little is known about the regulation of tissue size, despite it being a significant problem in both agriculture and medicine (Day and Lawrence, 2000; Gomer, 2001; Haldane, 1928; Potter and Xu, 2001). In the model system *Dictyostelium discoideum*, starving cells use relayed pulses of cAMP as a chemoattractant to aggregate in dendritic streams. On agar plates the streams of our laboratory strains break up into groups of a remarkably uniform size of 2×10^4 cells/group. Other strains similarly form even sized groups of up to 10^5 cells (Shaffer, 1957). Each group then develops into a fruiting body consisting of a 1-2 mm tall stalk supporting a mass of spore cells. The spores can then be dispersed by the wind to start new colonies of cells. The group formation appears to function to limit the size of fruiting bodies, since fruiting bodies with more than these numbers of cells tend to collapse or fall over (Brock and Gomer, 1999).

Using shotgun antisense transformation for mutagenesis, we found a *Dictyostelium* transformant we named *smlAas* that formed very small groups and fruiting bodies (Spann et al., 1996). The *smlAas* cells formed streams with normal morphology and size which then broke up into a large number of small groups. Disrupting the *smlA* gene using homologous recombination resulted in cells with a phenotype identical to that of *smlAas* (Spann et al., 1996). SmlA is a cytosolic protein with no strong similarity to any known protein. It appears to regulate some aspect of secretion, as the *smlA*$^-$ and *smlAas* cells secrete a soluble factor which when added to early developing wild-type cells causes them to form small groups (Brock et al., 1996).

One way in which cells can theoretically sense the size of a tissue or group of cells is if all the cells in the group secrete a diffusible factor. As the number of cells in the group increases, the concentration of the factor will increase, and thus the cells themselves or a different set of cells can sense the size of the group (Brock et al., 1996; Clarke and Gomer, 1995; Gomer, 2001). We hypothesized that the factor secreted by the *smlA*$^-$ cells might be an example of such a factor. Using conventional protein chromatography and the ability of this factor to cause wild-type cells to form small groups as a bioassay, we partially purified the factor and found that it appeared to be a ~450 kDa complex of polypeptides we named counting factor (CF). We found that CF was also secreted by wild-type cells, although to a lesser extent than by *smlA*$^-$ cells. This suggested that the

smlA^{-}cells oversecrete a factor that the wild-type cells use to sense the number of cells in a group, so that when there is a small number of *smlA*$^{-}$ cells in a group, the higher concentration of CF causes these cells to sense that there is a much larger number of cells (Brock and Gomer, 1999).

A SDS-polyacrylamide gel of CF showed that there are at least 5 different polypeptides in the complex (Brock and Gomer, 1999). We obtained N-terminal amino acid sequence for 3 of the proteins; another 2 were blocked. We isolated the cDNA encoding a 40 kDa component, which we named countin. The derived amino acid sequence of the countin protein has some similarity to amoebapores, a superfamily of proteins that bind to membranes (Zhai and Saier, 2000). A prediction of the 'secreted factor being used to count cells' model is that if the cells secrete little or no factor, huge groups will form. To test the hypothesis that countin is part of such a factor, we disrupted the *countin* gene using homologous recombination. The *countin*$^{-}$ cells did not secrete any measurable CF activity according to the bioassay, and the streams of *countin*$^{-}$ cells did not break into groups, resulting in the streams coalescing into huge mounds of cells which then formed huge fruiting bodies (Brock and Gomer, 1999). Addition of anti-countin antibodies to developing wild-type cells caused them to form large groups, again indicating that countin is used by wild-type cells for group size regulation. A secreted protein with ~40% identity to countin, countin2, also regulates group size (Okuwa et al., 2001).

To try to understand what could cause a stream of cells to break up into groups, we wrote a simple computer simulation of cells moving in a stream while secreting and sensing a diffusible factor (Roisin-Bouffay et al., 2000). This simulation indicated that if a secreted factor negatively regulates cell-cell adhesion, and/or increases random motility, the presence of too many cells in a stream results in a high level of the factor and thereby cause the stream to dissipate and subsequently break up. The subsequent lower concentrations of the factor amongst the dispersed cells will cause them to recoalesce, and the simulation indicated that they would coalesce into separate groups rather than in a continuous stream (Roisin-Bouffay et al., 2000). During early *Dictyostelium* development, there are two main cell-cell adhesion molecules: gp24 and gp80 (Loomis, 1988; Siu et al., 1987; Siu and Kamboj, 1990). Overexpression of gp80 caused the formation of unbroken streams and large aggregates, while blocking gp80 binding activity with monoclonal antibodies resulted in broken streams and many small aggregates (Kamboj et al., 1990; Siu and Kamboj, 1990). Because altering cell-cell adhesion can alter group size in *Dictyostelium*, we examined whether *countin*$^{-}$or *smlA*$^{-}$ cells have altered cell-cell adhesion. We found that *countin*$^{-}$cells have abnormally high cell-cell adhesion, while *smlA*$^{-}$cells have low adhesion (Roisin-Bouffay et al., 2000). Addition of highly purified preparations of CF to wild-type cells decreased cell-cell adhesion within 30 minutes. Decreasing adhesion with exogenous anti-gp24 antibodies also decreased group size. The simulations indicated that if a secreted factor regulates both adhesion and motility, the group size becomes more uniform (Tang et al., 2002). We found that CF increases cell motility by increasing actin polymerization and the levels of the actin-crosslinking protein ABP-120, and by decreasing levels of myosin polymerization (Tang et al., 2002). Motility and the expression of the adhesion molecules is regulated by the relayed pulses of cAMP, and we found that CF indeed regulates cAMP signal transduction (Tang et al., 2001). These results suggested that CF regulates cell-cell adhesion and motility, and this in turn can regulate the size of a group of cells.

All of the above work was done with either *countin*$^{-}$cells or exogenous anti-countin antibodies to inhibit countin function. However, CF contains several other proteins in addition to countin. To help understand why cell-number counting in *Dictyostelium* is mediated by a large complex of polypeptides, we have characterized the function of CF50, another protein component of CF.

MATERIALS AND METHODS

cDNA isolation, sequencing and generation of a knockout construct

To isolate fragments of cDNA, PCR was done with primers that matched the genomic sequence from the *Dictyostelium* sequencing project, using vegetative and developmental cDNA libraries as templates. PCR was also used to obtain fragments of genomic DNA, using Ax4 genomic DNA as a template. The DNA was ligated into pCR 2.1 (Invitrogen, San Diego, CA) and sequenced at Lonestar Labs (Houston, TX). Possible O-glycosylation sites were searched for using the DictyOGlyc 1.1 server http://www.cbs.dtu.dk/services/DictyOGlyc/ (Gupta et al., 1999). To generate a gene disruption construct, PCR was done on Ax4 genomic DNA with the primers CGATAATCATCCGCGGGAGGGATGTATCCTTAGC and GGTAAAGTCACCAGGGACTTGTCTAGAGCATGC, yielding a 437 bp fragment of the 5′ side of a gene designated *ctnB* that will be referred to as *cf50*. This was digested with Sac II and Xba I, and ligated into the same sites in pBluescript SK+ (Stratagene, La Jolla, CA) to generate p50L. PCR was then done with GCCAATGTAAGCTTGGCAGCAGAACAAGCTGG and CATGTGATATACACGAATTACGGGCCCAATGCG on Ax4 genomic DNA to generate a 771 bp fragment of the 3′ side of *cf50*. This was digested with *Hin*dIII and *Apa*I and ligated into the same sites in p50L to generate p50LR. The 1.4 kb *Xba*I-*Hin*dIII blasticidin resistance cassette from pUCBsrΔBam (Adachi et al., 1994; Sutoh, 1993) was then ligated into the same sites in p50LR to generate p50KO. This was digested with *Sac*II and *Apa*I and the insert was purified by gel electrophoresis and a Geneclean II kit (Qbiogene, Inc., Carlsbad, CA). This was used to transform *Dictyostelium* Ax2 cells following the method of Shaulsky et al. (Shaulsky et al., 1996). PCR was used to identify *cf50*$^{-}$clones with a disruption of *cf50*. The specific clone used in this study is HDB17-4 and is referred to as *cf50*$^{-}$.

Cell culture and group number assays

Cell culture was done according to Brock and Gomer (Brock and Gomer, 1999). The disruption of strain *ctnA* (also known as *countin*) used in this study was HDB2B/4 and will be referred to as *countin*$^{-}$. The *smlA* disruption strain used was HDB7YA and will be referred to as *smlA*$^{-}$. Slices of agar with adhering fruiting bodies were turned sideways and photographed on Kodak Ektachrome 160T with a light blue filter using a Nikon Microphot with a 4× lens stopped down with a 1 mm pinhole in front of the lens. Synergy assays were done according to Brock et al. (Brock et al., 1996). Production of conditioned starvation medium (CM), exposure of cells to conditioned medium, and exposure of cells to 1:500 anti-countin antibodies or 1:500 anti-CF50 antibodies was done according to Brock and Gomer (Brock and Gomer, 1999). Group size assays were done following the method of Brock et al. (Brock et al., 1996). To make conditioned growth medium, cells were inoculated into growth medium at 1×10^{6} cells/ml. After 20 hours, the medium was clarified by centrifugation

at 2100 ***g*** for 3 minutes, and the supernatant was further clarified by centrifugation at 12,000 ***g*** for 10 minutes. The supernatant was mixed with SDS sample buffer and heated to 100°C for 3 minutes for western blots stained with anti-CF50. For western blots stained with anti-countin antibodies, the supernatant was concentrated by a factor of 10 using an Ultrafree Biomax10 kDa cutoff spin filter (Millipore, Bedford, MA) and then treated as above. The procedure of Wood et al. (Wood et al., 1996) was used to determine the percentages of CP2-positive and SP70-positive cells at low cell density. For each cell type, cells were grown in shaking culture, and (where indicated) recombinant CF50 was added to the cells to 0.2 μg/ml at the time indicated. The cells were then collected by centrifugation, washed in starvation buffer and starved in duplicate wells of an 8 well slide at low cell density in monolayer culture in the presence of a 1:10 dilution

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   1  TTA TTT TTA TAC ATA TTT TAA AAA ATG AAT AAA ATG AAT AAT ATT TTT TTA ATA ATA TCA   60
                                      M   N   K   M   N   N   I   F   L   I   I   S

                                                          ↓
  61  AGT ATT ATT TTA TCT ATT GTT ATT TTC GTC AGT GGT GAA TGT GCC ATT GAT TTC TCA TCA   120
       S   I   I   L   S   I   V   I   F   V   S   G   E   C   A   I   D   F   S   S

 121  GAG ATT TCA GTT GGA ATT TCA GAT TCT CAA TGG TCA TGT TTA GCT AGT AAT AAT CAA AGA   180
       E   I   S   V   G   I   S   D   S   Q   W   S   C   L   A   S   N   N   Q   R

 181  GTT ATA ATT CAA GTG TGG TCA GGA GGT GGT CAA TAT AAT TCA AAT ATT TCA TCT GTT GTA   240
       V   I   I   Q   V   W   S   G   G   G   Q   Y   N   S   N   I   S   S   V   V

                      ←
 241  TCG GCA GCA GAA CAA GCT GGT TTC GAT AAT ATT GAT CTT TAT GCA TTT TTA TGT AGT GAA   300
       S   A   A   E   Q   A   G   F   D   N   I   D   L   Y   A   F   L   C   S   E

 301  TGT GAT GGT AAT TAT CCA GCA TCA AGT GCA ATT CAA AGT TTA GTT TCT AGT TTA AAA TCC   360
       C   D   G   N   Y   P   A   S   S   A   I   Q   S   L   V   S   S   L   K   S

 361  GAT GGT ATC AAT TTC AAT ATG TTA TGG ATT GAT GTT GAA CAA TGT GAT GGT TGT TGG GGA   420
       D   G   I   N   F   N   M   L   W   I   D   V   E   Q   C   D   G   C   W   G

 421  GCA GAG AGT GAT AAT GCC GAT TAT GTT CAA GAA GCT GTT GAA ACT GCA CAA GGT CTT GGT   480
       A   E   S   D   N   A   D   Y   V   Q   E   A   V   E   T   A   Q   G   L   G

 481  GTT CTA GTA GGT GTA TAC AGT AGT GAA GGA GAA TGG CCA CAA ACT GTT GGT AAT TTA TCA   540
       V   L   V   G   V   Y   S   S   E   G   E   W   P   Q   T   V   G   N   L   S

 541  ACT CTA AGT CAA TAT CCA CTT TGG TAT GCT CAT TAT GAT GAT AAT CCA TCT TTT TCC GAT   600
       T   L   S   Q   Y   P   L   W   Y   A   H   Y   D   D   N   P   S   F   S   D

 601  ACC GCT TTT TAT GAA TTT GGT GGT TGG ACT TCA CCA GCA ATG AAA CAA TAT ATT GGC AAT   660
       T   A   F   Y   E   F   G   G   W   T   S   P   A   M   K   Q   Y   I   G   N

                                                          ↓
 661  ACA AAT CAA TGT GGT GTT AGT GTT GAT TTA GAT TTT TAT GGA TCT GGT AGT GGT TGT TCA   720
       T   N   Q   C   G   V   S   V   D   L   D   F   Y   G   S   G   S   G   C   S

 721  ACA TCC TCT GGT AGT GCA TCT GGT AGT GCT TCT GGT AGT GCA TCT GGT AGT GCA TCT GGT   780
       T   S   S   G   S   A   S   G   S   A   S   G   S   A   S   G   S   A   S   G

 781  AGT AAT AGT GGA AGC AGT AAT AGT GGA AGT AGT AAT AGT GGA AGT AGT AAT AGT GGA AGT   840
       S   N   S   G   S   S   N   S   G   S   S   N   S   G   S   S   N   S   G   S

 841  AAT AGT GGA AGT AGT AAT AGT GGA AGT GGT AAT AGT GGA AGT AGT AAT AGT GGA AGT GCA   900
       N   S   G   S   S   N   S   G   S   G   N   S   G   S   S   N   S   G    S   A

 901  AGT GGA AGT GGT ACA GGA AGT GGA TCA TCC ATT TAA AAA AAT AAA TAA TTT TAT TTC TTT   960
       S   G   S   G   T   G   S   G   S   S   I   *
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Fig. 1. Sequence of the *cf50* gene. The sequence starts with genomic DNA; the 5′ end of known cDNA sequence starts at nt 28 and continues to 83 nt past the stop codon, and is an exact match to the genomic sequence. The first ATG is at nucleotide 25. The N-terminal amino acid sequence obtained from the purified 50 kDa protein is indicated with a heavy underline. Two potential N-linked glycosylation sites (Alexander, 1997) are indicated by shaded boxes. Vertical arrows mark the beginning and end of the sequence expressed in bacteria and then used to immunize rabbits for antibody production. An arrow pointing to the left over nucleotide 260 marks the 3′ end of the region that was replaced with a blasticidin resistance cassette to make a gene disruption transformant; the 5′ end of the region that was replaced was 681 bp upstream of the first ATG. A double underline indicates a potential AAUAAA poly(A) addition signal. The GenBank accession number is AF405695.

Table 1. CF50 shares sequence similarity with lysozyme, but it has very low lysozyme activity

Sample	Lysozyme activity in PBM, units/μg	Lysozyme activity in 0.1 M potassium phosphate, pH 6.4, units/μg
Hen egg white lysozyme	229±18	273±59
Recombinant CF50	0.003±0.003	0.009±0.006

Lysozyme activity was measured by the ability to cause a decrease in absorbance of a solution of lyophilized *Micrococcus lysodeikticus* following the method of Monchois et al. (Monchois et al., 2001). Values are the means ± s.e.m. from three separate experiments.

of Ax4 conditioned medium to supply CMF and thus allow CP2 and SP70 expression at low cell density. After 6 hours, cAMP was added to induce the expression of prestalk and prespore genes. 12 hours later, the cells were fixed and one well was stained for CP2 while the other was stained for SP70. The total number of cells per well and the number of positive cells were then counted. To examine differentiation in aggregates, cells were starved on filter pads (Brock et al., 1996) and were dissociated after 18 hours. Approximately 2500 cells in 200 μl of PBM were placed in the well of an 8 well slide. After allowing the cells to settle for 10 minutes, the cells were fixed and stained following Wood et al. (Wood et al., 1996).

Northern blots

RNA was isolated using a RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's directions with the exception that 2×10^7 cells were used. Northern blot analysis was as described previously (Brock et al., 1996).

Preparation of recombinant CF50 and lysozyme assay

To make recombinant CF50, a PCR reaction was done using a vegetative cDNA library and the primers GGCAGCCATATGGAATGTGCCATTGATTTCTC and GGATCCTCGAGTTAAATGGATGATCCACTTCC to generate a fragment of the *cf50* coding region corresponding to the entire polypeptide of the secreted protein, with a *Nde*I site on one end and a *Xho*I site on the other. To make a recombinant fragment of CF50 lacking the C-terminal serine-glycine rich region (which is very similar to a region in a 45 kDa component of CF; D. A. B., R. D. H. and R. H. G., unpublished), a PCR reaction was carried out using the first primer above and GCCGGATCCTCGAGTTAATAAAAATCTAAATCAACAC. After digestion with *Nde*I and *Xho*I, these were ligated into the corresponding sites of pET-15b (Novagen). The recombinant proteins were expressed in bacteria. The recombinant CF50 was found in inclusion bodies, so these were solubilized in 8 M urea and the recombinant CF50 was purified using a B-Per 6x His spin purification kit (Pierce, Rockford, IL) following the manufacturer's directions. To refold denatured recombinant CF50, a final concentration of 100 μg/ml protein was dialyzed in 20 mM Tris-HCl, pH 7.5, 0.1 mM DTT at 4°C for 24 hours with three changes of buffer. The protein was then dialyzed in the same buffer without DTT at 4°C for 48 hours with four changes of buffer, and stored as aliquots at –80°C. The purified protein was quantitated in two ways. First, dilution curves of known amounts of bovine serum albumin and various dilutions of recombinant CF50 were electrophoresed side-by-side on SDS-polyacrylamide gels and stained with Coomassie Blue, and the lanes were scanned. Second, a Bio-Rad protein assay (Bio-Rad, Hercules, CA) was performed. The two methods gave similar results. Both constructs generated proteins that had poly-histidine tags at the N terminus, a thrombin cleavage site, followed by the first amino acid of the secreted form of CF50 (Fig. 1, first vertical arrow). The first construct generated a protein that terminated after the last amino acid of the predicted sequence. The second construct generated a protein that terminated after the last amino acid before the serine/glycine-rich motif (Fig. 1, second vertical arrow). Proteins were assayed for lysozyme activity using the method of Monchois et al. (Monchois et al., 2001) using hen's egg white lysozyme (Sigma) as a standard.

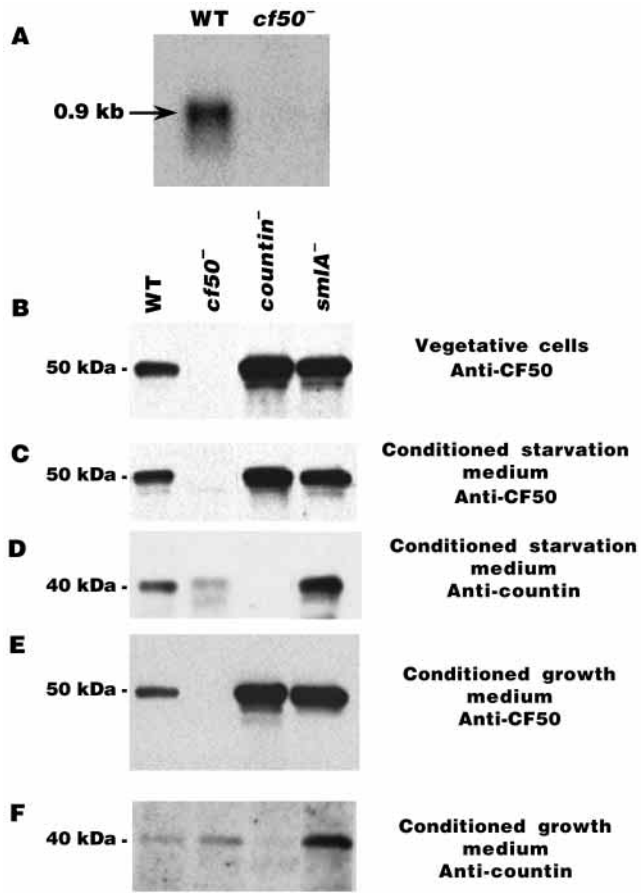

Fig. 2. *cf50*$^-$cells do not have *cf50* mRNA or CF50 protein. (A) A northern blot of RNA from vegetative cells was probed with a fragment of the cf50 cDNA. A 0.9 kB band (arrow) is present in Ax2 wild-type parental cells but not in the *cf50*$^-$cells. Aliquots of the samples were electrophoresed on separate gels and stained with ethidium bromide to verify equal loading and that the ribosomal bands were not degraded. (B) A western blot of vegetative cells was stained with anti-CF50 antibodies. A 50 kDa band is present in Ax2, *countin*$^-$and *smlA*$^-$cells, but is absent from *cf50*$^-$cells. (C) A western blot of conditioned starvation media from the above cell lines was stained with anti-CF50 antibodies. A 50 kDa band is present in Ax2, *countin*$^-$and *smlA*$^-$CMs, but is absent from *cf50*$^-$CM. (D) A similar blot stained with anti-countin antibodies, showing countin present in the wild-type and *smlA*$^-$CMs, absent from the *countin*$^-$CM and present at low levels with what appears to be a lower molecular mass band in the *cf50*$^-$CM. (E) A blot of conditioned HL5 growth media showing that CF50 is secreted from growing Ax2, *countin*$^-$and *smlA*$^-$cells, but not from *cf50*$^-$cells. (F) A similar blot of conditioned HL5 growth media stained with anti-countin antibodies. For B-F, SDS-polyacrylamide gels of aliquots of the samples used for the western blots were stained with Coomassie Blue to verify that equal amounts of protein were loaded for each blot.

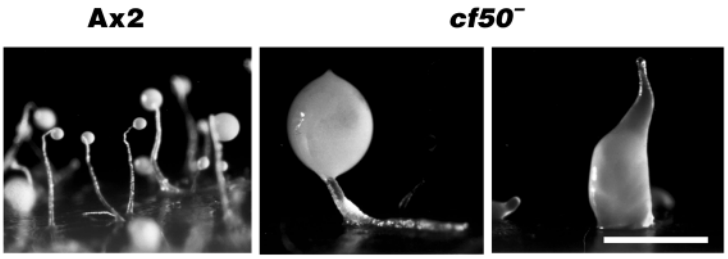

Fig. 3. Developing *cf50*$^{-}$cells form unusually large structures. Cells were grown on bacteria on agar plates, and as the cells consumed the bacteria they starved and formed fruiting bodies. Slices of agar with fruiting bodies or aggregates were tilted and photographed. The left panel shows the parental Ax2 cells; the center and right panels show examples of the structures formed by *cf50*$^{-}$cells. Bar is 1 mm.

Circular dichroism

Circular dichroism measurements were performed using a model 62ADS spectrometer (Aviv, Lakewood, NJ) with a 2 mm × 10 mm dual pathlength quartz cuvette (Starna Cells, Atascadero, CA) at 24°C. Data was collected from 260 to 190 nm at 1 nm intervals, 1 nm bandwidth, and a 1 second integration time. For these measurements, recombinant CF50 was diluted to a final concentration of 20 μg/ml in PBM.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed on a Beckman XL-A (Beckman, Palo Alto, CA) analytical ultracentrifuge with a four-position An60Ti rotor and double-sector centerpieces at 25°C. 1.0, 0.5, and 0.25 mg/ml of recombinant CF50 were examined in a six-channel centerpiece unit in which three channels on one side contained the different concentrations of protein and the three channels on the other side contained buffer. Samples were centrifuged at 10,000 rpm, 14,000 rpm, or 18,000 rpm. Analysis of data was accomplished using software provided by Beckman Instruments.

Antibody production, western blots and immunofluorescence

For antibody production, the recombinant truncated CF50 was additionally purified by SDS-polyacrylamide gel electrophoresis (Gomer, 1987). Immunization of a rabbit with the recombinant truncated CF50 and affinity purification of the antibodies was done by Bethyl Laboratories (Montgomery, TX). For western blots, 10^6 cells in 80 μl, or 80 μl of CM, was mixed with 20 μl of 5× Laemmli sample buffer and heated to 100°C for 3 minutes. 15 μl was electrophoresed on a 12% polyacrylamide Tris-HCl gel (Bio-Rad, Hercules, CA). Protein was transferred to Immobilon P and stained as described for the ubiquitin western blots by Lindsey et al. (Lindsey et al., 1998) with the exception that filters were not boiled, were blocked overnight at 4°C in PBS/5% BSA/1% Tween-20/1% Nonidet P-40/0.1% SDS, and the primary antibody was used at a 1:3000 dilution. Staining of western blots with anti-countin antibodies and size-exclusion gel chromatography was performed as described in Brock and Gomer (Brock and Gomer, 1999). For immunofluorescence, 200 μl of cells at a density of 5×10^5 cells/ml were placed in the well of a Lab-Tek eight-well slide (Nalge, Naperville, IL). After 30 minutes, the liquid was gently removed from the settled cells and replaced with 2% formaldehyde, 0.2% glutaraldehyde, 0.002% Triton X-100 in PBM. After 7 minutes, this was removed and the cells were washed with two changes of PBM and then incubated for 15 minutes in several changes of 4 mg/ml sodium borohydride. The cells were then stained with 10 μg/ml of affinity-purified anti-CF50 antibody (Gomer, 1987). Cells were examined with a Nikon Microphot Fx with a 1.4 NA 60× lens or a Deltavision (Applied Precision, Issaquah, WA) deconvolution microscope with a Zeiss 1.4 NA 100× lens. Sieving gel chromatography was done as described by Brock and Gomer (Brock and Gomer, 1999).

Cell-cell adhesion and motility

Adhesion was measured (Roisin-Bouffay et al., 2000) using cells developing on filter pads and the cells were then dissociated before doing the assay following (Tang et al., 2002). To measure motility, cells were starved in PBM as described above, diluted to 3×10^5 cells/ml in PBM and 400 μl was placed in the well of a Lab-Tek 155411 8 well chambered coverglass (Nalge, Naperville, IL). After 30 minutes, 250 μl of liquid above the cells was removed from the well. Cells were videotaped for 10 minutes, 5 to 6 hours after being placed in the well, using the method of Yuen et al. (Yuen et al., 1995) with the exception that a 20× objective was used.

RESULTS

CF50 shows some similarity to a lysozyme precursor

Dictyostelium cells use CF to sense the number of cells in an aggregation stream (Brock et al., 1996). We previously found that the factor is a complex of polypeptides, and that disruption of *countin*, the gene encoding the 40 kDa component of CF, essentially abolishes the activity of the factor. To elucidate why CF contains multiple polypeptides, we examined a second component of the complex. We previously determined the

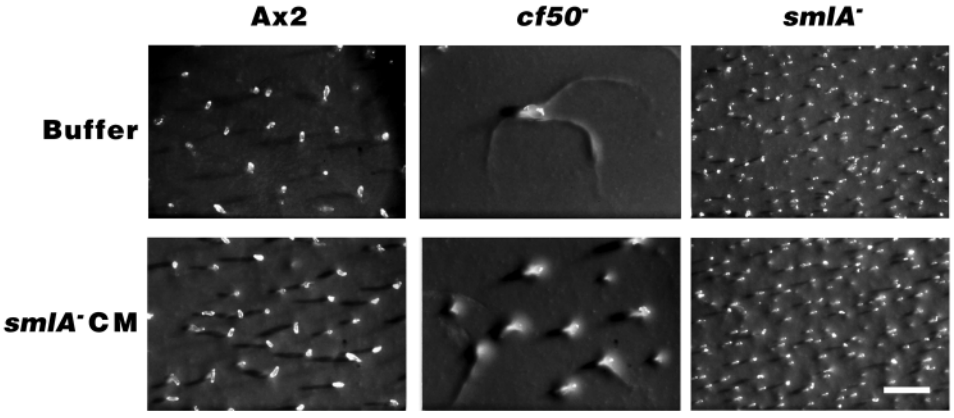

Fig. 4. Starvation of *cf50*$^{-}$cells in the presence of *smlA*$^{-}$conditioned starvation medium (CM) causes a decrease in group size. Cells were starved on filters sitting on pads soaked with either buffer or CM from *smlA*$^{-}$cells. Bar is 1 mm.

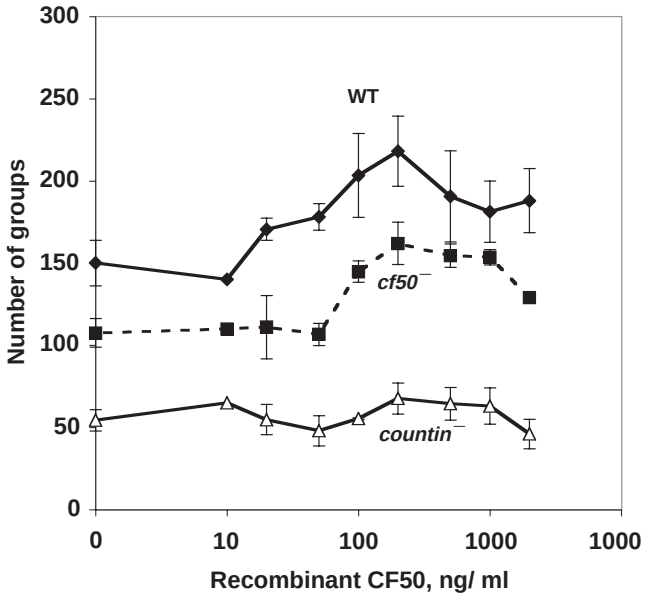

Fig. 5. Addition of recombinant CF50 causes an increase in group number. Cells were starved on filters sitting on pads soaked with the indicated concentration of recombinant CF50. The number of groups the streams broke up into was then counted 18 hours later. Values are means ± s.e.m. from at least 3 separate experiments.

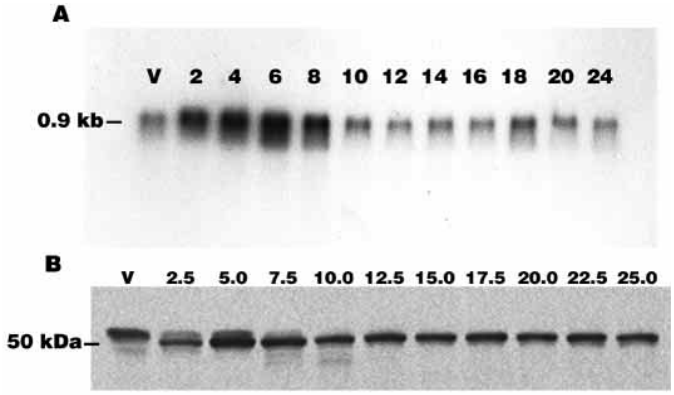

Fig. 6. *cf50* mRNA and CF50 protein are present in growing and developing cells. Ax2 wild-type cells were starved on filters, and samples were harvested at the indicated times (in hours) after starvation; V indicates vegetative cells. (A) A northern blot of mRNA extracted from cells; (B) a western blot of whole cells stained with anti-CF50 antibodies.

amino-terminal sequence of CF50, the 50 kDa component of the semi-purified factor (Brock and Gomer, 1999). A search of the *Dictyostelium* genomic sequence database revealed an open reading frame encoding a methionine followed by a predicted secretion signal, then an exact match to the sequence of the N terminus of purified CF50 (underline, Fig. 1), followed by an open reading frame, with an AATAAA polyadenylation signal 4 nucleotides after the stop codon (double underline, Fig. 1). Additional AATAAA sequences were observed beginning at 27, 33 and 37 nucleotides (nt) past the stop codon. Using PCR primers matching portions of the genomic sequence, *cf50* cDNAs were isolated from a vegetative and a developmental cDNA library. The sequence of both cDNAs matched the sequence of the genomic DNA between nucleotides 87 and 743. A search of *Dictyostelium* cDNA databases revealed sequences with exact matches to nucleotides 28 to 268, and 390 to 83 nt past the stop codon shown in Fig. 1. These cDNAs showed a poly(A) sequence beginning at 84 nt past the stop codon.

Table 2. The effect of anti-countin and anti-CF 50 antibodies on cells

	Percentage reduction in group number caused by treatment with	
Cell	anti-countin antibodies	anti-CF50 antibodies
Ax2	34±4	39±10
countin$^-$	ND	10±4
cf50$^-$	35±2	2±6

Cells were starved in the presence of preimmune or immune antibodies and the number of groups formed was counted. For each antibody, the number of groups formed in the presence of immune antibodies was subtracted from the number formed in the presence of preimmune antibodies, and this number was then divided by the number formed in the presence of preimmune antibodies to obtain a percentage reduction in group number caused by the immune antibodies. Values are means±s.e.m. from five separate experiments. There was no significant difference between the number of groups formed in the presence of preimmune antibodies and buffer controls. Previous experiments indicated that anti-countin antibodies had no significant effect on the number of groups formed by *countin*$^-$cells (Brock and Gomer, 1999).

The first 24 amino acids of the predicted CF50 protein appear to be a signal sequence (typically a lysine followed by a hydrophobic region), and are missing from the purified native protein (Fig. 1). This suggests that CF50 is a secreted protein. The predicted molecular mass of the 279 amino acid protein starting from the observed N terminus is 28.2 kDa. There are two potential N-linked glycosylation sites (shaded boxes, Fig. 1) and no significant O-linked glycosylation sites. We previously observed that other proteins secreted by *Dictyostelium* cells are extensively glycosylated, resulting in polypeptide backbones that are ~70% of the total mass of the protein (Brock and Gomer, 1999; Jain and Gomer, 1994). The first 200 amino acids of CF50 show a 34% identity and a 51% similarity to the *Entamoeba histolytica* lysozyme II precursor, and the last 70 amino acids contain 13 copies of (A/N)SGS motifs. There are no concentrations of charged amino acids, only 3 positively charged amino acids, and the predicted pI is 3.5. There are no large regions of hydrophobicity. Recombinant CF50 (which as described below has CF bioactivity) appeared to contain a mixture of α helix, β sheet and random coil as determined by circular dichroism (data not shown), and ultracentrifugation indicated a molecular mass of 35 kDa, suggesting that the recombinant protein behaves as a monomer. Lysozyme activity assays indicated that recombinant CF50 has more than 10^4-fold lower specific enzymatic activity than hen's egg-white lysozyme (Table 1), suggesting that despite the sequence similarity, CF50 is not a lysozyme.

Cells lacking CF50 form large fruiting bodies

To help understand the function of CF50, we examined the effect of disrupting its expression. A northern blot showed the presence of a 0.9 kb *cf50* mRNA in Ax2 vegetative cells (Fig. 2A). This RNA was absent in *cf50*$^-$cells, suggesting that these cells lack the *cf50* mRNA. Western blots of total cell protein from vegetative or 6-hour starved cells showed a 50 kDa band staining with anti-CF50 in wild-type, *smlA*$^-$and *countin*$^-$cells;

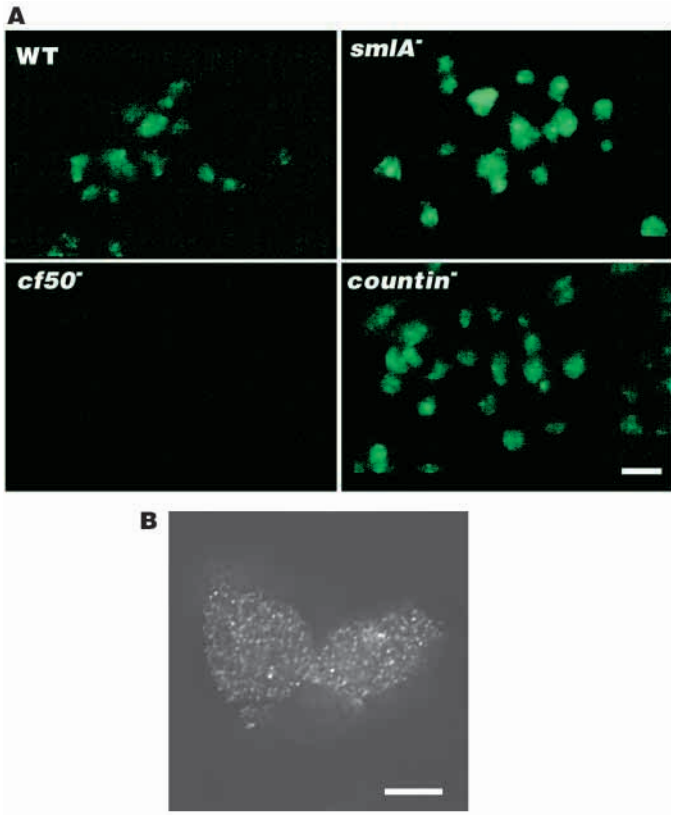

Fig. 7. Distribution of CF50 in cells. (A) Cells of the indicated type were starved on filters for 6 hours, harvested, allowed to settle on glass slides, fixed, and stained for CF50 by immunofluorescence and observed by conventional immunofluorescence microscopy. (B) Wild-type cells were further examined by deconvolution fluorescence microscopy. Bar in A is 20 μm and bar in B is 5 μm.

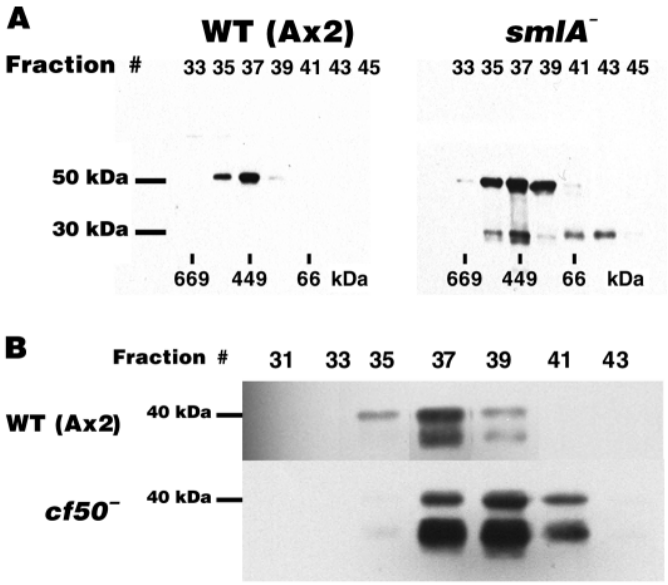

Fig. 8. CF50 is present in an ~450 kDa complex in CM. CM was prepared from Ax2 and *smlA*⁻ cells, and concentrated ~10-fold. (A) The material was size-fractionated on a sieving gel column, and the fractions were assayed for CF50 by western blotting. The 50 kDa CF50 band from both wild-type and *smlA*⁻CM elutes at approximately 450 kDa; a 30 kDa band eluting below 66 kDa is also present in the *smlA*⁻CM. The elution position of size markers is shown at the bottom. (B) CM from Ax2 and *cf50*⁻ cells was similarly size fractionated and the fractions were assayed for countin by western blotting. As previously observed, countin in Ax2 CM elutes at ~450 kDa (Brock and Gomer, 1999), while the countin in *cf50*⁻ CM elutes primarily at a lower molecular weight. The position of size markers is the same as in A.

this band was absent from *cf50*⁻cells (Fig. 2B and data not shown). Compared to parental cells, there appeared to be more CF50 protein in *countin*⁻and *smlA*⁻cells. Western blots of conditioned starvation medium indicated that CF50 is secreted by developing wild-type, *smlA*⁻and *countin*⁻cells, but not from *cf50*⁻cells (Fig. 2C). Compared to the amount of CF50 in wild-type CM, there appeared to be higher levels of CF50 in the CM from both *countin*⁻and *smlA*⁻cells. As previously described, there was a higher levels of countin protein in *smlA*⁻CM than in wild type CM, and no countin in *countin*⁻CM (Brock and Gomer, 1999). We observed variable but low levels of countin protein in *cf50*⁻CM, with bands at ~35 and ~30 kDa reacting with the anti-countin antibodies (Fig. 2D and data not shown). Sequencing of tryptic peptides from bands purified from crude preparations of CF indicate that there can exist degradation products of countin at these molecular masses. In support of the idea that countin is degraded in *cf50*⁻CM, we observed that *cf50*⁻cells occasionally revert to a phenotype very similar to wild type. Each time this happened, we observed that concomitantly the countin protein in the CM was no longer being degraded. Western blots of conditioned growth medium indicated that wild-type, *smlA*⁻and *countin*⁻cells but not *cf50*⁻ cells secrete CF50, with higher levels in growth media conditioned by *countin*⁻ and *smlA*⁻ cells (Fig. 2E). When similar samples were stained for countin, this protein was observed in growth medium from wild-type, *smlA*⁻, and *cf50*⁻ cells but not *countin*⁻cells (Fig. 2F). Compared to media from wild-type cells, growth media conditioned by *cf50*⁻ cells contained somewhat more countin, while media from *smlA*⁻ cells contained very high levels. The above results suggest that *cf50*⁻cells lack the *cf50* mRNA and the CF50 protein, that vegetative as well as developing wild-type and *smlA*⁻ cells secrete countin and CF50, and that countin is degraded in *cf50*⁻ CM.

The *cf50*⁻cells grew normally on plates with bacteria as well as in shaking culture. When the cells starved, they formed normal aggregation ‘spiders’ which either coalesced without breaking up, or broke up infrequently, both resulting in the formation of very large groups and fruiting bodies (Fig. 3). The large groups and fruiting bodies were observed when the *cf50*⁻cells were allowed to develop on filter pads, on water agar, on SM/5 plates with or without bacteria, and a variety of other conditions (data not shown). Although *cf50*⁻ and *countin*⁻cells share a similar phenotype of abnormally large aggregates and fruiting bodies, the developing structures of *cf50*⁻are much more aberrant. Along with irregularly-shaped, large aggregates, extremely long, very extended slug or finger-like structures can be seen which even appear to fruit horizontally (data not shown). Together, the data suggest that like *countin*⁻cells, *cf50*⁻cells form large groups and fruiting bodies, but that *cf50*⁻cells have additional defects such as extended structures.

Extracellular CF50 affects group size

To determine if the phenotype of the *cf50*$^{-}$ cells is due to a defect in a signal transduction pathway or is instead due to the lack of a secreted factor, we first performed synergy assays. As we previously observed (Brock et al., 1996), when wild-type cells were mixed with 10% *smlA*$^{-}$ cells, they formed 1.7±0.2-fold more groups than wild-type cells alone. When *cf50*$^{-}$ cells were mixed with 10% *smlA*$^{-}$ cells, they formed 2.9±1.1-fold more groups than *cf50*$^{-}$ cells alone. This suggested that the *cf50*$^{-}$ cells were able to change their group size in response to an extracellular signal provided by the *smlA*$^{-}$ cells. To determine if this signal is soluble, *cf50*$^{-}$ cells were starved in the presence of buffer or various conditioned media. As previously observed, *smlA*$^{-}$ conditioned medium (CM) caused Ax2 cells to form a larger number of smaller groups (Brock et al., 1996). *smlA*$^{-}$ CM also caused *cf50*$^{-}$ cells to form larger number of smaller groups, suggesting that the factor is indeed soluble (Fig. 4). We also measured the CF activity in conditioned starvation medium from *cf50*$^{-}$ cells. In a low cell density assay, wild-type cells formed 52±10 groups in the presence of PBM, 228±8 groups in the presence of *smlA*$^{-}$ CM, 73±4 groups in the presence of *cf50*$^{-}$ CM, and 75±1 groups in the presence of *countin*$^{-}$ CM. This suggests that *smlA*$^{-}$ CM causes wild-type cells to form a large number of groups compared to buffer alone, whereas *countin*$^{-}$ or *cf50*$^{-}$ CM cause a much smaller increase in group number. Together, the data suggest that *cf50*$^{-}$ cells can respond to a secreted factor, which regulates group size, and that decreased levels of a secreted factor causes the large group size in *cf50*$^{-}$ cells.

A simple explanation for the above results is that CF50 is a necessary or at least important component of CF. To test this hypothesis, recombinant CF50 was added to starving cells. A dilution curve of recombinant CF50 showed that for both parental Ax2 and *cf50*$^{-}$ cells, the optimal concentration of recombinant CF50 for increasing the number of groups was ~200 ng/ml. At this concentration the *cf50*$^{-}$ cells formed fruiting bodies that were indistinguishable in size and morphology from untreated wild-type cells (Fig. 5 and data not shown). Under these conditions 0.2 μg/ml recombinant CF50 caused a 46±9% (mean ± s.e.m., n=3) increase in group number for Ax2 cells, an 89±18% increase in group number for *cf50*$^{-}$ cells, and a 24±6% increase for *countin*$^{-}$ cells (Fig. 5). When recombinant CF50 was added to *cf50*$^{-}$ cells starving in shaking culture, the countin levels in the CM increased and there were less countin degradation products. Together, the data indicate that addition of recombinant CF50 rescues the defects observed in *cf50*$^{-}$ cells.

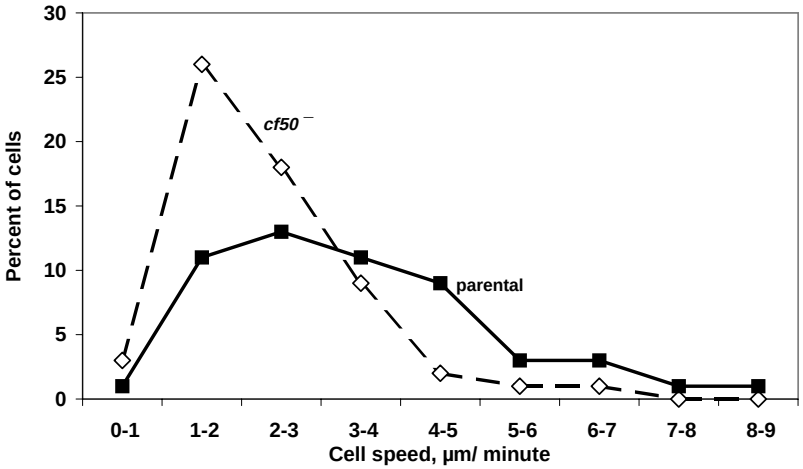

Fig. 9. Developing *cf50*$^{-}$ cells move more slowly than parental Ax2 cells. Cells were starved in submerged culture for 6 hours, and then fields of cells were observed by time-lapse videomicroscopy. For each cell, the approximate center of the cell was tracked for 5 minutes, and the cell speeds were then binned in 1 μm/minute increments. The average speeds were 3.3±0.2 μm/minute for Ax2 and 2.3±0.1 μm/minute for *cf50*$^{-}$ (means ± s.e.m. for 60 cells each, combining the observations from 20 cells in three separate experiments).

In the group number assay shown in Fig. 5, 30 μl of cells at 5×10^6 cells/ml were spotted onto a filter. For untreated wild-type cells there were ~150 groups (Fig. 5) and thus ~1000 cells/group. This number is much smaller than the number of cells per group when the cells are on agar, and we attribute this partially to CF and other factors being trapped by the filter. When 1 μl of either Ax2 or *cf50*$^{-}$ cells at 1×10^7 cells/ml were spotted onto filters, both cell types formed 1 group per spot, and thus roughly 10^4 cells/group. Since the small spots have a greater ratio of periphery to surface area, we hypothesize that under these conditions there is less trapping of secreted factors and group sizes approach those seen on agar.

cf50^{-} cells secrete bioactive countin activity

We previously observed that when wild-type cells were starved on filters soaked with anti-countin antibodies, the number of groups decreased and the group size increased (Brock and Gomer, 1999). To determine the effect of depleting countin and

Table 3. Differentiation of cells into CP2-positive prestalk and SP70-positive prespore cells at low cell density

		Percent positive cells		
Cell	Marker	No addition	rCF50 added 1 hour before starvation	rCF50 added 6 hours before starvation
Ax2	SP70	31.8±0.3	32.6±0.1	32.2±0.3
Ax2	CP2	11.0±0.1	11.3±0.1	11.5±0.0
countin^{-}	SP70	32.8±0.3	32.6±0.3	32.3±0.1
countin^{-}	CP2	11.6±0.1	11.7±0.2	11.5±0.1
cf50^{-}	SP70	48.4±1.5	40.7±2.0	33.7±0.7
cf50^{-}	CP2	0.8±0.2	4.9±0.4	9.6±0.2

The total number of cells in a well (typically 2000) was counted, and the number of positive cells. Values are the means±s.e.m. from three separate experiments.

Table 4. Differentiation of cells into CP2-positive prestalk and SP70-positive prespore cells at low cell density in the presence or absence of anti-CF50 antibodies

			Percentage positive cells			
		Addition to growth medium:	nothing	nothing	recombinant CF50	recombinant CF50
Cell	Marker	Addition to starvation buffer:	nothing	anti-CF50 antibodies	nothing	anti-CF50 antibodies
Ax2	SP70		31.8±0.3	40.6±4.1	33.3±3.2	33.2±3.1
Ax2	CP2		11.3±0.5	10.1±0.6	11.0±0.6	11.0±1.2
cf50$^-$	SP70		49.8±1.3	49.6±0.9	39.4±4.6	51.3±0.8
cf50$^-$	CP2		1.3±0.5	1.6±0.3	7.4±2.2	1.1±0.2

Cells were grown and starved as described in the text, with recombinant CF50 added for 6 hours when indicated. Where indicated, anti-CF50 antibodies were added to the starved cells. Values are the means±standard deviations from 9 independent experiments.

CF50 simultaneously, we starved Ax2 parental and *cf50*$^-$cells on filters for 2 hours and then transferred the filters with cells to pads soaked with buffer, preimmune or immune anti-countin antibodies. There was no significant difference in the number of aggregates formed on buffer as opposed to preimmune sera. Anti-countin antibodies decreased the number of groups formed by *cf50*$^-$cells, and anti-CF50 antibodies decreased the number of groups formed by wild-type cells but not *cf50*$^-$cells (Table 2). The anti-CF50 antibodies also caused a slight reduction in the number of groups formed by *countin*$^-$cells. The data suggest that there is some residual CF activity secreted by *cf50*$^-$cells, and that depleting extracellular countin can decrease this activity. The data also indicate that anti-CF50 antibodies are able to deplete CF activity from the extracellular medium, and that *countin*$^-$cells secrete a small amount of CF activity that can be neutralized with anti-CF50 antibodies.

Like countin, CF50 shows a punctate distribution in cells and is part of a 450 kDa complex when secreted

To elucidate the developmental regulation of *cf50* expression, a northern blot of RNA from different developmental stages was probed with a section of the *cf50* gene. As shown in Fig. 6A, *cf50* mRNA is present in vegetative cells and throughout development, with somewhat higher levels from 2 to 8 hours of development. A western blot of protein from cells at various stages of development showed that CF50 was present in cells at all stages of development, with a slight increase in amount between 5 and 7.5 hours of development (Fig. 6B). Interestingly, in vegetative cells and up to 7.5 hours, there was a 53 kDa band that stained with anti-CF50 (Fig. 6B). Together, the data suggest that CF50 is present throughout development and that like the secreted signal CMF (Jain et al., 1997; Yuen et al., 1991), its synthesis involves a slightly larger precursor form.

Immunofluorescence with anti-CF50 antibodies showed a punctate distribution in wild-type, *countin*$^-$and *smlA*$^-$cells during both growth and development (Fig. 7 and data not shown). The punctate staining was absent during growth and development in *cf50*$^-$cells (Fig. 7 and data not shown). There was somewhat brighter staining with anti-CF50 antibodies of the *smlA*$^-$and *countin*$^-$cells compared to wild type, similar to the higher levels of CF50 in these transformants observed on western blots (Fig. 2). The punctate distribution of CF50 is similar to that observed previously when cells were stained with anti-countin antibodies (Brock and Gomer, 1999), and suggests that before it is secreted, CF50 is located in vesicles.

We previously identified CF50 as a protein present in the CF preparation. To determine whether all or just some of the secreted CF50 is in the 450 kDa CF complex, CM was size-fractionated by gel chromatography, and the fractions were stained for CF50 by western blotting. As shown in Fig. 8, CF50 protein from Ax4 CM appears in a single peak around 450 kDa, in the same fractions where the countin peak appears (Brock and Gomer, 1999). Similar chromatography of CM from *smlA*$^-$cells also shows a peak at ~450 kDa, and in addition a band at 30 kDa that is present in both the 450 kDa fraction as well as a fraction representing material with molecular mass less than 66 kDa. Very long exposures did not reveal any of the 30 kDa protein in the sieve column fractions of Ax4 CM, suggesting that the 30 kDa protein that binds the anti-CF50 antibodies is specific to the *smlA*$^-$CM. These results suggest that in *smlA*$^-$CM some of the CF50 is broken down.

Although both countin and CF50 are found in the fraction with CF activity after purification through several columns and a non-denaturing gel, there is a possibility that the two proteins are in different 450 kDa complexes. To test the hypothesis that the two proteins do not co-associate, we determined whether deletion of CF50 would affect the apparent molecular weight of the complex containing countin, because if the two proteins are in different complexes one would expect that deletion of CF50 would not affect the size of the complex containing countin. As shown in Fig. 8B, the countin in wild-type CM elutes at approximately 450 kDa, whereas the countin in *cf50*$^-$CM elutes at a lower molecular weight. As mentioned before, there appears to be increased degradation of countin in the *cf50*$^-$CM. The observation that CF50 causes the apparent molecular weight of the complex containing countin to decrease disproves the hypothesis that CF50 and countin are in different complexes, thus suggesting that countin and CF50 are part of the same complex.

Like *countin*$^-$cells, *cf50*$^-$cells have high cell-cell adhesion and low motility

Computer simulations predicted that a higher cell-cell adhesion and/or a lower random cell motility would keep a stream intact, and thus by preventing stream breakup lead to larger groups (Roisin-Bouffay et al., 2000). We previously observed that *countin*$^-$cells have a higher cell-cell adhesion than their parental cells. Compared to wild-type parental, *cf50*$^-$cells had 5.9±1.5% higher adhesion at 2 hours of development, 3.0±1.3% higher at 4 hours, and 2.2±0.9% higher at 6 hours (means ± s.e.m. from 5 separate experiments). These experiments were done on cells that were forming streams on filter pads. The streams were morphologically similar up to about 8 hours of development, and had not begun to coalesce.

Table 5. Differentiation of cells into CP2-positive prestalk and SP70-positive prespore cells in aggregates

Cell	Marker	Percentage positive cells	
		No addition	rCF50 added
Ax2	SP70	31.9±0.2	35.5±0.9
Ax2	CP2	11.3±1.7	10.7±1.9
cf50$^-$	SP70	49.8±0.4	46.7±1.4
cf50$^-$	CP2	1.3±0.3	2.1±0.4

Cells were grown as described in the text, with recombinant CF50 added for 6 hours before starvation, when indicated. The cells were then starved and allowed to develop on filter pads. At 18 hours the aggregates were dissociated and cells were placed on slides, fixed, and stained for CP2 or SP70. Values are the means±standard deviations from 9 independent experiments.

Thus whereas during later development the *cf50*$^-$ cells form huge groups and cells in the interior might be starved for oxygen, in these assays the cells were in structures of a similar size. In addition to having abnormally high cell-cell adhesion, *countin*$^-$ cells have an abnormally low cell motility during the time wild-type streams are breaking up (Tang et al., 2002). *cf50*$^-$ cells also have significantly decreased cell motility at 6 hours after starvation (Fig. 9). These assays were done with cells developing in submerged monolayer culture, so, as above, these differences are not due to factors such as a lack of oxygen. Together, the data indicate that disruption of *cf50* has qualitatively the same effect on cell-cell adhesion and cell motility as disruption of *countin*.

Extracellular CF50 affects initial cell-type choice

We previously observed that *countin*$^-$ cells show a normal initial differentiation into CP2-positive prestalk and SP70-positive prespore cells (Brock and Gomer, 1999). CP2 is a protein which is a marker for an initial population of prestalk cells that gives rise to the first set of cells expressing other markers such as *ecmA* (Clay et al., 1995; Gomer et al., 1986), while SP70 is expressed in a subset of prespore cells and later appears on spore coats (Gomer et al., 1986). A similar assay showed that an abnormally low percentage of *cf50*$^-$ cells initially differentiate into CP2-positive cells while, compared to parental, a higher percent differentiate into SP70-positive cells (Table 3 and data not shown). These assays were done with cells developing at very low cell density in submerged monolayer culture, so these differences are not due to differences in group size. The initial decision to differentiate into a CP2-positive, SP70-positive or null cell is made at the time cells starve (Gomer and Firtel, 1987). Because we observed that wild-type cells secrete CF50 into the growth medium, we determined if the abnormal initial cell-type differentiation of *cf50*$^-$ cells is due to a lack of extracellular CF50 just before the cells starve. Cells growing in shaking culture were treated with recombinant CF50 for various times, starved, and allowed to differentiate at low cell density. Recombinant CF50 had little effect on Ax2 or *countin*$^-$ cells when added for 1, 3, or 6 hours before starvation (Table 3 and data not shown). However, a 1-hour exposure of vegetative *cf50*$^-$ cells to recombinant CF50 caused a partial rescue of the abnormal differentiation, while a 6-hour exposure caused the percentages of *cf50*$^-$ cells differentiating into CP2-positive and SP70-positive cells to be similar to the percentages seen with wild-type cells. Adding recombinant CF50 to growing cells for 8 hours, and then washing and starving the cells caused a 2.1±0.7 (mean ± s.e.m., n=4) increase in the number of groups formed by *cf50*$^-$ cell, but no significant change in the number of groups formed by Ax2 cells. To determine if the rescue of the abnormal differentiation of *cf50*$^-$ cells by exogenous CF50 was due to exogenous CF50 affecting something during vegetative growth or whether it was being sequestered by the cells and then affecting something during later development, we examined the effect of starving the CF50-treated cells in the presence of anti-CF50 antibodies. As shown in Table 4, adding anti-CF50 antibodies reversed the effect of the recombinant CF50 on the differentiation of cf50$^-$ cells. These data suggest that during vegetative growth *cf50*$^-$ cells can apparently sequester exogenous CF50 and then when starved release the CF50, which then causes them to alter the percentages of cell types that they would differentiate into if starved.

To determine if *cf50*$^-$ cells developing in aggregates also have an abnormal initial cell-type differentiation, we starved cells on filter pads and then dissociated them at 18 hours, when there is maximal accumulation of CP2 and SP70. As shown in Table 5, *cf50*$^-$ cells developing under conditions where they form aggregates also have an abnormal cell-type differentiation. This abnormal differentiation was observed for cells developing in spots of either 10^4 cells in a 1 μl spot or 5×10^5 cells in a 50 μl spot. When the cells were grown in the presence of recombinant CF50 and then starved, there was a slight increase in the percentage of CP2-positive cells, albeit much lower than the increase seen when these cells were starved at low cell density. The data thus suggest that *cf50*$^-$ cells developing in aggregates have an abnormal cell-type differentiation.

DISCUSSION

One of the ways cells could theoretically sense the number of cells in a group or tissue is by secreting and simultaneously sensing a characteristic diffusible factor (Gomer, 2001). We identified such a factor in *Dictyostelium*, and found that it is a relatively large complex of polypepetides (Brock and Gomer, 1999). We have found here that disrupting the expression of *cf50* has essentially the same effect as disruption of *countin* with respect to group size, adhesion and motility, but that unlike the effect of disrupting *countin*, disrupting *cf50* affects the initial cell-type choice.

Native CF50 is a 50 kDa molecule, whereas the polypeptide backbone is only 28 kDa. Since many secreted proteins in *Dictyostelium* are glycosylated, one possibility is that the 22 kDa of non-polypeptide mass is post-translational glycosylation. Our observation that bacterially synthesized CF50 polypeptide backbone has CF activity when added to wild-type or *cf50*$^-$ cells suggests that the functional domain of CF50 is in this backbone. *countin*$^-$ cells did not exhibit a strong response to the recombinant CF50, possibly because they already secrete large amounts of CF50 (Fig. 2C). For a different secreted polypeptide factor, CMF, we found that the presence of post-translational glycosylation appears to protect the protein from proteases (Jain and Gomer, 1994; Yuen et al., 1991). Thus a possible explanation for the existence of the CF50 glycosylation is that it protects CF50 from degradation. We previously observed that highly purified preparations of CF could cause a 3-fold increase in group number, and that a 1.5-fold increase could occur at concentration as low as 0.3 μg/ml

(Brock and Gomer, 1999). The recombinant CF50 occasionally will cause a 2-fold increase in group number, but a 1.5-fold increase tends to be the norm and the lowest concentration this occurs is at ~0.1 μg/ml; Fig. 5 and data not shown). Assuming that the CF50 is roughly one-fifth of the CF, the specific activity of CF50 is roughly that of CF, although recombinant CF50 does not seem to increase group number to the extent that CF can.

Both *countin*$^{-}$ and *cf50*$^{-}$ cells produce conditioned medium that causes a slight increase in group number compared to buffer alone, suggesting that there is some residual CF activity secreted in cells lacking just countin or just CF50. The antibody depletion experiments indicated that *cf50*$^{-}$ cells, which form large groups, will form even larger groups when countin is depleted from the extracellular environment with anti-countin antibodies, and *countin*$^{-}$ cells similarly form slightly larger groups when CF50 is depleted with antibodies (Table 2). Thus both the CF production assays and antibody depletion experiments suggest that neither CF50 nor countin is the sole effector molecule in the CF complex, but that both molecules can separately affect group size.

During early development, *countin*$^{-}$ cells have a considerably higher cell-cell adhesion than parental cells. We found that *cf50*$^{-}$ cells have only a slightly higher adhesion than parental cells. This higher adhesion is probably not sufficient to strongly affect group size. However, the *cf50*$^{-}$ cells have a significantly lower motility than parental cells, and most importantly far fewer highly motile cells than wild type (Fig. 9). Our computer simulations suggest that streams break when strongly motile cells break adhesive bonds and tear away from other cells (Roisin-Bouffay et al., 2000; Tang et al., 2002). The highly motile cells thus have the greatest influence on stream breaking, and we hypothesize that the greatly reduced numbers of these cells seen in *cf50*$^{-}$ is the reason *cf50*$^{-}$ cells form streams that rarely break and thus form large groups.

Vegetative *countin*$^{-}$ cells have high cell-cell adhesion and high levels of the adhesion molecule gp24 compared to wild-type cells, while vegetative *smlA*$^{-}$ cells have lower levels (Roisin-Bouffay et al., 2000) (Roisin-Bouffay and Gomer, unpublished). Vegetative cells contain smlA and countin proteins (Brock et al., 1996; Brock and Gomer, 1999), and we found here that vegetative cells also contain CF50. If we assume that vegetative cells can respond to CF, our observation that countin and CF50 are secreted by vegetative cells suggests that the differences in gp24 levels and cell-cell adhesion in vegetative *smlA*$^{-}$, wild-type and *countin*$^{-}$ cells could be due to low CF activity being secreted by vegetative *countin*$^{-}$ cells and high levels of CF being secreted by *smlA*$^{-}$ cells. This could then result in different levels of gp24 and different levels of cell-cell adhesion in vegetative wild-type cells compared to *smlA*$^{-}$ or *countin*$^{-}$ cells.

Wild-type cells all appear to have the same amount and distribution of CF50 (Fig. 7), so the altered cell-type differentiation of *cf50*$^{-}$ cells (Tables 3, 4, and 5) does not appear to be due to CF50 levels or distribution specifying a cell fate. This then suggests that CF50 somehow affects a process that is however heterogeneous in the cells. Although recombinant CF50 can rescue the abnormal initial cell-type differentiation when added to vegetative *cf50*$^{-}$ cells that are subsequently starved at low cell density, anti-CF50 antibodies added during starvation can block this rescue (Table 4). In addition, when the CF50-treated cells are starved at high cell density, there is very little change in the initial cell-type differentiation (Table 5). Together, the data suggest that the recombinant CF50 added to growing *cf50*$^{-}$ cells affects initial differentiation at a step after growth. One possibility is that the *cf50*$^{-}$ cells are able to sequester the added CF50 and then release it during development, and that this released CF50 can rescue the altered initial cell-type differentiation of *cf50*$^{-}$ cells when they are starved at low density but is degraded when the cells are starved at high cell density. Recombinant CF50 affects group size when added to vegetative cells that are then washed and starved at high cell density. Under these same conditions there is little effect on initial cell-type choice. A possible explanation for this is that during growth CF50 affects the expression of genes involved in adhesion and/or motility, and the altered levels of the associated proteins persist into development and affect stream breakup.

It is unclear why recombinant CF50 affects group size in Ax4 cells, which secrete CF50 as part of the CF complex. One possibility is that there are limiting amounts of CF50 in CM, and the recombinant CF50 allows CF complexes to form and or become active. However, western blots of gel filtration column fractions of whole conditioned medium from wild-type cells indicate that all of the detectable countin and CF50 are both present in a roughly 450 kDa complex, suggesting that there are no incomplete complexes. In addition, the removal of CF50 causes much of the countin present in CM to elute at a lower molecular weight (Fig. 8B). However, we cannot exclude the possibility that some fraction of countin was present in ~450 kDa complexes that lack CF50, and that exogenous CF50 increased the activity of this subset of complexes. Another possibility is that the exogenous CF50 (and thus the CF50 in the CF complex) binds to and activates a cell surface receptor.

There are several examples of signals that are present in complexes. One example is a ternary complex of Twisted gastrulation (TSG)/Short gastrulation (SOG or chordin)/Bone morphogenetic protein (BMP) (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). TSG and SOG act synergistically to repress signaling by BMP and TSG appears to enhance a proteolytic cleavage of SOG. Insulin-like growth factors, which can regulate the size of developing tissues, are found in ternary complexes with two specific binding proteins (Boisclair et al., 2001). Another example is TGFβ, which is stored in platelets and secreted as a complex with TGFβ masking protein, a 440 kDa complex of polypeptides. TGFβ forms a homodimer, and the masking protein contains 4 to 6 subunits (Nakamura et al., 1986; Okada et al., 1989). For all three of these examples, one protein in the complex can act as a signal, and other proteins regulate its activity. Another signal that is normally in a complex is thrombospondin, a trimeric ~450 kDa secreted extracellular matrix protein with binding sites for a variety of factors and receptors (Bornstein et al., 2000; Chen et al., 2000). Like CF, thrombospondin inhibits cell adhesion and increases cell motility, allowing cells to move and thus reshape structures (Mansfield et al., 1990; Murphy-Ullrich, 2001). CF appears to be somewhat different from these examples of multi-subunit factors insofar as having subunits that have similar as well as distinct effects on cells. This then suggests the possibility that for unknown reasons there may be different receptors for different components of the cell-number counting factor.

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