A Cell Number-Counting Factor Regulates Levels of a Novel Protein, SsLA, as Part of a Group Size Regulation Mechanism in Dictyostelium

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Developing Dictyostelium cells form aggregation streams that break into groups of ~2 × 10^4 cells. The breakup and subsequent group size are regulated by a secreted multisubunit counting factor (CF). To elucidate how CF regulates group size, we isolated second-site suppressors of smlA^-, a transformant that forms small groups due to oversecretion of CF. smlA^- ssa11(CR11) cells form roughly wild-type-size groups due to an insertion in the beginning of the coding region of ssa11, one of two highly similar genes encoding a novel protein. The insertion increases levels of SsIA. In wild-type cells, the ssa11(CR11) mutation forms abnormally large groups. Reducing SsIA levels by antisense causes the formation of smaller groups. The ssa11(CR11) mutation does not affect the extracellular accumulation of CF activity or the CF components countin and CF50, suggesting that SsIA does not regulate CF secretion. However, CF represses levels of SsIA. Wild-type cells starved in the presence of smlA^- cells, recombinant countin, or recombinant CF50 form smaller groups, whereas ssa11(CR11) cells appear to be insensitive to the presence of smlA^- cells, countin, or CF50, suggesting that the ssa11(CR11) insertion affects CF signal transduction. We previously found that CF reduces intracellular glucose levels. sla1(CR11) does not significantly affect glucose levels, while glucose increases SsIA levels. Together, the data suggest that SsIA is a novel protein involved in part of a signal transduction pathway regulating group size.

Much remains to be understood about tissue size regulatory mechanisms, such as how during development or regeneration a tissue grows to a certain size and then stops growing. The simple eukaryote Dictyostelium discoideum is an excellent model for studying tissue size regulation, as during development, Dictyostelium forms structures of specific sizes, with our laboratory strains forming groups of ~20,000 cells. Dictyostelium is a unicellular soil organism that uses bacteria as a food source. When the food source in an environment is exhausted and the cells begin to starve, they secrete a cell density-sensing factor, conditioned medium factor (20, 24, 25, 42, 70, 71). When a high number of the cells have starved, as indicated by a high concentration of conditioned medium factor, the cells begin to make multicellular structures. Using relayed pulses of cyclic AMP (cAMP) as a chemoattractant, the cells form streams moving toward an aggregation center. The streams either coalesce into a group of cells or, if there are a large number of cells in the streams, break up into groups (53). Each group then develops into a fruiting body made up of a mass of spore cells supported on top of a column of stalk cells (31, 41). There is an optimal number of stalk and spore cells in a fruiting body, so that a maximal number of spores are held as high as possible above the soil surface for dispersal to a new food source. When there are too few stalk cells, the result is a short stalk, and the spores will then be too close to the ground for optimal dispersal. A fruiting body with too many stalk or spore cells gives rise to a stalk that cannot support the spore mass, which falls to the ground, where it is not easily dispersed (4).

In our laboratory strains, fruiting bodies with more than ~3 × 10^4 cells tend to fall over or have the spore mass slide down the stalk (4). The formation of an optimal fruiting body is thus dependent upon the streams either not breaking up (if the number of cells streaming toward a given center is less than ~2 × 10^4) or breaking up into groups if there are more than that number of cells streaming toward a center. We have found that one determinant of stream breakup and aggregate size is counting factor (CF), a secreted complex of polypeptides with a molecular mass of ~450 kDa (4). Four of the components of CF are countin, CF45-1, CF50, and CF60. Disrupting the gene or decreasing mRNA levels by antisense for either countin, CF45-1, CF50, or CF60 results in the formation of fewer but larger aggregates and fruiting bodies that are huge and either misshapen to begin with or about to collapse (4–7). Cells with a disruption of the smlA gene or smlA antisense (smlAas) cells, where levels of the SmlA protein have been severely decreased by antisense, oversecrete CF. The cells oversecreting CF or wild-type cells exposed to high levels of purified CF form streams that break up into large numbers of small aggregates, and these then form small, short fruiting bodies (3, 58). Wild-type cells secrete CF, and diffusion calculations indicated that as the number of cells in a stream increases, the concentration of CF increases (4). Together, these observations suggested that wild-type cells sense the number of cells in a stream by monitoring the levels of CF and that when there are too many

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cells in a stream, as indicated by a high level of CF, the stream breaks to prevent the formation of an excessively large group.

Computer simulations indicated that a stream will begin to dissipate and then break if the cell-cell adhesion decreases and/or the random cell motility increases (51, 61). The simulations predicted that the broken stream segments would coalesce into groups if the adhesion subsequently increased and/or the random motility decreased. We found that CF decreases cell-cell adhesion and the expression of gp24, a key adhesion molecule (51). CF also increases cell motility and, in agreement with an increase in cell motility, increases actin polymerization and the expression of AQP-120, an actin cross-linking protein, and decreases myosin polymerization (61).

During aggregation, each pulse of extracellular cAMP activates G protein-coupled cAMP receptors. These receptors activate a signal transduction pathway that does two things (11, 15, 16, 28, 32, 35, 39, 43, 46–48, 66, 67, 69). First, the cells regulate their motility to move toward the source of the cAMP pulse. This movement toward the source of the extracellular cAMP pulse is accompanied by a brief increase in intracellular cyclic GMP (cGMP) levels. Second, the cells produce and secrete a pulse of cAMP to relay the chemotactic signal. CF regulates cAMP signal transduction at a point downstream of the cAMP receptor and G protein activation (60).

The CF signal transduction pathway is unclear. Garrod and Ashworth (18) found that growing cells in the presence of high concentrations of glucose causes the formation of larger fruiting bodies, and we found that CF appears to negatively regulate intracellular glucose levels (26, 27). Increasing intracellular glucose levels mimics the effect of decreasing extracellular CF levels with respect to CF’s effects on adhesion and motility. Although CF regulates both the cAMP and the cGMP pulses (60), glucose appears to mediate CF’s effect on the cAMP but not the cGMP pulse (26). Since CF regulates group size by regulating several different signal transduction pathways, and since little is known about size regulation in general, we initiated a second-site-suppressor screen of smlAas cells to further study the CF signal transduction pathway.

MATERIALS AND METHODS

Cell culture, group number assays, and Western blots. Dictyostelium discoideum Ax-4 wild-type, smlAas (strain HDB1647-1), and smlA- (strain HDB77YA) cells and cells with a disruption of the ctnA gene (strain HDB2B/4 cells, referred to in this paper and previous papers as counter− cells) were grown as previously described (3, 4). Photography of fruiting bodies followed Brock et al. (5). Cells were developed on filter pads as described in Jain et al. (25). Cell-mixing (synergy) experiments were performed according to Brock et al. (3). Production of conditioned starvation medium (CM) and starvation of cells in the presence of CM were done according to Brock and Gomer (4). Group number assays were done according to Brock et al. (3). Staining of Western blots with anti-CF50 and anti-counter antibodies was performed as previously described (5). Depletion of extracellular ctnA by using anti-counter antibodies was performed as described in Tang et al. (61). For cells developed in the presence of recombinant ctnA or recombinant CF50, log-phase (1 × 10^6 to 2 × 10^6 cells/ml) vegetative cells were collected by centrifugation and resuspended in PBM (20 mM KH_2PO_4, 10 μM CaCl_2, 1 mM MgCl_2 [pH 6.1 with KOH]) to 5 × 10^6 cells/ml. Thirty microliters of the cells was spotted onto AABP04700 filters (Millipore, Billerica, MA) placed on two layers of a 3 filter paper (Whatman, Maidstone, United Kingdom) soaked with PBM. For some experiments, 2 ml of the cells was used to cover the entire 47-mm-diameter filter. After 1 h, the filters were transferred to filter paper soaked with either PBM, 200 ng/ml recombinant ctnA in PBM, or 200 μg/ml recombinant CF50 in PBM. For starvation in shaking culture, cells were collected by centrifugation, washed twice in PBM, and resuspended to 5 × 10^6 cells/ml in PBM and were then shaken at 140 rpm.

REM1 mutagenesis and cell transformation. Restriction enzyme-mediated integration (REMI) mutagenesis was essentially done as described by Adachi et al. (1), with the exception that the smlAas strain was used as the parental line for REM1 mutagenesis. After transformation, the REM1-mutagenized cells were grown in HL-5 in submerged culture with 2.5 μg/ml blasticidin. Clones were selected on SM/5 agar plates with Klebsiella aerogenes bacteria. Since smlAas cells differentiate into small groups and few tiny fruiting bodies, REM1 transformants that formed normal-sized or large groups were selected.

Restriction enzyme sites flanking the REM1 vector insertion sites were mapped using Southern blots of transformant genomic DNA probed with a fragment of the REM1 vector blasticidin resistance cassette (36). Integrated plasmids and flanking sequences were excised from Dictyostelium cells by digestion with StyI, ligated under dilute conditions to form circular DNA, and then cloned into Escherichia coli. Recombination of mutations of multiple recombinations into smlA (these smlA− cells were made using a ura disruptor, and the resulting smlA− cells were sensitive to blasticidin) as well as disruption of smlA in wild-type cells was performed as described in Kuspa and Loomis (36). Insertion of the construct into smlA− (as opposed to insertion into smlA2, an smlA pseudogene, or a random insertion) was verified by Southern blotting using a fragment of the blasticidin resistance cassette and a fragment of smlA as probes. The phenotypes of smlAas and smlA− were indistinguishable, and the phenotypes of smlAas smlA(CR11) and smlA− smlA(CR11) were also indistinguishable. Because of problems with cell culture, in some experiments smlAas was compared to smlAas smlA(CR11), and in other experiments smlAas was compared to smlA− smlA(CR11).

Sequencing, RACE-PCR, and Southern and Northern blots. DNA sequencing was performed by Lone Star Laboratory (Houston, TX), and the sequences were compiled and analyzed using software from the Genetics Computer Group (Madison, WI). The 5′ and 3′ regions of the cDNA sequence were obtained using a SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (Clontech, Palo Alto, CA), followed by PCR using an Advantage 2 PCR kit (Clontech) with Clontech universal primer mix and the gene-specific primers (3, 4). Sequencing, RACE-PCR, and Southern and Northern blot analyses were performed as described in Wood et al. (68). An ~1 kb DNA fragment obtained from smlA cDNA by PCR with the primers 5′-GAGAAGTGGCGTACTGTTCTCAGA-3′ (to obtain the 3′ region of the cDNA) and 5′-CAAATGTGTTCCCCATGCGTGCTCTCACC-3′ (to obtain the 5′ region) (primers not predicted SslA sequence) were evaluated with the TMPPRED program (http://www.ch.embnet.org/software /TMPPRED_form.html). Molecular weights and pIs were calculated using the ExPaSy server at http://ca.expasy.org/tools/pi_tool.html, and secondary structure was evaluated using a variety of algorithms available at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page =NPSA/npsa_seccons.html. Potential glycosylation sites were identified using algorithms at http://www.cbs.dtu.dk/services /YinYong/ and http://www.cbs.dtu.dk/services/NetNGlyc/. Southern and Northern blot analyses were performed as described in Wood et al. (68). An ~1 kb DNA fragment obtained from smlA cDNA by PCR with the primers 5′-ATGCCCAAAATTGCTACCAAAATGAG-3′ and 5′-TCTTCTCCTGGTCTAGGATAAAATACGAC-3′ was used as a probe.

Antibody preparation, Western blots, and immunofluorescence. The peptide GPDIVPSCKSYDPNKL, corresponding to amino acids 217 to 232 from SslA (this sequence is also present in the predicted amino acid sequences of SslA2), was synthesized, and an affinity-purified antibody was produced by Bethyl Laboratories Inc. (Montgomery, TX). To examine the levels of SslA during development, cells were developed on filter pads as described by Jain et al. (25). For each time point, 1 × 10^6 cells were collected by centrifugation, resuspended in 100 μl of sodium dodecyl sulfate (SDS) sample buffer, and boiled for 5 min. Ten microliters of each sample was then analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Western blots were done using an ECL Western blotting kit (Amersham Bio- sciences, Piscataway, NJ), following the manufacturer’s phosphate-buffered saline–Tween protocol and staining the blots with 0.5 μg/ml anti-SslA in phosphate-buffered saline–0.05% NP-40 was done according to Gomer (19). Where indicated, deconvolution microscopy was done as previously described (5).

Cell fractionation. Wild-type cells were developed on filter pads in PBM for 3 h as described above. Cells (1 × 10^6) were collected and resuspended to 1 × 10^9 cells/ml in ice-cold MESUS buffer (20 mM MES [morpholineethanesulfonic acid], pH 6.5, 1 mM EDTA, and 0.25 M sucrose). Cells were lysed through a 5-μm-pore 25N syringe filter (Osmonics, MSI, Westbranh, CA). Cell fractionation was done using centrifugation according to Brock et al. (3).

smlA−. To generate a fragment of cDNA for antisense, PCRs were performed using combinations of primers AGCGCTGCAGGATTTAACCAGATATACCTGTGSGC (3032), GTGTGATCGTCCCCACAACTTTGCTGCTTTC (BAM3709), TTGGGTCGAGGTCAAGCTCAGTGGAACAC (SLA7587), and TTGGG GACCGCCGGATTACGTTTGGGAT (BAM4403), using an Advantage 2 PCR kit. RNAs used as templates were isolated from vegetative or developmen-
tal cells by using an RNasey mini kit (QIAGEN, Valencia, CA). Three sslA cDNA fragments with different lengths were generated, with fragment A1 containing nucleotides 464 to 1844 (SAL3024/BAM4403), fragment A2 nucleotides 464 to 1150 (SAL3024/BAM3709), and fragment A3 nucleotides 1361 to 1844 (SAL3875/BAM4403), each with a SalI site at the 5' end and a BamHI site at the 3' end. The PCR products were purified with a Genclean III kit (Qbiogene, Carlsbad, CA), digested with SalI and BamHI, and then ligated into the SalI and BamHI sites of the antisense vector pV18neo (58). The ligation was then transformed into Sure electroporation-competent cells (Stratagene, La Jolla, CA), and plasmid preps of the antisense constructs were used to transform wild-type cells (34, 58). Cells were cloned on SM5 plates spread with a mixture of killed Klebsiella aerogenes and G418. Killed K. aerogenes bacteria from three plates were collected and resuspended in 1 to 1.5 ml of PBM, incubated at 60°C for 15 min, and then immediately placed on ice. Three hundred microliters of the dead K. aerogenes was then mixed with transformed Dictyostelium cells and G418 to a final concentration of 10 μg/ml and then spread onto a fresh SM5 agar plate. Immunofluorescence was used to screen cells from individual colonies for reduced levels of SslA. No transformants were obtained with the A1 construct, while transformants with the A2 and A3 constructs had identical phenotypes and similar reductions in levels of SslA. The results describe a clone generated with the A3 construct designated sslAasA3-1 and referred to as sslA antisense (sslAas) in this report.

Glucose assay. Glucose levels were measured according to Jang et al. (26). To measure the effect of glucose on SslA levels, log-phase Ax-4 cells were collected by centrifugation, resuspended in HL-5 media with or without 1 mM glucose at 1 × 10⁶ cells/ml, and grown for 16 h. The cells were then harvested as previously described and stored in either PBM or PBM with 1 mM glucose at 5 × 10⁶ cells/ml in shaking culture. Cells (1 × 10⁶) were taken at 0, 3, and 6 h of starvation, collected by centrifugation, suspended in 100 μl SDS sample buffer, and boiled for 5 min. Proteins were separated on a 7.5% SDS-PAGE gel, and a Western blot was stained with anti-SsslA antibodies.

cAMP and CGMP production and cell type differentiation. The measurement of CGMP production in response to 1 μM extracellular cAMP was performed as previously described (30, 60). cAMP production was assayed according to Tang et al. (60), with the exception that cells were lysed at 0, 3, and 5 min. Cell type differentiation at low cell density was assayed according to Wood et al. (68).

Cell adhesion and motility. Cells (5 × 10⁶) were starved on filter pads as described above. After 0.5, 2, 4, 6, or 8 h of starvation, cells were harvested and adhesion was assayed as described in Roisin-Bouffay et al. (51), following Desbarats et al. (13). Cells in doublets or larger groups were counted as aggregated cells. Cell motility was measured according to Tang et al. (61).

RESULTS

Identification of a second-site suppressor of sslAas. To identify genes whose products may be involved in group size regulation, we used REMI to identify possible second-site enhancers and suppressors of sslAas (54). From approximately 3,000 independent REMI transformants of sslAas cells, we identified 27 that formed fruiting bodies that were larger than sslAas fruiting bodies. Wild-type cells form small groups and small fruiting bodies when mixed with sslAas or sslAas cells, due to the high levels of CF secreted by the sslAas or sslAas cells (3). To determine if a given REMI transformant was similarly sensitive to high levels of CF, an aliquot of each of the above-mentioned 27 transformants was mixed 85:15 with sslAas cells. Twenty transformants formed smaller groups and fruiting bodies, indicating that they still had functional CF signal transduction pathways, whereas the group and fruiting body sizes of 7 transformants were unaffected by the presence of sslAas cells, suggesting that these 7 transformants had defects in their CF signal transduction pathways (data not shown). The REMI vector, along with flanking genomic DNA, was excised from one of these transformants, designated sslAas sslA1(CR11), with sslA1(CR11) indicating “suppressor of sslA allele CR11.” The plasmid was linearized and used to transform wild-type and sslAas cells. This generated sslAas sslA1(CR11) and sslA1(CR11) cells. As shown in Fig. 1, the sslAas sslA1(CR11) cells formed larger fruiting bodies than the sslAas parental cells, and the fruiting bodies formed by sslA1(CR11) cells were somewhat larger than the fruiting bodies formed by the wild-type parental cells. Together, the data indicated that the insertion of the REMI vector into the sslA1 gene caused the formation of larger groups and fruiting bodies.

There are three closely related sslA genes in the Dictyostelium genome. Southern blot analysis of genomic DNA from wild-type cells also suggested that there is more than one copy of the sslA gene in the Dictyostelium genome (data not shown). In addition, Northern blots of RNA from both vegetative and developmental wild-type cells occasionally resolved two closely spaced bands when the sslA probe was used (Fig. 2A and data not shown). 5' RACE-PCR using mRNA from wild-type cells identified two sslA cDNAs. One cDNA (DDB0218600; GenBank accession no. AF508975) matched sslA1, while the other (DDB020502; GenBank accession no. EU003987) matched sslA2. The cDNA sequences of sslA1 and sslA2 contain upstream AUG codons. This has been observed in other eukaryotic cDNAs (23, 37, 44, 50). Besides sslA1 and sslA2, we found two additional sslA-like sequences in the Dictyostelium genome. DDB0205363 is located on chromosome 3. The derived amino acid sequence of DDB0205363 is the same as that of sslA1. RACE-PCR using mRNA from sslA1(CR11) cells produced no cDNA product from DDB0205363, suggesting that DDB0205363 is a pseudogene. DDB0168839, located on chromosome 2, is more similar to sslA2 than sslA1. However, DDB0168839 is a truncated sslA gene. There was no DDB0168839 cDNA product as determined by reverse transcription (RT)-PCR using DDB0168839-specific primers. This suggests that DDB0168839 is also a pseudogene.

The derived amino acid sequence of SslA1 (Fig. 2B) shows no significant similarity to known sequences. There is a possible transmembrane domain between amino acids 509 and 529.

FIG. 1. An insertion in the sslA1(CR11) locus increases the sizes of sslAas fruiting bodies. The indicated cell lines were grown on agar plates spread with bacteria. Photographs show fruiting bodies that formed as the cells overgrew the bacteria and starved. WT indicates wild-type cells. Bar, 0.5 mm.
(according to the SsA1 numbering; this domain is present in both of the predicted SsA1s). A transmembrane prediction program suggested that if the domain crosses a membrane, the orientation of the protein is with the N terminus in the extracellular space and the C terminus in the cytosol. The predicted SsA1 backbone is 66.8 kDa with a pI of 8.6, and the predicted SsA2 backbone is 49.6 kDa with a pI of 6.8. There are no large regions of positively or negatively charged residues. There are three potential N glycosylation sites and three potential O glycosylation sites in SsA1 and SsA2.

RACE-PCR using mRNA from sslA1(CR11) cells indicated that, as expected, the 5′ end of the sslA2 mRNA was unaffected by the insertion into sslA1. There was a modified sslA1 transcript present in sslA1(CR11) cells, beginning with the 87 nucleotides starting with the sequence ACGCGGGGAGATTT from the opposite strand of the actin 15 promoter contained in the REMI vector (thus, it appears that in this context the actin 15 promoter is acting as a bidirectional promoter, driving the expression of the blasticidin resistance gene in its normal orientation and driving the expression of sslA1 in the opposite direction). The mRNA sequence continued through the insertion site (6) (Fig. 2B) into the sslA1 sequence. The first AUG in the large open reading frame in the sslA1 mRNA from the sslA1(CR11) cells corresponds to amino acid 138 in the SsA1

![Diagram](image)

FIG. 2. Sequences of two sslA genes. (A) A Northern blot of RNA from vegetative cells was probed for sslA. Molecular mass markers are shown at left. (B) The predicted amino acid sequences encoded by the two sslA transcripts. The REMI vector insertion site in sslA1 is represented by ▼. The peptide highlighted in gray was used for antibody production. The cDNA sequences are available in GenBank under accession numbers AF508975 and EU003987.

| Medium for starvation of wild-type cells | No. of groups |
|---------------------------------------|---------------|
| Buffer                                 | 52 ± 3        |
| Wild-type CM                          | 68 ± 3        |
| sslA1(CR11) CM                        | 67 ± 3        |
| smlA-CM                                | 181 ± 5       |
| sslA1(CR11) CM                        | 178 ± 9       |

a Wild-type cells were starved on filter pads soaked with PBM buffer or 20-h CM from each indicated cell line. Aggregate numbers were counted after 16 h of starvation. Values are means ± SEMs from three separate experiments. The difference between wild-type CM and sslA1(CR11) CM and smlA-CM is significant at P values of <0.001; the difference between wild-type CM and smlA(CR11) CM is not significant, as is the difference between smlA-CM and smlA sslA1(CR11) CM (one-way ANOVA, Tukey’s test).
sequence. This would generate a protein with a predicted backbone size of 50.5 kDa, similar to the predicted backbone size of SslA2. These data suggest that the sslA(CR11) insertion does not affect the extracellular accumulation of countin or CF50. CM was prepared from the indicated cell types. Western blots of the CMs were then stained with anti-countin or anti-CF50 antibodies. WT, wild type.

The sslA(CR11) mutation appears to affect CF signal transduction. There are three general ways that a second-site mutation could cause smlA cells (which oversecrete CF) to form normal-size groups. The first is by repressing the oversecretion of CF, the second is by inhibiting cells from sensing the oversecreted CF, and the third involves some mechanism unrelated to CF. To test the hypothesis that the sslA mutation affects the abilities of cells to accumulate extracellular CF, bioassays for CF activity were performed by starving wild-type cells in the presence of starvation medium conditioned by cell starving for 20 h (CM) from sslA(CR11) or wild-type parental cells. As previously observed, wild-type CM causes a slight increase in group number compared to buffer alone (Table 1); sslA(CR11) CM also caused an increase in group number similar to that seen with wild-type CM. Compared to wild-type CM, smlA CM caused a significant increase in group number (3), as did smlA sslA(CR11) CM. However, there was no significant difference between smlA CM and smlA sslA(CR11) CM. In addition, Western blots of CM stained with anti-countin or anti-CF50 antibodies showed that sslA(CR11) cells secreted as much countin and CF50 as parental wild-type cells, and smlA smlA(CR11) cells secreted as much countin and CF50 as smlA cells (Fig. 3). Together, the bioassays and Western blots suggested that the sslA(CR11) mutation does not affect the accumulation of extracellular CF.

The second possible way that the sslA(CR11) mutation could cause cells to form larger groups is by affecting the ability to sense CF, and as described above, this can be crudely assessed in mixing experiments. Mixing wild-type cells 85:15 with smlA cells caused a large increase in the number of groups formed during development, indicating that the wild-type cells were sensitive to the CF oversecreted by smlA cells (Table 2). Mixing smlA sslA(CR11) cells 85:15 with smlA cells or mixing sslA(CR11) cells 85:15 with smlA cells had relatively little effect on the number of groups formed during development (Table 2). This suggested that smlA sslA(CR11) and sslA(CR11) cells were relatively insensitive to the presence of smlA cells. Recombinant countin and recombinant CF50

### TABLE 2. Effect of the presence of smlA cells on the number of groups formed by a cell line

| Cell line                | No. of groups |
|-------------------------|---------------|
|                         | Alone | With 15% smlA cells |
| Wild type               | 156 ± 26 | 228 ± 26 |
| sslA(CR11)              | 95 ± 10  | 100 ± 10 |
| smlA                    | 336 ± 26 | NA |
| smlA sslA(CR11)         | 165 ± 7  | 180 ± 15 |

*The indicated cell lines were starved on filter pads, and after 16 h, the number of groups was counted. Values are means ± standard deviations for three separate experiments. NA, not applicable. The effect of adding smlA cells is significant for wild-type cells (P < 0.05) but not significant for sslA(CR11) cells or smlA sslA(CR11) cells (two-way ANOVA, Bonferroni posttest).*

### TABLE 3. Differentiation of cells into CP2-positive and SP70-positive cells at low cell density

| Cell line               | % CP2-positive cells | % SP70-positive cells |
|-------------------------|---------------------|-----------------------|
| Wild type               | 11.4 ± 0.4          | 32.2 ± 1.1            |
| sslA(CR11)              | 12.1 ± 0.6          | 32.1 ± 0.7            |
| smlA                    | 11.7 ± 0.4          | 31.9 ± 0.5            |
| smlA sslA(CR11)         | 12.3 ± 0.4          | 32.9 ± 0.6            |

*Approximately 2,000 cells of each cell type were starved in duplicate wells of an eight-well slide, and cAMP was added 6 h later to induce accumulation of the SP70 and CP2 antigens. Eighteen hours after starvation, the cells were fixed, and the cells in one well were stained for CP2 while the cells in the other well were stained for SP70. The total number of cells in a well and the number of positive cells were counted. Values are means ± SEMs from three separate experiments. There was no significant difference in percent CP2-positive cells (or SP70-positive cells) between any two cell lines (one-way ANOVA, Tukey’s test).*
both decrease group size when added to developing wild-type cells (5, 17). To further examine whether sslA1(CR11) cells are sensitive to these components of CF, sslA1(CR11) and its parental cell line were starved in the presence of recombinant countin or CF50. As shown in Fig. 4, both proteins caused wild-type cells to form more (and thus smaller) groups. However, neither of these recombinant proteins significantly affected sslA1(CR11) group size or number. Although we cannot rule out the possibility that the sslA1(CR11) mutation also affects something other than the CF pathway, the data suggest that the sslA1(CR11) mutation makes cells insensitive to CF or the components of CF. This suggests that the mutation affects or overrides the CF signal transduction pathway.

Like countin- cells, sslA1(CR11) cells have a normal initial differentiation. Disruption of the genes encoding countin or CF50 causes the formation of abnormally large groups (4, 5). A clear difference between these transformants is that while countin- cells have a normal initial differentiation of cells expressing CP2 (a marker for an initial subset of prestalk cells) and SP70 (a marker for a subset of prespore cells), the differentiation of cf50- cells is abnormal, with a very low percentage of CP2-positive cells (5). Both the sslA1(CR11) and the sslA1/SssA1(CR11) cells showed normal percentages of cells expressing these markers (Table 3). By this criterion, the sslA1(CR11) mutation mimics the disruption of countin more than it mimics the effect of disrupting cf50.

SslA is present during early development. To determine when during development SslA is present, wild-type cells

![FIG. 5. SslA is present during early development. (A) A Western blot of total cell lysates from wild-type (WT) cells at the indicated times of development (in hours) was stained with anti-SslA antibodies. V indicates vegetative cells. (B) A Western blot of total cell lysate and 100×-concentrated 20-h CM from starved cells was stained with anti-SslA antibodies.](image-url)

FIG. 6. Subcellular distribution of SslA. Three-hour-starved wild-type cells were lysed and then fractionated by centrifugation. Nuclei and unbroken cells were pelletted with a low-speed spin, and the supernatant was then centrifuged at a medium (med) speed to pellet organelles. The supernatant from this spin was then centrifuged at high speed to pellet microsomes. Samples of the pellets (P) and supernatants (S) were boiled in Laemmli sample buffer and electrophoresed on an SDS-polyacrylamide gel. A Western blot of the gel was then stained with anti-SslA antibodies.

![FIG. 6. Subcellular distribution of SslA.](image-url)

![FIG. 7. sslA1(CR11) transformants have increased amounts of sslA mRNA and SslA proteins. (A) A Northern blot of total RNA from the indicated vegetative cells was probed with a fragment of sslA. The lower panel shows an ethidium bromide-stained gel of the RNA used for the Northern blot. WT, wild type. (B) Western blots of total cell lysates collected from the indicated cell lines at 0 and 3 h of development were stained with anti-SslA antibodies. On longer exposures (not shown), the SslA band was visible in the sslA- samples. The lower panels show aliquots of the protein samples electrophoresed on a duplicate gel, which was stained with Coomassie to verify that there was equal loading of protein. A small region of the gel is shown; on the entire gel, one could see that essentially all the bands were of the same intensity. (C) The same cell lines were starved for 3 h on filter pads, dissociated, allowed to adhere to a glass slide, fixed, and stained for SslA by immunofluorescence. On longer exposures (not shown), the sslA- cells showed staining with the anti-SslA antibodies. Bar, 20 μm.](image-url)
were starved on filter pads and harvested at different times. A Western blot stained with affinity-purified anti-SslA antibodies showed a band at 90 kDa (Fig. 5A). This molecular mass is higher than the mass predicted from both of the sslA cDNA sequences and could be due to posttranslational modification of SslA or anomalous migration of SslA on gels. There are many examples of proteins that migrate at anomalously high molecular masses on SDS-polyacrylamide gels (2, 33, 38, 55, 63). Besides the 90-kDa band, other bands occasionally appeared on Western blots stained with the anti-SslA antibodies. For unknown reasons, from experiment to experiment there were variable levels of SslA in vegetative cells (Fig. 5A and data not shown). The SslA levels were consistently high in early development and then significantly decreased after 10 h of starvation. SslA is undetectable in wild-type CM (Fig. 5B). The data thus suggest that SslA is not secreted and is present in cells at the time that streams form and begin to break up (approximately 6 h after starvation).

Subcellular localization of SslA. Since SslA is not secreted, we used cell fractionation as an initial way to determine its subcellular location. Three-hour-starved wild-type cells were lysed, and cell fractions were obtained by differential centrifugation. A Western blot (Fig. 6) shows that SslA is undetectable in the low-speed pellet (containing nuclei [10] and cytoskeletons), the medium-speed pellet (containing large organelles such as mitochondria [64, 65] and lysosomes [8, 9, 22, 59]), and the high-speed supernatant, which contains cytosolic proteins (21). SslA, however, is present in the high-speed pellet fraction (containing microsomes [52, 62], small vesicles [21], and ribosomes [45, 57]). The fractionation thus suggests that SslA is associated with small subcellular structures.

The sslA(CRII) mutation increases SslA levels. Northern blots were probed with a fragment of the sslA coding region to determine the effect of the insertion of the REMI vector into sslA1. The cDNA sequences indicate that sslA1 mRNA is about 2.3 kb and sslA2 mRNA is about 2.2 kb, which is difficult to separate on RNA gels. These bands were observed in RNA from wild-type and smlA/H11002 cells (Fig. 2A and 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRII) and smlA~ sslA1(CRII) cells (Fig. 7A). The cDNA sequences predict that the truncated sslA1 mRNA in cells with the sslA1(CRIII) insertion should be 1.5 kb, and strong hybridization to an RNA at this size was seen in RNA from sslA1(CRIII) and smlA~ sslA1(CRIII) cells (Fig. 7A). In addition, weak expression of an ~2.2-kb mRNA was also observed in cells with the sslA1(CRIII) insertion. This could be the sslA2 mRNA, as RT-PCR as well as RACE-PCR detected sslA2 mRNA in sslA1(CRIII) and smlA~ sslA1(CRIII) cells (data not shown). As shown in Fig. 7B, the sslA1(CRIII) insertion causes cells to express more SslA protein than their parental cell lines.

FIG. 8. Decreasing SslA protein levels by antisense correlates with a decrease in fruiting body size. (A) A Western blot of total cell lysates from 3-hour-developing wild-type (WT) and sslAas cells was stained with anti-SslA antibodies. The lower panel shows aliquots of the protein samples electrophoresed on a duplicate gel, which was stained with Coomassie to verify that there was equal loading of protein. (B) Immunofluorescence of 3-hour-developing wild-type and sslAas cells stained with anti-SslA antibodies. Bar, 20 μm. (C) Fruiting bodies of wild-type and sslAas cells. Bar, 0.5 mm.
However, there did not appear to be any change in the apparent molecular mass of SslA in sslA1 (CR11) or smlA/H11002 sslA1 (CR11) cells. Immunofluorescence using affinity-purified anti-SslA antibodies to stain cells starved for 3 h showed the presence of SslA in wild-type cells and much lower levels in smlA/H11002 cells (Fig. 7C). sslA1 (CR11) cells had levels of SslA roughly comparable to or higher than those seen in wild-type cells, while smlA− sslA1 (CR11) cells had levels of SslA that were comparable to those seen in wild-type cells and much higher than those seen in smlA− cells. Similar results were observed in vegetative cells and cells starved for 6 h (data not shown). For each cell line at each time point, immunofluorescence did not reveal any obvious variation in the amount of SslA per cell, and there was no obvious difference in the distribution of SslA for the different cell lines or time points. Together, the data indicate that the sslA1 (CR11) insertion causes cells to overexpress SslA2 or a large portion of SslA1.

**Decreasing SslA levels results in small fruiting bodies.** Since the sslA1(CR11) mutation causes increased levels of SslA and larger fruiting bodies, we examined the hypothesis that decreasing SslA levels might cause the formation of smaller fruiting bodies. Multiple attempts to replace the coding region of sslA1(CR11) with a selectable marker by homologous recombination were unsuccessful, as were attempts to overexpress SslA (data not shown). Therefore, to decrease the levels of SslA, we transformed wild-type cells with an sslAas construct. Antisense can be used to repress the expression of a protein such as discoidin I, which is encoded by three separate genes (12). A Western blot of total cell protein stained with anti-SslA indicated that there was less SslA protein in the sslAas cells than in wild-type cells (Fig. 8A). Immunofluorescence staining also indicated that the sslAas cells had less SslA protein (Fig. 8B). The sslAas cells formed smaller fruiting bodies than parental cells (Fig. 8C). This suggests that although SslA levels can differ within a range, excessively high or low levels of SslA may reduce viability. Together, the data indicated that compared to wild-type cells, sslAas cells have reduced levels of SslA protein and form small fruiting bodies, suggesting that SslA levels regulate fruiting body size.

**CF represses SslA levels.** To determine if CF affects SslA, cells secreting different amounts of CF activity were stained for SslA. Wild-type cells show punctate staining, with a higher concentration of the staining near one side of the cell (Fig. 9A). There was no significant cell-to-cell heterogeneity in the amount of staining. A similar but less intense staining pattern for SslA was observed in smlA− cells. countin− cells showed a much brighter staining, more widespread in the cell, than the wild type. Western blots showed that compared to those in wild-type cells, SslA protein levels were decreased in smlA− cells and increased in countin− and cf50− cells (Fig. 9B). When
added to cells, recombinant countin protein has many of the observed effects of semipurified CF, while adding anti-countin antibodies blocks CF activity (4, 17). To determine if altering countin levels can affect SslA levels, wild-type cells were starved in the presence of recombinant countin, preimmune sera, or anti-countin antibodies for 3 h. Treatment with recombinant countin decreased the levels of SslA in wild-type cells (Fig. 9C), while anti-countin antibodies increased SslA levels (Fig. 9D). Together, the data suggest that CF represses the amount of SslA in cells.

Glucose levels affect levels of SslA. CF appears to regulate cytosolic glucose levels, and these in turn affect adhesion and motility, suggesting that glucose levels are part of the CF signal transduction pathway (26). To determine whether SslA might be part of the CF-to-glucose signal transduction pathway, we measured the levels of internal glucose in smlA−, smlA− sslA1(CR11), wild-type, and sslA1(CR11) cells. As previously observed, smlA− cells have lower glucose levels than wild-type cells (26) (Fig. 10A and B). During vegetative growth and development, both smlA− sslA1(CR11) and sslA1(CR11) cells had glucose levels similar to those in their parental strains except at 2 h [smlA− sslA1(CR11)] and 4 h [sslA1(CR11)] (Fig. 10A and B). However, at 6 h of development, the internal glucose levels were essentially the same in transformants and

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FIG. 10. Altered SslA levels do not affect glucose levels, but glucose levels affect SslA levels. (A) sslA1(CR11) and parental wild-type (WT) cells were starved by shaking them in PBM and harvested at the times indicated, and the level of internal glucose was measured. (B) smlA− sslA1(CR11) and parental smlA− cells were starved by shaking them in PBM and harvested at the times indicated, and the level of internal glucose was measured. Values in panels A and B are means ± SEMs from three independent assays. A paired t test indicated that the values at 4 h in panel A were different at P values of 0.17, and the values at 2 h in panel B were different at P values of 0.15; the differences are thus not formally statistically significant. (C) Wild-type cells were grown in HL-5 medium with and without 1 mM glucose; then, cells from each treatment were starved in PBM buffer with or without 1 mM glucose. Samples were taken at 0 and 3 h of starvation, and a Western blot of the lysates was stained with anti-SslA antibodies.
their parental strains. Together, the data suggest that CF does not regulate glucose levels via regulation of SslA levels.

Growth and/or starvation in the presence of 1 mM glucose causes cells to express more of the gp24 adhesion protein and form larger groups (26). To determine if glucose levels regulate SslA levels, wild-type SslA cells were starved for 6 h in shaking culture with or without 1 mM glucose. The cells were then harvested and starved in either buffer alone or buffer with 1 mM glucose in shaking culture. A Western blot stained with anti-SslA antibodies showed that treatment with glucose did not change SslA protein expression in vegetative cells (Fig. 10C). However, the addition of glucose to developing cells increased the expression of SslA at 3 h, suggesting that glucose levels regulate SslA levels.

SslA regulates the cAMP-induced cAMP and cGMP pulses. Countin potentiates the cAMP-induced cAMP pulse and inhibits the cAMP-induced cGMP pulse, with glucose levels mediating the regulation of the cAMP but not the cGMP pulses (17, 26, 60). To determine if SslA levels affect the cAMP-stimulated cAMP and cGMP pulses, wild-type and sslA1(CR11) cells were starved for 6 h and stimulated with 10 μM 2′-deoxy-cAMP. In sslA1(CR11) cells, the cAMP-stimulated cAMP pulse size is decreased (Fig. 11A), similar to what was observed in countin cells and in cells treated with glucose. However, the decreased cGMP pulse in sslA1(CR11) cells (Fig. 11B) is different from the large cGMP pulse seen in countin cells and the delayed cGMP pulse seen in cells treated with glucose (26, 60). This indicates that the sslA1(CR11) mutation affects both the cAMP-stimulated cAMP and cGMP pulses.

SslA regulates adhesion and motility. CF affects group size by decreasing cell-cell adhesion and increasing motility (51, 61). To determine if SslA affects adhesion, cells were starved and adhesion was measured at different times during development. The adhesion of wild-type cells increases during development (40, 56) (Fig. 12A). The adhesions that we measured, especially during early development, were higher than what we previously observed for these cells in one set of experiments (51) but lower than what we observed approximately 2 years later (17). However, as we previously observed, the adhesion of smlA− cells was less than that of the wild-type cells (Fig. 12A). The adhesion of the sslA1(CR11) cells was essentially indistinguishable from that of the parental wild-type cells, while the adhesion of smlA− sslA1(CR11) cells was higher than that of smlAas cells but lower than that of wild-type cells (Fig. 12A). At 6 h after starvation, the motility of smlA− cells was higher than that of wild-type cells (Fig. 12A). SslA regulates adhesion and motility.

DISCUSSION

SslA may be a component of the CF signal transduction pathway. We used REMI to isolate second-site suppressors of smlA− (with the formation of larger fruiting bodies as the selection criterion) and found that one of these suppressors was due to an insertion in a novel gene, sslA1. There are two highly similar sslA genes encoding highly similar proteins, and the sslA1(CR11) insertion either increased the expression of a truncated SslA1, increased the expression of SslA2, or increased both.

SslA is expressed during development when streams are forming and breaking into groups. In a wild-type background, the sslA1(CR11) insertion causes the formation of larger fruiting bodies, suggesting that SslA regulates group size. In both wild-type and smlA− backgrounds, the sslA1(CR11) insertion does not affect the extracellular accumulation of CF activity or the CF components countin and CF50. This indicates that the sslA1(CR11) mutation either affects the CF signal transduction pathway or causes larger groups to form through alteration of a mechanism unrelated to the CF signal transduction pathway. Mixing experiments and exposing developing cells to recombinant countin or recombinant CF50 also indicated that either
the sslA1(CRI11) cells have defects in their CF signal transduction pathways or the sslA1(CRI11) insertion affects some mechanism which forms large groups in a way that overrides the CF signal transduction mechanism. smlA− sslA1(CRI11) and sslA1(CRI11) cells form groups that are smaller than those formed by countin− or cf50− cells, suggesting that the sslA1(CRI11) insertion does not cause a complete block of the CF signal transduction pathway.

**Amount of SslA positively affects group size.** The RT-PCR and RACE-PCR data indicate that both sslA1 and sslA2 are expressed as mRNAs, suggesting that the corresponding proteins are expressed. At the resolution of our Western blots, we observed a single band staining with anti-SslA antibodies in wild-type cell lysates, despite the fact that the predicted sizes of SslA1 and SslA2 are different. This suggests that one of the SslAs is not translated or is degraded or that both proteins are processed to molecules of similar electrophoretic mobilities. This band is larger than the predicted backbone size of either SslA, suggesting that there may be anomalous migration of SslA on gels or posttranslational modifications of SslA. Both SslA proteins have possible transmembrane domains, and the SslA band is associated with a fraction that includes small vesicles in agreement with the punctate distribution of SslA seen by deconvolution immunofluorescence. If SslA is a vesicle-associated transmembrane protein, it could be glycosylated. Both SslAs have potential N and O glycosylation sites. It is thus conceivable that glycosylation increased the apparent mass of SslA to 90 kDa.

In most transformants generated by REMI, the insertion of the vector DNA causes the encoded protein to be either not expressed or expressed as a truncated variant (49). In both
wild-type and smlA− backgrounds, the sslA1(CRI1) insertion caused expression of higher levels of SslA and increases in the sizes of groups. Conversely, decreasing SslA levels by antisense decreased group size. Our working hypothesis is thus that the level of SslA in cells positively affects group size (Fig. 13).

**CF decreases SslA levels.** Both immunofluorescence and Western blots indicated that smlA− cells have less SslA than parental cells, while countin+ cells have more. Adding recombinant countin to wild-type cells decreased SslA levels, while inhibiting CF with anti-countin antibodies increased SslA levels. This would suggest that if SslA does seem to have a major effect on glucose levels, while glucose positively affects group size (Fig. 13).

**SslA1 may be in a CF-regulated pathway between glucose and cAMP pulse size.** An unusual aspect of the CF signal transduction pathway is that it appears to involve levels of intracellular glucose (26). The sslA1(CRI1) mutation does not seem to have a major effect on glucose levels, while glucose levels increase SslA levels. This would suggest that if SslA is in a CF signal transduction pathway, the pathway involves glucose levels partially regulating SslA levels (Fig. 13).

**CF decreases the cAMP-stimulated cGMP pulse by regulating guanylyl cyclase and increases the cAMP pulse by regulating adenylyl cyclase (17, 60).** If SslA mediates the effects of CF on both the cGMP and cAMP pulses, we expect increased SslA levels to result in decreased cAMP and increased cGMP pulse sizes. However, our data show that increasing SslA levels results in reductions in both cAMP-induced cGMP and cAMP pulse sizes. A possible explanation is that the sslA1(CRI1) mutation causes an increased level of a truncated SslA protein, and this mimics the effect of increased levels of the endogenous SslA protein on the cAMP pulse but does not mimic the effect of increased levels of the endogenous SslA protein on the cGMP pulse. Alternatively, since SslA levels are regulated by glucose, and glucose appears to mediate the effects of CF on the cAMP pulse but not the cGMP pulse (26), SslA might be in a CF-glucose-cAMP pulse size pathway but not in the CF-cGMP pathway (Fig. 13).

Computer simulations predicted that increasing adhesion and/or decreasing motility would result in the formation of larger groups (51). Decreasing the cAMP pulse size, increasing adhesion, and decreasing motility all increase group size (29, 60, 61). The sslA1(CRI1) insertion caused a decrease in the cAMP pulse size, an increase in adhesion (although only in smlA− cells), and a decrease in motility. We hypothesize that this is why the sslA1(CRI1) and smlA− sslA1(CRI1) cells form larger groups than their respective parental cells.

CF, glucose, and cAMP pulses affect motility, and CF and glucose (but not cAMP pulses) affect adhesion (26, 51, 61). In wild-type cells, the sslA1(CRI1) insertion affects cAMP pulses and motility. This suggests that SslA might function downstream of CF and glucose and upstream of cAMP and motility in a pathway that affects group size. The sslA1(CRI1) insertion affects adhesion in smlA− but not wild-type cells, whereas CF and glucose do affect adhesion in wild-type cells. Together with the abnormal effect on the cAMP-stimulated cGMP pulse, this suggests that the sslA1(CRI1) insertion affects part, but not the entirety, of the CF signal transduction pathway. This in turn suggests that the multisubunit CF signal regulates group size through a branching pathway.

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