

A *Dictyostelium* mutant with defective aggregate size determination

Debra A. Brock¹, Greg Buczynski², Timothy P. Spann^{1,*}, Salli A. Wood¹, James Cardelli² and Richard H. Gomer^{1,†}

¹Howard Hughes Medical Institute, Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892, USA

²Department of Microbiology and Immunology, LSU Medical Center, Shreveport, LA 71130, USA

*Current address: Department of Cell and Molecular Biology, Northwestern University Medical School, 303 E. Chicago Ave. Chicago IL 60611, USA

†Author for correspondence (e-mail: richard@bioc.rice.edu)

SUMMARY

Starved *Dictyostelium* cells aggregate into groups of roughly 10^5 cells. We have identified a gene which, when repressed by antisense transformation or homologous recombination, causes starved cells to form large numbers of small aggregates. We call the gene *smlA* for small aggregates. A roughly 1.0 kb *smlA* mRNA is expressed in vegetative and early developing cells, and the mRNA level then decreases at about 10 hours of development. 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 indicates that SmlA is a $35 \times 10^3 M_r$ cytosolic protein present in all vegetative and developing cells and is absent from *smlA* cells. The absence of SmlA does not affect the growth rate, cell cycle, motility, differentiation, or developmental

speed of cells. Synergy experiments indicate that mixing 5% *smlA* cells with wild-type cells will cause the wild-type cells to form smaller fruiting bodies and aggregates. Although there is no detectable SmlA protein secreted from cells, starvation medium conditioned by *smlA* cells will cause wild-type cells to form large numbers of small aggregates. The component in the *smlA*-conditioned media that affects aggregate size is a molecule with a molecular mass greater than $100 \times 10^3 M_r$ that is not conditioned media factor, phosphodiesterase or the phosphodiesterase inhibitor. The data thus suggest that the cytosolic protein SmlA regulates the secretion or processing of a secreted factor that regulates aggregate size.

Key words: *Dictyostelium*, *smlA*, tissue size, cell counting, slug formation, aggregate

INTRODUCTION

The basic mechanisms that determine the size of a biological structure during development or regeneration are currently unknown. One of the best eukaryotic systems for studying developmental mechanisms such as size determination is the slime mold *Dictyostelium discoideum*. *Dictyostelium* is a unicellular amoeba 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 (Gomer et al., 1991; Jain and Gomer, 1994; Jain et al., 1992b; Yuen and Gomer, 1994; Yuen et al., 1995). As more and more cells starve, the local concentration of CMF increases and, when there is a high density of starving cells and thus a high concentration of CMF, the cells aggregate using relayed pulses of cAMP as a chemoattractant (reviewed in Robertson and Grutsch, 1981). Starving cells secrete phosphodiesterase, which causes the levels of cAMP to return to a baseline level in the interval between pulses (Dicou and Brachet, 1979; Faure et al., 1988, 1989; Franke and Kessin, 1981; Hall et al., 1993; Kessin et al., 1979; Orlow et al., 1981; Riedel and Gerisch, 1971; Tsang and Coukell, 1979; 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 (Devreotes, 1989; Firtel, 1995; Loomis, 1975, 1993; Schaap, 1991 for review).

Dictyostelium aggregates contain approximately 10^5 cells. Little is known about the molecular mechanism that determines this number. There exist mutants that give rise to large numbers of small fruiting bodies (Gerisch, 1968; Hohl and Raper, 1964; Sussman and Sussman, 1953). A high level of phosphodiesterase, which decreases the effective range of the cAMP chemotactic signal, can cause small aggregates. Riedel et al. (1973) isolated a mutant with an abnormally high level of extracellular phosphodiesterase, which forms increased numbers of small fruiting bodies, and a 30-fold overexpression of phosphodiesterase in *Dictyostelium* cells causes small aggregates to form (Faure et al., 1988). Other mutants with a small aggregate phenotype have little or no detectable phosphodiesterase inhibitor (Riedel et al., 1973). However, factors other than phosphodiesterase must be involved in aggregate size regulation, since some mutants with increased numbers of small fruiting bodies have normal phosphodiesterase and phosphodiesterase inhibitor levels (Riedel et al., 1973).

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 different mechanisms. The first mechanism is the ability to aggregate, and mutants with defects in this mechanism could be rescued by crowding the cells together so that aggregation became unnecessary. The second mechanism is a critical mass sensor, which in *Dictyostelium* and other systems (Spratt Jr. and Haas, 1961) regulates aggregate size and causes the aggregate to split in two if it exceeds a critical size. Hohl and Raper (1964) also found mutants of this type, since some mutant cells when starved at very high cell densities still formed small aggregates and fruiting bodies. A possible reason for the existence of a mechanism that is used to limit the size of aggregates and thus fruiting bodies might be that there are limits to the strength of the stalk and of the adhesion of the spores in the cell mass, so that if a large aggregate were to form and try to make a fruiting body, either the stalk would fall over or the spore mass would slide down the stalk.

To isolate genes involved in aspects of *Dictyostelium* morphogenesis such as size determination, we developed a technique in which *Dictyostelium* cells are transformed with an antisense vector containing a library of cDNAs (Spann et al., 1996). A transformant that develops at a normal speed but which forms very small fruiting bodies was designated *smlAas* for small aggregates. The antisense cDNA was isolated by PCR and 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, caused a repeat of the original *smlAas* phenotype. Cells transformed with a *smlA* gene disruption construct, designated *smlA* cells, had no detectable *smlA* mRNA on northern blots and had a small-fruiting-body phenotype (Spann et al., 1996). In this report, we show that the absence of the SmlA protein causes an increased amount of an extracellular factor which in turn causes a decrease in aggregate size.

MATERIALS AND METHODS

Isolation of DNA: northern and Southern blots

RACE-PCR was done following Jain et al. (1992a). DNA sequencing was done at the University of Texas Medical School at Houston core sequencing facility. Total genomic DNA was isolated from wild-type Ax-4 cells using a Blood and Cell Culture DNA Kit (Qiagen, Chatsworth, CA) and using 1 to 1.5×10^8 cells/ml. Southern blots were done using Bio-Rad Zeta-Probe GT membranes. The hybridizations were performed at 60°C in 0.125 M Na₂HPO₄, pH 7.2, 0.25 M NaCl, 5% SDS, 0.001 M EDTA, 10% PEG (average molecular mass 8×10^3 M_r) overnight. The washes were done with 0.05 M Na₂HPO₄, pH 7.2, 0.5% SDS one time at room temperature for 15 minutes, then 2 × 15 minutes each at 55–60°C. Autoradiography was done on Kodak X-OMAT AR5 film at –70°C overnight to one week. RNA isolation and northern blot analysis followed Jain et al. (1992a) with the exception that blotting, hybridization and washing were done as for the Southern blots above. The probe for both northern and Southern blots was the 1.2 kb cDNA.

Cell culture

Cell culture, development of cells and preparation of conditioned medium followed Jain et al. (1992b). In all assays, the *smlAas* and *smlA* antisense cells were compared to their parental line, Ax4,

and the *smlA* knockout cells were compared to their parental line, DH1; unless noted, Ax4 and DH1 cells behaved identically. The growth rate of cells was determined following Gomer and Ammann (1996). Cell motility assays were done as described in Yuen et al. (1995). For synergy experiments, mixtures of mutant and parental cells were starved on filter pads following Sussman (1987) with the exception that cells were starved in PBM (20 mM KH₂PO₄, 10 μM CaCl₂, 1 mM MgCl₂, pH 6.1 with KOH), the pads were soaked with PBM and 100 μl of cells at 7×10^7 or 1.5×10^7 total cells/ml were used. For starvation in the presence of exogenous conditioned medium (CM), a pad of two stacked 15 mm square pieces of Whatman 3 was soaked with PBM buffer or CM and a 10 mm square piece of AABP 04700 black filter (Millipore, Bedford, MA) was placed on the pad. Cells were washed and resuspended in PBM as for preparation of conditioned medium, and 30 μl of cells at 5×10^6 cells/ml was placed on the Millipore filter. For starvation in the presence of activated beef heart phosphodiesterase type P0134 (PDE; Sigma, St Louis MO), 20 serial factor-of-three dilutions of PDE were made in PBM starting with 10 mg/ml, and the dilutions of PDE were used to soak pads as described above. Phosphodiesterase was assayed by a radiometric technique following Kessin et al. (1979). Cells developing on pads were examined and photographed as described in Jain and Gomer (1994) with the exception that a piece of aluminum foil with a 1 mm diameter hole punched in it was placed over the 4× lens to increase the depth of field. For submerged monolayer culture assays, cells were harvested, washed and resuspended to 6×10^5 cells/ml in PBM as described above. 330 μl of the cell suspension was placed in the well of a Falcon 3047 24-well plate (Becton Dickinson, Lincoln Park NJ) and mixed with 150 μl of PBM or CM. Submerged cells were examined with a Nikon TMS inverted scope with a 4× lens. Fractionation of CM with Microcon (Amicon, Beverly, MA) and Ultrafree-MC (Millipore) spin filters followed Yuen et al. (1991).

Preparation of recombinant SmlA and anti-SmlA antibodies

To make a recombinant fragment of the *smlA* protein, mRNA was prepared from vegetative Ax-4 cells with a QuickPrep kit (Pharmacia, Piscataway, NJ). cDNA was made following Clay et al. (1995) using the primer V163 (CGGGATCCTCATGGTAAACCACCAGAAA-ACC). Following Clay et al. (1995), the cDNA was treated with RNase H and a PCR reaction was done on the cDNA using V163 and V165 (GGAATTCCATATGTCGTATCCAATTGTTGGAAGCTG) as primers. The 485 bp PCR fragment was then digested with *Bam*HI and *Nde*I and ligated into the *Bam*HI and *Nde*I sites of pET 15b (Novagen, Madison, WI). The construct was sequenced to verify that the fusion was in frame and the recombinant *smlA* protein fragment was purified following Clay et al. (1995). Actin and bovine serum albumin (BSA) were from Sigma, and protein concentrations were measured with the Biorad assay. SmlA protein concentration was estimated by comparison of the SmlA band with a dilution series of known quantities of actin and BSA on a Coomassie-stained SDS-polyacrylamide gel. A rabbit was immunized by injecting 300 μg of fusion protein in complete Freund's adjuvant in the popliteal lymph nodes and subcutaneously. Boosts were done subcutaneously and intramuscularly with 300 μg of fusion protein in incomplete Freund's adjuvant split among multiple sites. Six inoculations were done and the immune sera used for the experiments was collected 10 days after the sixth inoculation. Injections and serum collection were done by Cocalico Laboratories (Reamstown, PA). Antibodies were purified with an EZ Sep kit (Pharmacia).

Protein gels, western blots and immunofluorescence

Western blotting followed Jain and Gomer (1994) with the following exceptions: samples being stained for SmlA were electrophoresed on 20% SDS-polyacrylamide gels. Blots were blocked overnight at 4°C in 5% BSA and were then incubated with a 1:2000 dilution of anti

SmlA antibodies at room temperature for 1 hour. Bound antibody was detected with the ECL western blotting kit (Amersham, Arlington Heights, IL) following Pampori et al. (1995) using horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham). For cell fractionation, 10^9 Ax-4 cells were collected by centrifugation and resuspended to 3×10^8 cells/ml in ice-cold MESES buffer (20 mM MES, pH 6.5, 1 mM EDTA, and 0.25 M sucrose). Cells were broken with a tight fitting Dounce homogenizer and centrifuged at 3,000 *g* for 5 minutes in a SS34 rotor (DuPont-Sorvall, Newtown CT) to remove nuclei. Postnuclear supernatants were centrifuged again at 10,000 *g* for 10 minutes, and the supernatant from this centrifugation was respun at 200,000 *g* for 30 minutes in an 80Ti rotor (Beckman, Palo Alto CA). Immunofluorescence was done as described in Gomer (1987), and videotaping and motility assays were done as described in Yuen et al. (1995). Silver staining was done as described in Gomer et al. (1991).

Exocytosis assays

Fluid phase exocytosis measurements were performed as previously described (Temesvari et al., 1996). Cells loaded to steady state with FITC-dextran were recovered by centrifugation, washed and resuspended in phosphate buffer. At the indicated times, cells were harvested, washed and resuspended in 5 mM glycine, 100 mM sucrose, 0.5% Triton X-100, pH 8.5. FITC fluorescence was measured using a Hitachi 4010 fluorescence spectrophotometer (excitation wavelength 492 nm; emission wavelength 525 nm). The percent of FITC-dextran retained in the cells was calculated by dividing the intracellular fluorescence at the indicated times by the fluorescence at 0 minutes. To examine the secretion of α -mannosidase and acid phosphatase, cells growing in HL5 were harvested by centrifugation, washed and resuspended in PBM. At the indicated times, cells were separated from the conditioned medium by centrifugation and the activity of the two enzymes in the conditioned medium was measured following Cardelli et al. (1987). The secretion of acid proteinases active on gelatin was determined following North et al. (1990). Conditioned medium samples prepared as described above were electrophoresed on 11% polyacrylamide gelatin-SDS gels. Following electrophoresis, the gels were soaked in 2.5% Triton X-100 for 30 minutes, fixed overnight in 0.1 M acetic acid, pH 4.0, and stained with Coomassie brilliant blue.

RESULTS

Disruption of *smlA* causes small aggregates without affecting cell growth, the cell cycle or cell motility

We previously isolated a transformant designated *smlA*s from a shotgun antisense mutagenesis screen. Repression of *smlA* expression by antisense or gene disruption resulted in cells that develop at normal speed but which form large numbers of small fruiting bodies (Spann et al., 1996). To examine whether the small fruiting bodies might be a consequence of a general defect in metabolism, we assayed the growth of cells in liquid and on lawns of bacteria. We found that the growth rates for the antisense and the gene disruption transformants were indis-

1	TTA ATT TTA TTT AGT CAT TAA AAA ATT TGT AAT TTA GCC CAT AAA AAA AAA AAA AAA AAA	60
	↓	
61	AAA AAA AAA AAA AAA AAA AGT TTT TTG CAA CCT ATA AAA AAT TAT AAA TAG AAA TTG TTT	120
121	TGT TGT GTT TTT TTA TTT TAT TTT ATT TTA TTT TTA TTT TTT AAA GAA ATA ATT TTT TAT	180
181	AAA AAA AGA ATG GAA GAA ATA AAA GAA ACT GTT GTA AAA AAA AAT AAA GAA AAA AAG TAT	240
	M E E I K E T V V K K N K E K K Y	
241	TTA AAT GAT TAT AAA ATA ATT TCT GAA GGT AAT AAT ATT AAA GTT TTA CCA AAA AAA GAT	300
	L N D Y K I I S E G N N I K V L P K K D	
301	TTC AAA CCA AAA TTT TCA TTG GTA TCA TAT AAA AAT TAT GAA CCA AAT TTG GGA TTT GGG	360
	F K P K F S L V S Y K N Y E P N L G F G	
361	AAA ATG CTA AAA TTG TTT CAA CCA ATT TGG GCC ATC GAT CGT CAT ATA AAG ATG AAG AAT	420
	K M L K L F Q P I W A I D R H I K M K N	
421	CCA CCA GAT CCA TTT GTA TTT ATT AAA ATG CCA TCT TCA AAG CTT GTT CAA AGT CAG TTA	480
	P P D P F V F I K M P S S K L V Q S Q L	
481	AAA CTT TAC TAT GAA AAT GAA GGT TAT AAG GAT ATT CCA GTA TGG ATT ACA CCA TGT TCA	540
	K L Y Y E N E G Y K D I P V W I T P C S	
541	GCA TTT ATG AGA TAC ATT AAA TCG TAT CCA ATT GTT GGA ACT GTA ACA GAG ACA ATG GAA	600
	A F M R Y I K S Y P I V G T V T E T M E	
601	AAG AAA AAA GGT TTC ACC AAT AGA TTC TTA TCA ACT AGT GAA ACC AAA GCA TCA GTT TCA	660
	K K K G F T N R F L S T S E T K A S V S	
661	GTC GGA TTC TTT GGT TGT GAA TCA TCG ATG GAA GTT ACA AGT GGT TAT GAA TAT GAA GAC	720
	V G F F G C E S S M E V T S G Y E Y E D	
721	ACT GTT ACA TCT GAA GAA ACT AGA AGT TGG AGT CAA ACT TTA AAT GAA GGT TCT TAT ATT	780
	T V T S E E T R S W S Q T L N E G S Y I	
781	GTT TAT CAA AAC GTT TTA GTT TAT GCT TAT ATA ATC TAT GGT GGT AGT TAT AAA CCA TAC	840
	V Y Q N V L V Y A Y I I Y G G S Y K P Y	
841	ATT GAT CAA ATG AAT AAA TTT AAT CCT GGA TTA AAT ATC AAA TTC TTT AGA GAA GAT AAA	900
	I D Q M N K F N P G L N I K F F R E D K	
901	TGT ATC TTT TTT GTA CCA ATT AAT CGT GAT GAT GCC TTC ACT CTT CGT TAT CAA GAT AAT	960
	C I F F V P I N R D D A F T L R Y Q D N	
961	ACA TGG GAT CCA GTT GAA TAT GAT GTT TTA ATT GAT TAC TTG GGT CAA AAT CAA GAT AAA	1020
	T W D P V E Y D V L I D Y L G Q N Q D K	
1021	TGG TTT TCT GGT GGT TTA CCA TGA AAA TAA TAA TAA TAA TAA TAA TAA TTA TAT AAA	1080
	W F S G G L P *	
1081	TTA CAT AAA GGT TTC ACT ATT GGT GTT TTA ATC CTG GTA TAA ATA AAA TTC TAA AAA TAA	1140
1141	AAA ATA AAA AAC TAT ATA AAA NTT AAA AAA AAA AAA AAA AAA	1182

Fig. 1. Sequence of *smlA* DNA. The sequence starts with genomic sequence; the 5' end of the cDNA is indicated by an arrow at nucleotide 82. The sequence from nt 82 to nt 1100 is cDNA sequence (which matches genomic sequence until just before the poly(A) tail), and the remaining sequence is from the cDNA only. The amino acid sequence of the single large open reading frame is also shown. The DNA sequence is available as Genbank accession number U48706.

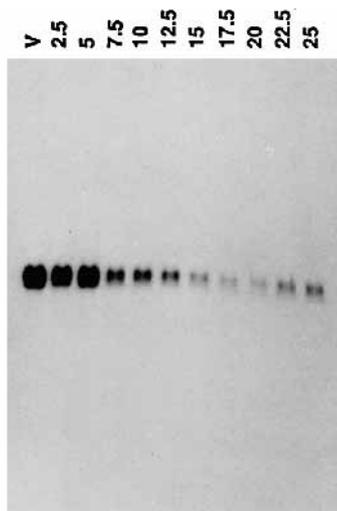


Fig. 2. Expression of *smlA* mRNA during development of wild-type cells. RNA was isolated from axenically growing vegetative Ax-4 cells (V) and from Ax-4 cells starved on filter pads for the indicated times in hours. A duplicate gel of the RNA was stained to verify that equal amounts of RNA were loaded.

tinguishable from that of the respective parental cell lines. The appearance of vegetative *smlA*asr and *smlA* cells by phase-contrast and Hoffman modulation contrast microscopy was indistinguishable from that of the respective parental cell lines. Starvation of *smlA*as, *smlA*asr and *smlA* cells at low cell density in CMF, with the addition of cAMP after 6 hours, caused normal percentages of CP2-positive prestalk and SP70-positive prespore cells to differentiate. These results suggest that disruption of *smlA* does not cause general defects in differentiation, CMF-sensing, or sensing of high continuous levels of cAMP. The percentage of cells in S, and the percentage of cells in M phase were also assayed for the *smlA*asr transformant (Wood et al., unpublished data) and were identical to the percentages for other antisense transformants. The flow cytometry profile of vegetative *smlA*asr and *smlA* cells fixed and stained for DNA content was examined and was also indistinguishable from that of Ax-4 cells (Wood et al., unpublished data). The motility of vegetative cells in submerged culture in HL-5 and the motility of starving cells in PBM and in conditioned medium on a plastic surface were measured for both *smlA*asr and *smlA* cells. Although DH1 cells have a much lower motility than Ax-4 cells, the distribution of cell speeds for the transformants was indistinguishable from that of the parental cell lines (data not shown). In submerged culture, the *smlA* cells form aggregation streams, which break up into small clumps of cells. Under the same conditions, wild-type cells form essentially identical streams, which coalesce into large aggregates instead of breaking up. The above results thus suggest that SmlA is specifically involved in a mechanism that regulates the size of the final aggregate.

SmlA is a novel protein

The cDNA isolated by PCR from the *SmlA* antisense transformant was a 275 bp fragment. To obtain additional sequence information, 3' and 5' RACE PCR were performed. The 3' RACE fragment contained 44 bp of perfect overlap with the original fragment and extended to the poly(A) tail. The 5'

RACE fragment extended the sequence an additional 215 bp. The sequence of an isolated 1.2 kb cDNA matched the RACE and original antisense cDNA sequence, and gave additional sequence past the 5' end of the 5' RACE sequence. When Southern blots of genomic DNA digested with restriction enzymes that do not digest the cDNA were probed with the *smlA* cDNA, a single band was detected, suggesting that there is a single *smlA* gene. A 5 kb *EcoRI-PstI* fragment of genomic DNA containing the *smlA* coding region was isolated and was partially sequenced. Fig. 1 shows the compiled sequence. An 851 nt reading frame is present in the cDNA. This open reading frame starts with the first ATG in the cDNA and continues to a stop codon which is then followed by a series of 8 additional in-frame TAA stop codons. An AAUAAA poly(A) addition signal is located 21 nt upstream of the poly(A) tail. The predicted open reading frame encodes a hydrophilic protein with a molecular mass of $33.2 \times 10^3 M_r$ and predicted pI of 8.7. Database comparisons of the *smlA* nucleotide and derived amino acid sequences indicate that *smlA* is the open reading frame that was found 750 bp downstream from the pseudogene pLK109-3 (Giorda et al., 1989), but otherwise has little similarity to any known gene. Motif searches and examination of the derived amino acid sequence indicate that SmlA has no obvious motifs or large regions of hydrophobicity or charge.

We then examined the expression pattern of *smlA*. As shown in Fig. 2, there is a relatively high level of *smlA* mRNA in vegetative and early developing cells. Between 5 and 7.5 hours after starvation, the level drops; however, *smlA* message is present through the rest of development including the fruiting body stage (25 hours after starvation).

SmlA protein is present throughout development in the cytosol of all cells

To determine the distribution of the SmlA protein, we made antibodies against a recombinant $23 \times 10^3 M_r$ fragment of SmlA. A western blot of vegetative wild-type and *smlA* cells stained with the preimmune serum showed no bands, while a similar blot stained with the immune serum showed a single band at roughly $35 \times 10^3 M_r$ in the wild-type cells; the *smlA* cells do not contain any detectable SmlA protein (not shown). Similarly, there was no detectable immunofluorescence staining of vegetative cells with the preimmune serum (not shown). The immune sera showed no staining of *smlA* cells, and showed a diffuse cytosolic staining of vegetative wild-type cells (Fig. 3). Western blots of vegetative and developing cells indicated that SmlA is present in vegetative cells and throughout early development and then levels decrease after about 10 hours (Fig. 4). To determine the amount of SmlA protein in the vegetative cells, we electrophoresed protein from different numbers of cells and known amounts of recombinant SmlA on SDS-polyacrylamide gels. Western blots of these gels were stained with anti-SmlA antibodies. We found that the staining intensity of 1×10^5 cells was roughly the same as the staining intensity of 2 ng of recombinant SmlA, and the staining of 2×10^5 cells was roughly the same as 4 ng, etc. This indicated that there are of the order of 5×10^5 molecules of SmlA per cell.

To examine further the subcellular location of SmlA protein, vegetative cells were lysed and fractionated by centrifugation. After a low speed spin to remove nuclei, SmlA was present in the supernatant (Fig. 5). The supernatant was then recentrifuged to pellet large organelles, and after this centrifugation

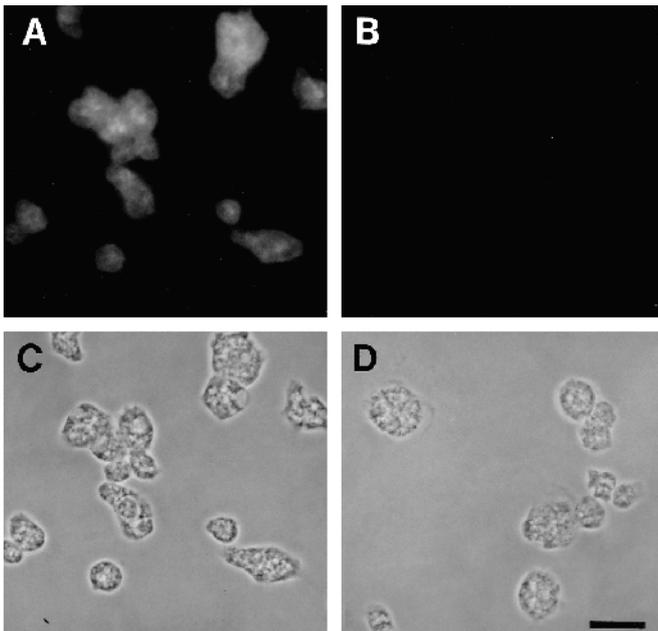


Fig. 3. Distribution of SmlA protein in cells. Vegetative Ax-4 (A) and *smlA* (B) cells were fixed and then stained with anti-SmlA antibodies. Corresponding phase images are in C and D. Bar in D is 20 μ m.

SmlA was still present in supernatant. After centrifugation of this second supernatant for 6×10^6 g-minutes, most of the SmlA remained in the supernatant. In conjunction with the diffuse staining seen by immunofluorescence, this suggests that SmlA is predominantly cytosolic.

The effect of SmlA on aggregate size involves changes in secreted factors

To investigate the role of extracellular factors in the *smlA* phenotype, we performed cell mixing experiments. *smlA* cells were mixed with Ax-4 wild-type cells and *smlA* cells were mixed with DH1 or Ax-4 cells. When the wild-type cells were 5 or 10% of the mixture, the *smlA* phenotype was not rescued. However, with only a 5% addition of either *smlA* cells to Ax-4 wild-type cells or 5% *smlA* knockout cells to DH1 or Ax-4 cells, there was a significant increase in aggregate number compared to Ax-4 or DH1 cells alone (Fig. 6). The density and size of the aggregates in these cell mixtures were indistinguishable from those of the mutants (data not shown). Because only a very small addition of mutant cells can affect the number of aggregates formed by wild-type cells, it is likely that there is an alteration in a secreted factor(s) in the *smlA* mutants.

SmlA is secreted at a very low rate if at all

To determine if SmlA is secreted during development, conditioned medium was made from wild-type cells starved at 5×10^6 cells/ml in shaking culture for 20 hours. The conditioned medium was concentrated 10-fold, and 18 μ l of the concentrate was electrophoresed on a SDS-polyacrylamide gel along with various amounts of recombinant SmlA. A western blot of the gel was then immunostained with anti-SmlA antibodies. No SmlA could be detected in the conditioned medium, whereas 0.1 ng of recombinant SmlA could be detected (data not

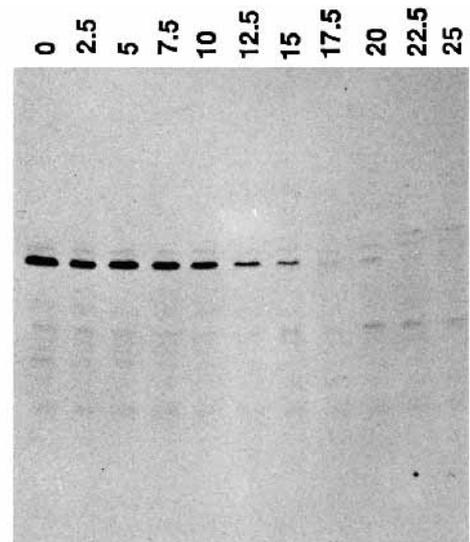


Fig. 4. Expression of SmlA protein during development. Axenically growing vegetative Ax-4 cells (0) and Ax-4 cells starved on filter pads for the indicated times in hours were solubilized in Laemmli sample buffer and electrophoresed on an SDS-polyacrylamide gel. A Western blot of the gel was stained with anti-SmlA antibodies.

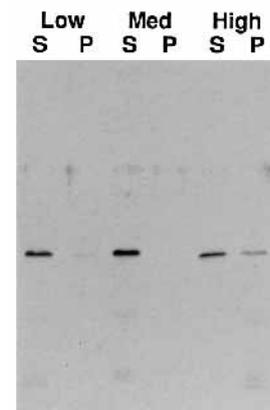


Fig. 5. Subcellular distribution of SmlA protein. Vegetative Ax-4 cells were broken open with a Dounce homogenizer and fractionated by centrifugation. A low speed spin was used to pellet nuclei and unbroken cells. The supernatant from this spin was centrifuged at a higher speed to pellet organelles. The supernatant from the second spin was centrifuged to pellet microsomes. Samples of the pellets (P) and supernatants (S) from the three spins were boiled in Laemmli sample buffer and electrophoresed on an SDS-polyacrylamide gel. A western blot of the gel was then stained with anti-SmlA antibodies.

shown). Since roughly 2.5×10^9 molecules of recombinant SmlA were detected, but no SmlA could be detected from the medium conditioned by approximately 9×10^5 cells, well less than ~3000 molecules of SmlA per cell had accumulated in the extracellular medium in 20 hours. We also added 100, 10, 1 or 0.1 ng/ml recombinant SmlA to starving cells at 1 hour after starvation, and then harvested the CM at 20 hours. No recombinant SmlA could be detected in the CM, suggesting that this portion of the SmlA protein is sensitive to the proteases present in CM. Addition of recombinant SmlA to Ax4 or *smlA* CM also did not

alter the effect of either CM on aggregate size. The above results suggest that SmlA is not secreted during development.

These results were surprising, since the cell mixing experiments suggest that the defect in *smlA* involves an extracellular molecule. One explanation is that SmlA could be regulating the secretion of an extracellular factor or the expression of a cell surface factor. To distinguish between these possibilities and to determine if the extracellular molecule causing small aggregates is soluble, Ax-4 wild-type cells were starved in submerged culture in buffer or conditioned starvation buffer (also known as conditioned medium or CM) from Ax-4 or *smlA* cells. CM contains CMF, which then allows starved cells to quickly begin aggregation and, as shown in Fig. 7, cells starved in wild-type or *smlA* CM began clustering quickly, whereas cells starved in buffer could not begin aggregation until the level of accumulated extracellular CMF had reached a threshold. The cells starved in buffer or wild-type CM formed large aggregates, whereas when wild-type cells were starved in *smlA* CM, they formed small aggregates (Fig. 7F). We also examined the behavior of wild-type cells starved at an air-liquid interface on filter pads soaked with buffer, wild-type CM or *smlA* CM. As shown in Fig. 8, normal size aggregates are formed when wild-type cells are allowed to develop on pads soaked with buffer alone or wild-type CM, whereas many small aggregates form when the same cells develop on a pad soaked with *smlA* CM. CM from *smlA* cells also caused Ax-4 cells to form small aggregates on pads or in submerged culture. These results suggest that the absence of SmlA causes changes in the conditioned media with the cascade effect of causing small aggregate formation. The small aggregate-forming activity of *smlA* CM is quite labile, losing most of its activity after 24 hours at 0°C and losing some activity after being frozen and thawed.

PDE activity is reduced in *smlA* mutants, but exogenous PDE does not restore normal aggregate size

Since very high levels of *Dictyostelium* phosphodiesterase can decrease aggregate size (Faure et al., 1988), we examined whether SmlA affects the level of extracellular phosphodiesterase activity during development. *smlA* CM consistently contained approximately 4-fold less phosphodiesterase activity than did DH1 or Ax-4 CM. To determine if the decrease in phosphodiesterase activity is responsible for the *smlA* phenotype, we starved cells in the presence of activated beef heart phosphodiesterase. Although concentrations above 0.1 mg/ml somewhat affected the aggregation speed of Ax4 and *smlA* cells, concentrations of beef heart phosphodiesterase ranging from 10 mg/ml to 3 pg/ml had no significant effect on the size or number of aggregates formed by Ax-4 or *smlA* cells (Wier, 1977 and data not shown).

The factor secreted by *smlA* cells that causes small aggregates is not CMF or cAMP

Another extracellular factor that regulates

aggregation is the density-sensing factor CMF. Bioassays of CM from Ax-4, DH1, *smlA*sr and *smlA* cells indicated that similar amounts of CMF activity are produced by all four cell types, and Western blots of CM stained with anti-CMF antibodies indicated that *smlA* and Ax-4 cells secrete indistinguishable amounts of CMF protein (data not shown). In

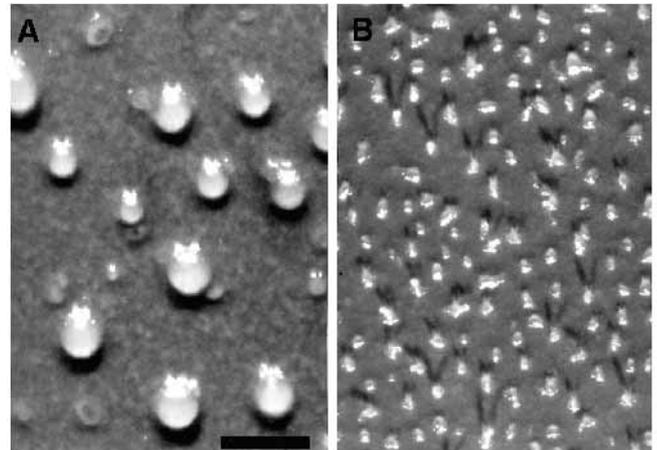


Fig. 6. Synergy assays of *smlA* cells. Ax-4 wild-type cells (A) or a mixture of 95% Ax-4 cells with 5% *smlA* cells (B) were starved on filter pads and photographed 12 hours later. Bar in A is 0.5 mm.

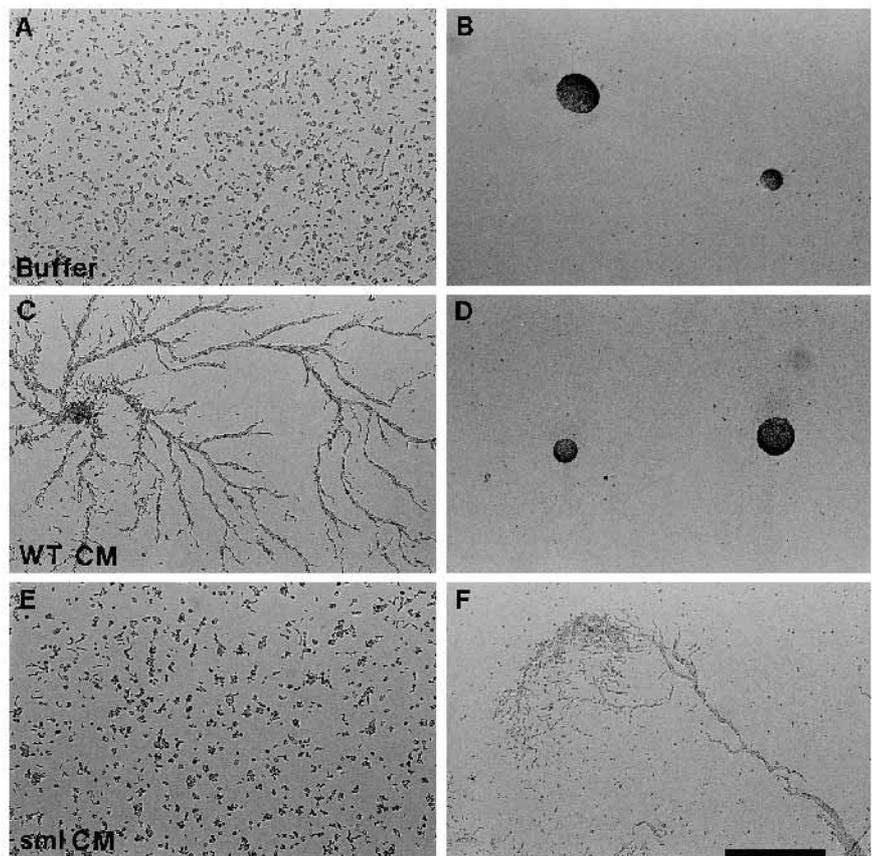


Fig. 7. Effect of *smlA*-conditioned starvation medium (CM) on wild-type cells in submerged monolayer culture. Ax-4 cells were starved in the presence of PBM (A,B), Ax-4 CM (C,D) or *smlA* CM (E,F). Cells were photographed after 7 hours (A,C,E) and after 19 hours (B,D,F). Bar in F is 0.5 mm.

addition, we have found that varying the level of exogenous purified or recombinant CMF added to CMF antisense cells starved on filter pads affects whether or not the cells aggregate, but does not significantly affect aggregate size (Jain, Yuen and Gomer, unpublished results). This suggests that the effect of SmlA on aggregate size does not involve altered secretion of CMF.

To determine if the factor secreted by the *smlA* cells is cAMP or the small ($<5 \times 10^3 M_r$), highly potent breakdown products of CMF (Yuen et al., 1991), we used spin filtration to size fractionate *smlA* CM. Under conditions where cAMP or the small breakdown products of CMF pass through $30 \times 10^3 M_r$, $50 \times 10^3 M_r$ or $100 \times 10^3 M_r$ cutoff spin filters, we found that

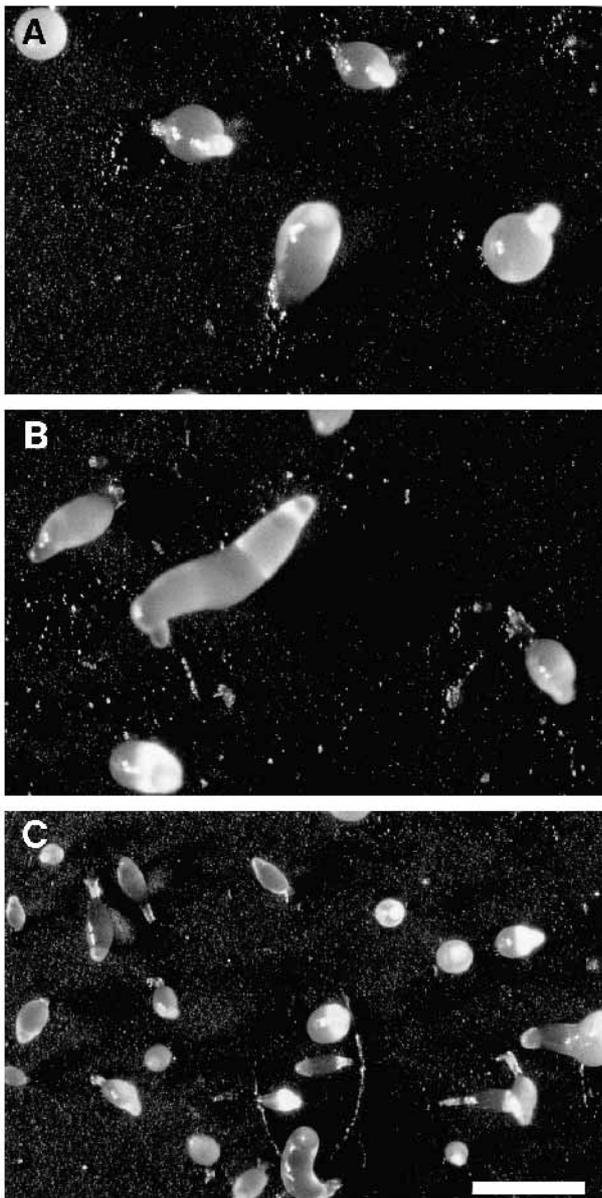


Fig. 8. Effect of *smlA*-conditioned medium on wild-type cells starved on filter pads. Ax-4 cells were washed and resuspended in buffer, and then placed on filter pads soaked with buffer (A), Ax-4 CM (B), or *smlA* CM (C). The aggregates were photographed 14 hours after starvation. Bar in C is 0.5 mm.

the activity was retained by and could be recovered from all three types of spin filters. Thus neither cAMP nor the CMF breakdown products appear to be directly involved in the decrease in aggregate size caused by *smlA* CM. In addition, these results suggest that the factor secreted by the *smlA* cells, which causes cells to form small aggregates, is associated with or is a molecule with a molecular mass greater than $100 \times 10^3 M_r$.

Loss of *smlA* alters the extracellular concentrations of multiple secreted proteins

We then examined whether SmlA affects the pattern of protein secretion during development. Parental DH1 and *smlA* cells were starved in buffer in shaking culture, and the CM was harvested at different times after starvation. A silver-stained SDS-polyacrylamide gel shows that the total amounts of protein secreted by DH1 or *smlA* cells starved for 5 minutes are roughly the same (Fig. 9, lanes marked 0). The exocytosis rate of endosomally/lysosomally localized fluid phase markers is also identical for both strains (Fig. 10A). Comparing individual protein bands through development, the level of many proteins in the *smlA* CM is the same as in the DH1 CM (Fig. 9). However, the level of some proteins is clearly increased, while the amount of other proteins is clearly decreased. For instance, at 5 minutes there is less of a $21 \times 10^3 M_r$ band in *smlA* CM compared to DH1 CM, while a $34 \times 10^3 M_r$ band is more intense in the *smlA* CM. The differences also change during development; for instance, at 3 hours, an $80 \times 10^3 M_r$ band becomes more prominent in the *smlA* CM and, at 6 hours, a $38 \times 10^3 M_r$ band appears in the *smlA* CM. These differences can also be seen by comparing Ax-4 and *smlA* CMs (not shown). We also measured the rate of secretion of the lysosomal hydrolases α -mannosidase and acid phosphatase. After 3 hours of incubation in starvation buffer, *smlA* cells secreted 60% the amount of both enzymes secreted from wild-

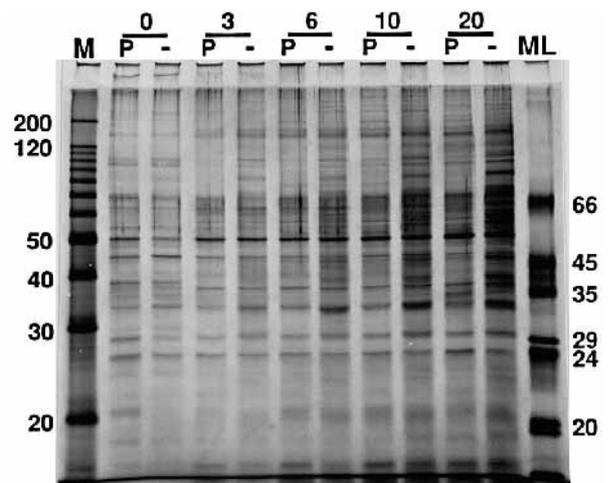
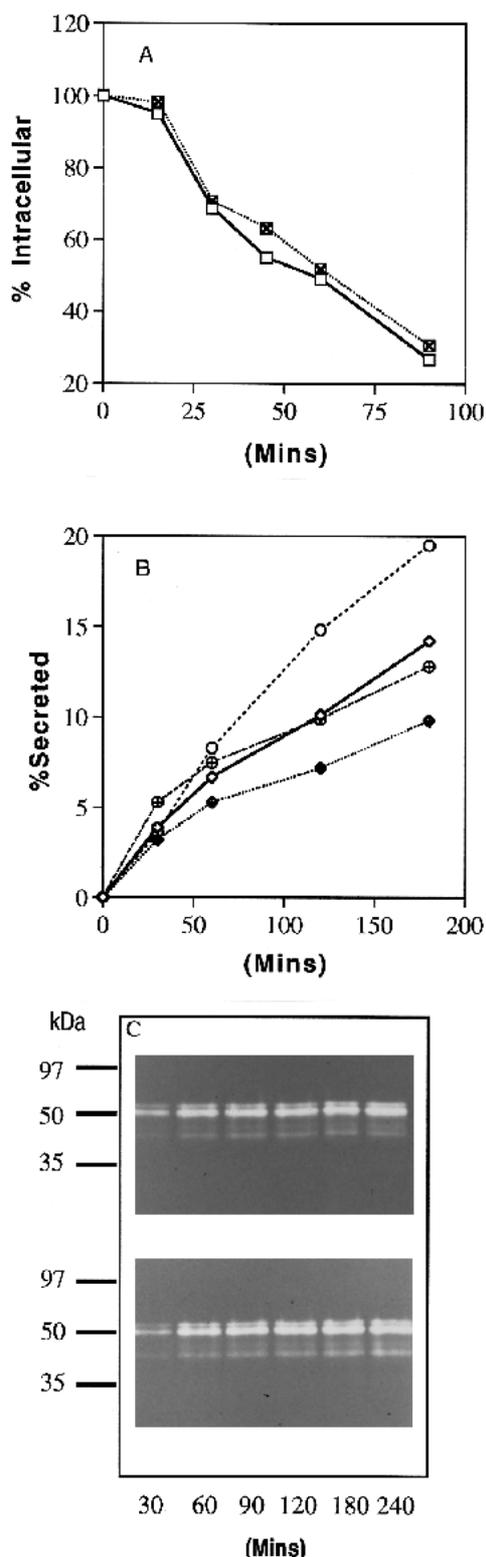


Fig. 9. SDS-polyacrylamide gel of proteins secreted by DH1 parental and *smlA* cells. Cells were starved in shaking culture. At the times in hours indicated at the top, an aliquot of the culture was removed and the conditioned starvation medium freed of cells by centrifugation; 0 represents starvation medium that was conditioned by cells for approximately 5 minutes. The CM samples were electrophoresed on a SDS-20% polyacrylamide gel, which was then stained for proteins by the silver stain procedure.



type cells (Fig. 10B). In contrast, the rate and extent of secretion of multiple acid proteases was identical between the two strains (Fig. 10C). The altered secretion of some proteins from cells lacking SmlA protein is consistent with the observation that these cells secrete a factor(s) that causes the formation of small aggregates.

Fig. 10. Secretion of endosomal/lysosomal enzymes and fluid phase material from DH1 parental and *smlA* cells. (A) Fluid phase exocytosis was measured by loading cells with FITC-dextran, washing them and resuspending them in phosphate buffer. At the indicated times, cells were washed and the amount of FITC-dextran that was retained in the cells was determined. The open squares are DH1 parental cells and the closed squares are *smlA* cells. (B) DH1 parental (open symbols) and *smlA* cells (closed symbols) growing in HL5 were harvested by centrifugation, washed and resuspended in PBM. At the indicated times, samples were taken and cells were separated from the supernatant by centrifugation. The activity of alpha mannosidase (circles) and acid phosphatase (diamond symbols) was measured. (C) To examine the activities of several proteases, supernatant samples prepared as described in B were electrophoresed on 11% polyacrylamide gelatin SDS gels. After allowing the proteases to hydrolyze the gelatin, the gels were stained with Coomassie. Areas where the gelatin was hydrolyzed appear white. The upper panel represents the conditioned medium from the DH1 parental cells and the lower panel represents *smlA*-conditioned medium.

DISCUSSION

One of the most fascinating aspects of development is that large numbers of cells can organize themselves into structures of specific sizes and shapes. We have identified a *Dicystoteliium* mutant, *smlA*, which has a defect in an aggregate size-determination mechanism; this defect causes the formation of an unusual high density of aggregates, each containing an abnormally small number of cells. We find that even when *smlA* cells are starved at very high densities, they form small aggregates, so, following the classification system of Hohl and Raper (1964), they have a defect in a size-determination mechanism as opposed to a defect in the ability to aggregate. To a first approximation, the defect in the *smlA* cells appears to be limited to a size-determination mechanism, since *smlA* cells have normal growth rates, cell cycles, motility, developmental time courses, differentiation into prespore and prestalk cells, CMF secretion and sensing, and cAMP sensing during late development. The small aggregates are due to an increased amount of a diffusible factor secreted by the *smlA* cells since the *smlA* phenotype can be mimicked by developing wild-type cells in the presence of *smlA* CM, but cannot be rescued by starving *smlA* cells in the presence of wild-type cells or CM. This diffusible factor appears to be a molecule with molecular mass greater than 100×10^3 Mr. The ability of 5% *smlA* cells in a population of wild-type cells to cause the entire population to form small aggregates, and the inability of 10% wild-type cells to rescue *smlA* cells, suggests that the *smlA* phenotype is not due to the lack of a secreted factor.

Loss of SmlA alters the extracellular concentration of several secreted proteins during development. Some proteins become more abundant, others less abundant. Although most of these proteins remain to be identified, we have found that *smlA* cells secrete less lysosomal acid phosphatase and α -mannosidase compared to wild-type cells. However, the secretion of most proteins during development, including CMF and multiple acid proteases is not altered in *smlA* cells. The absence of SmlA affects the profile of the secreted proteins within five minutes after starvation. Since the absence of SmlA does not seem to affect growth, the presence of SmlA in vegetative cells might be because SmlA is needed immediately after starvation.

CMF is another example of a protein that is made and sequestered in vegetative cells but appears to be used by the cells only after they have starved (Jain and Gomer, 1994; Jain et al., 1992b). SmlA itself does not appear to be secreted; it is a cytosolic protein without significant homology to any known protein. Sequence analysis reveals no known functional motifs, so the mechanism by which SmlA affects the concentration of only some extracellular proteins remains to be discovered.

An increased amount of phosphodiesterase or a decreased amount of phosphodiesterase inhibitor can cause a decreased aggregate size. The *smlA* phenotype is not caused by increased phosphodiesterase activity, since *smlA* cells have a 4-fold decrease in secreted phosphodiesterase activity. The failure of an excess of exogenous phosphodiesterase to rescue the *smlA* phenotype indicates that the decreased aggregate size in *smlA* cells does not result from a loss of phosphodiesterase inhibitor activity.

There are several possible mechanisms by which the factor(s) secreted by *smlA* cells could cause a decrease in aggregate size. We have proposed a mechanism that can be used to sense the total number of cells in a group (Clarke and Gomer, 1995; Yuen and Gomer, 1994). This mechanism involves cells simultaneously secreting and sensing the concentration of an extracellular factor that diffuses out of the group of cells, so that the concentration of the factor in the group is thus dependent on the total number of cells in the group. If the secretion rate of the factor increases or the exogenous extracellular concentration of the factor increases, a smaller than normal number of cells would be sensed as being the proper group size. If SmlA were part of such a mechanism, the factor whose secretion rate is increased by disrupting *smlA* is behaving like the factor in the above mechanism. A different mechanism that can generate patterns in fields of cells was proposed by Turing (1952). This mechanism uses two extracellular factors with different diffusion coefficients, which affect the concentration of each other. The factor(s) secreted by the *smlA* cells could also be part of this type of mechanism. Further characterization of the secreted factor(s) affected by *smlA* will lead to a better understanding of how an even field of starving cells breaks up into groups of a specific size.

We thank Stephen Richards for assistance with the preparation of the *smlA* expression vector, Robin Ammann for assistance with cell differentiation assays, Ed Frank for cDNA library screening, Jason Katz for assistance with filter pad assays, Bill Deery for doing the anti-CMF western blot, Diane Hatton for assistance with sequence analysis, David Lindsey for RNA and protein time-course samples, Yoshi Tonegawa, Bill Loomis and Gunther Gerisch for helpful discussions, and Maureen Price for supervision and guidance. R. H. G. is an assistant investigator of the Howard Hughes Medical Institute. This paper is dedicated to the memory of Jason Cardelli.

REFERENCES

- Cardelli, J. A., Golubeski, G. S., Woychik, N. A., Ebert, D. L., Mierendorf, R. C. and Dimond, R. L. (1987). Defining the intracellular localization pathways followed by lysosomal enzymes in *Dictyostelium discoideum*. In *Methods in Cell Biology* (ed. J. A. Spudich), pp. 139-155. Orlando, FL: Academic Press.
- Clarke, M. and Gomer, R. H. (1995). PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. *Experientia* **51**, 1124-1134.
- Clay, J. L., Ammann, R. A. and Gomer, R. H. (1995). Initial cell type choice in a simple eukaryote: Cell-autonomous or morphogen-gradient dependent? *Dev. Biol.* **172**, 665-674.
- Devreotes, P. (1989). *Dictyostelium discoideum*: A model system for cell-cell interactions in development. *Science* **245**, 1054-1058.
- Dicou, E. L. and Brachet, P. (1979). Multiple forms of an cyclic-AMP phosphodiesterase from *Dictyostelium discoideum*. *Biochim Biophys Acta* **578**, 232-242.
- Faure, M., Podgorski, G. J., Franke, J. and Kessin, R. H. (1988). Disruption of *Dictyostelium discoideum* morphogenesis by overproduction of cAMP phosphodiesterase. *Proc. Natl. Acad. Sci. USA* **85**, 8076-8080.
- Faure, M., Podgorski, G. J., Franke, J. and Kessin, R. H. (1989). Rescue of a *Dictyostelium discoideum* mutant defective in cyclic nucleotide phosphodiesterase. *Dev. Biol.* **131**, 366-372.
- Firtel, R. A. (1995). Integration of signaling information in controlling cell-fate decisions in *Dictyostelium*. *Gene Dev.* **9**, 1427-1444.
- Franke, J. and Kessin, R. H. (1981). The cyclic nucleotide phosphodiesterase inhibitory protein of *Dictyostelium discoideum* Purification and characterization. *J. Biol. Chem.* **256**, 7628-7637.
- Gerisch, G. (1968). Cell aggregation and differentiation in *Dictyostelium*. *Curr. Top. Dev. Biol.* **3**, 157-197.
- Giorda, R., Ohmachi, t. and Ennis, H. L. (1989). Organization of a gene family developmentally regulated during *Dictyostelium discoideum* spore germination. *J. Mol. Biol.* **205**, 63-69.
- Gomer, R. H. (1987). A strategy to study development and pattern formation: use of antibodies against products of cloned genes. In *Methods in Cell Biology* (ed. J. A. Spudich), pp. 471-487. Orlando, FL: Academic Press.
- Gomer, R. H. and Ammann, R. (1996). A cell-cycle phase-associated cell-type choice mechanism monitors the cell cycle rather than using an independent timer. *Dev. Biol.* **174**, 82-91.
- Gomer, R. H., Yuen, I. S. and Firtel, R. A. (1991). A secreted $80 \times 10^3 M_r$ protein mediates sensing of cell density and the onset of development in *Dictyostelium*. *Development* **112**, 269-278.
- Hall, A. L., Franke, J., Faure, M. and Kessin, R. H. (1993). The role of the cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum* during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the *pde*. *Dev. Biol.* **157**, 73-84.
- Hohl, H. R. and Raper, K. B. (1964). Control of sorocarp size in the cellular slime mold *Dictyostelium discoideum*. *Dev. Biol.* **9**, 137-153.
- Jain, R. and Gomer, R. H. (1994). A developmentally regulated cell surface receptor for a density-sensing factor in *Dictyostelium*. *J. Biol. Chem.* **269**, 9128-9136.
- Jain, R., Gomer, R. H. and Murtagh Jr, J. J. (1992a). Increasing specificity from the PCR-RACE technique. *BioTechniques* **12**, 58-59.
- Jain, R., Yuen, I. S., Taphouse, C. R. and Gomer, R. H. (1992b). A density-sensing factor controls development in *Dictyostelium*. *Gene Dev.* **6**, 390-400.
- Kessin, R. H., Orlow, S. J., Shapiro, R. I. and Franke, J. (1979). Binding of inhibitor alters kinetic and physical properties of extracellular cyclic AMP phosphodiesterase from *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* **76**, 5450-5454.
- Loomis, W. F. (1975). *Dictyostelium discoideum: A Developmental System*. New York: Academic Press
- Loomis, W. F. (1993). Lateral inhibition and pattern formation in *Dictyostelium*. *Curr. Top. Dev. Biol.* **28**, 1-46.
- Nanjundiah, V. and Malchow, D. (1976). A theoretical study of the effect of cyclic AMP phosphodiesterase during aggregation in *Dictyostelium*. *J. Cell Sci.* **22**, 49-58.
- North, M. J., Franek, K. J. and Cotter, D. A. (1990). Differential secretion of *Dictyostelium discoideum* proteinases. *J. Gen. Micro.* **136**, 827-833.
- Orlow, S. J., Shapiro, I., Franke, J. and Kessin, R. H. (1981). The extracellular cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum*. Purification and characterization. *J. Biol. Chem.* **256**, 7620-7627.
- Pampori, N. A., Pampori, M. K. and Shapiro, B. H. (1995). Dilution of the chemiluminescence reagents reduces the background noise on Western blots. *BioTechniques* **18**, 588-589.
- Riedel and Gerisch (1971). Regulation of extracellular c-AMP-PDE activity during development of *Dictyostelium discoideum* cAMP-phosphodiesterase. *Biochem. Biophys. Res. Commun.* **42**, 119,124.
- Riedel, V., Gerisch, G., Muller, E. and Beug, H. (1973). Defective cyclic adenosine-3,5'-phosphate-phosphodiesterase regulation in morphogenetic mutants of *Dictyostelium discoideum*. *J. Mol. Biol.* **74**, 573-585.
- Robertson, A. D. J. and Grutsch, J. F. (1981). Aggregation in *Dictyostelium discoideum*. *Cell* **24**, 603-611.

- Schaap, P.** (1991). Intercellular interactions during *Dictyostelium* development. In *Microbial Cell-Cell Interactions* (ed. M. Dworkin), pp. 147-178. Washington, DC: Am. Soc. Microbiol.
- Spann, T. P., Brock, D. A., Lindsey, D. F., Wood, S. A. and Gomer, R. H.** (1996). Mutagenesis and gene identification in *Dictyostelium* by shotgun antisense. *Proc. Natl Acad. Sci. USA* **93**, 5003-5007.
- Spratt Jr., N. T. and Haas, H.** (1961). Intergrative mechanisms in development of the early chick blastoderm. III. Role of cell population size and growth potentiality in synthetic systems larger than normal. *J. Exp. Zool.* **147**, 271-293.
- Sussman, M.** (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. In *Methods in Cell Biology* (ed. J. A. Spudich), pp. 9-29. Orlando, FL: Academic Press.
- Sussman, R. R. and Sussman, M.** (1953). Cellular differentiation in Dictyosteliaceae: heritable modifications of the developmental pattern. *Ann. NY Acad. Sci.* **56**, 949,960.
- Temesvari, L., Bush, J., Peterson, M., Titus, M. and Cardelli, J.** (1996). Examination of the endosomal and lysosomal pathways in *Dictyostelium discoideum* myosin I mutants. *J. Cell Sci.* (in press)
- Tsang, A. S. and Coukell, M. B.** (1979). Biochemical and genetic evidence for two extracellular adenosine 3':5'-Monophosphate phosphodiesterases in *Dictyostelium purpureum*. *Eur. J. Biochem.* **95**, 407-417.
- Turing, A. M.** (1952). The chemical basis of morphogenesis. *Phil. Trans R Soc. Lond.* **237**, 37-72.
- Wier, P. W.** (1977). Cyclic AMP, cyclic AMP phosphodiesterase, and the duration of the interphase in *Dictyostelium discoideum*. *Differentiation* **9**, 183-191.
- Wu, L., Franke, J., Blanton, R. L., Podgorski, G. J. and Kessin, R. H.** (1995). The phosphodiesterase secreted by prestalk cells is necessary for *Dictyostelium* morphogenesis. *Dev. Biol.* **167**, 1-8.
- Yuen, I. S. and Gomer, R. H.** (1994). Cell density-sensing in *Dictyostelium* by means of the accumulation rate, diffusion coefficient and activity threshold of a protein secreted by starved cells. *J. Theoret. Biol.* **167**, 273-282.
- Yuen, I. S., Jain, R., Bishop, J. D., Lindsey, D. F., Deery, W. J., Van Haastert, P. J. M. and Gomer, R. H.** (1995). A density-sensing factor regulates signal transduction in *Dictyostelium*. *J. Cell Biol.* **129**, 1251-1262.
- Yuen, I. S., Taphouse, C., Halfant, K. A. and Gomer, R. H.** (1991). Regulation and processing of a secreted protein that mediates sensing of cell density in *Dictyostelium*. *Development* **113**, 1375-1385.

(Accepted 7 June 1996)