A cell-adhesion pathway regulates intercellular communication during *Dictyostelium* development

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Abstract

Cell adhesion molecules play an important physical role in shaping the structure of multicellular organisms. Recent studies show that they also play a role in intracellular and intercellular signaling. We describe a cell adhesion pathway that is mediated by the intercellular communication genes *comC*, *lagC*, and *lagD* during *Dictyostelium* development. Disruptions of these genes result in strains that are unable to generate spores when developed in a pure population but are capable of sporulation when developed in chimerae with wild-type cells. In contrast, any pair-wise chimera of the three mutants fails to form spores. We postulate that the wild-type cells supply the mutant cells with a signal that partially rescues their sporulation. We also propose that the three mutants are deficient in the production of that signal, suggesting that the three genes function in one signaling pathway. In support of that notion, the mutant cells share common non-cell-autonomous prespore and prestalk-specific defects and a common pattern of developmental progression and regression. We provide transcriptional and functional evidence for a network in which *comC* inhibits *lagC* and activates *lagD* expression, *lagC* and *lagD* are mutually inductive, and the cell adhesion gene *lagC* is the terminal node in this signaling network.

Keywords: Cell-cell adhesion; Intercellular communication; Dictyostelium development

Introduction

Cell-cell communication orchestrates the developmental process by which a mass of individual cells differentiates into a multicellular organism. As few as seven eukaryotic signaling pathways regulate development, each controlling the transcription of target genes during differentiation (Barolo and Posakony, 2002). Cell adhesion molecules mediate cell-cell and cell-intercellular matrix interactions that have been described in numerous structural processes. Recent work has demonstrated that adhesion molecules play a role in signaling as well (Juliano, 2002). For example, integrins are cell-adhesion molecules that have both direct and indirect roles in signaling. The direct role involves signaling through the focal adhesion kinase (FAK), MAP kinase, and Rho GTPase. The indirect roles involve the modulation of MAP kinase cascades and G protein-coupled receptor cascades by interaction of integrin with other membrane receptors (Juliano, 2002). Another example is the N-CAMs, a family of homophilic, Ca$$^{2+}$$-dependent cell adhesion molecules that function in neural cells. They have a role in signaling through FGF (fibroblast growth factor) and through PKA (cAMP-dependent protein kinase A) (Povlsen et al., 2003). Cadherins are probably the best known example of cell-adhesion/signaling molecules due to their interaction with the Wnt/wingless signaling cascade through competitive interactions with β-catenin, but also through direct signaling via the Rho family of GTPases (Yap and Kovacs, 2003). Other examples include the signaling roles of Ig-CAMs, selectins, and proteoglycans (Juliano, 2002).

In *Dictyostelium*, cell adhesion has been correlated to signaling in two cases. First, mutations in the cell adhesion gene *lagC* lead to non-cell-autonomous defects in cell type divergence (Dynes et al., 1994). Second, mutations in the
cell adhesion gene csaA result in an alteration in social behavior (Queller et al., 2003). These examples illustrate the role that cell adhesion molecules play in intercellular signaling. Dictyostelium is especially suitable for studying these roles because of the vast knowledge we have on adhesion and signaling (Coates and Harwood, 2001; Maeda et al., 1997). Because many eukaryotic signaling pathways have homologues in Dictyostelium (Aubry and Firtel, 1999), we examined the relationship between cell adhesion and communication in this model system.

During Dictyostelium development, cell communication coordinates the differentiation of 100,000 cells into a fruiting body. Intercellular interactions can be analyzed in chimera of wild-type and mutant strains (Sussman, 1954; Sussman and Lee, 1955). We have isolated mutant strains that develop spores in chimera but not in pure populations, pointing to defects in signal production (Kibler et al., 2003). Mutants that synergize with wild-type cells, but fail to synergize with each other, are presumed defective in elements of one signaling pathway. Here, we focus on three such genes, comC, lagC, and lagD.

The lagC gene has been described as being required for proper Dictyostelium development. lagC− development arrests at the loose aggregate stage, and the cells do not sporulate. They do not express the prespore gene cotC or the prestalk gene ecmA in a pure population, but express these genes in chimerae with wild-type cells. These findings first demonstrated that the lagC function is partially non-cell-autonomous, but failed to show that lagC− cells can sporulate in chimerae (Dynes et al., 1994).

lagC encodes gp150, a glycoprotein localized at the plasma membrane and at cell-cell contact sites (Geltosky et al., 1976, 1980; Wang et al., 2000). lagC mRNA and gp150 (LagC) are developmentally regulated, with high expression at 10 h and sustained expression until 18 h of development (Geltosky et al., 1979; Wang et al., 2000). LagC mediates Ca2+−independent cell-cell adhesion during aggregation and postaggregative stages and cell sorting during morphogenesis (Gao et al., 1992; Geltosky et al., 1979; Siu et al., 1983; Wang et al., 2000). Although LagC is an adhesion molecule, lagC− cells adhere to each other and aggregate if another cell adhesion mechanism, mediated by csaA (gp80), is intact (Wang et al., 2000). LagC is involved in heterophilic cell-cell adhesion through an unidentified, developmentally regulated receptor (Wang et al., 2000).

Our genetic screen revealed two strains that failed to synergize (sporulate in chimerae) with each other, lagC−, and comC−. We also mutated lagD, a lagC homologue, and observed that the mutants exhibit a similar non-cell-autonomous defect. lagD− cells fail to synergize with lagC− and comC−, suggesting that the three genes participate in one pathway. We describe the phenotypes of lagC−, lagD−, and comC− and provide evidence for a role of the genes in an intercellular signaling pathway.

Materials and methods

Strains and growth conditions

Dictyostelium discoideum strains: AX4 (wild type) (Knecht et al., 1986), TL1 (AX4 [cotBlacZ]), TL35 (AX4 [act15/lacZ]) (Shaulsky and Loomis, 1993), AK127 (Dynes et al., 1994), and lagC−[lagClagC] (kindly provided by C.H. Siu). Cells were grown and maintained as described (Kibler et al., 2003).

In the Dictyostelium nomenclature, lagC− indicates that the lagC gene is knocked out, LagC indicates the protein encoded by lagC, square brackets indicate a vector insertion, and a slash indicates a promoter/coding region fusion. For example, [cotBlacZ] means that the strain carries a vector in which the lacZ gene is expressed from the cotB promoter (http://dictybase.org/Nomenclature%20proposal.htm).

Development

Cells were washed with 20 mM potassium phosphate buffer, pH 7.1 (K/K2), resuspended in 20 mM KCl, 5 mM MgCl2, 9 mM K2HPO4, 13 mM KH2PO4, 0.3 mM streptomycin sulfate, pH 6.4 (PDF), deposited on nitrocellulose filters at a density of 3 × 106 cells/cm2, and developed.

For agar development, cells were washed, plated at 4.4 × 105 cells/cm2 on K/K2 buffered 1% agar and filmed with transmitted light for 24 h, using NIH Image 1.61 to record images every 5 min as integrated sums of 24 frames/s.

Mutagenesis and synergy

REMI mutagenesis and spore selection were performed as described (Kibler et al., 2003). For synergy, strains were mixed in equal proportions, codeveloped, spores were selected and plated on nutrient agar in association with bacteria as described (Kibler et al., 2003). Genotypes were determined from the plaque phenotypes. Assays were repeated at least three times.

Gene cloning and disruption

Genes were cloned as described and insertions verified by Southern blots and recapitulation into fresh hosts (Kuspa and Loomis, 1992; Shaulsky et al., 1996). lagD cDNA was cloned from a Lambda-Zap cDNA library (Shaullsky et al., 1995) and with a 5‘ RACE kit (GibcoBRL) according to the manufacturer’s protocol from wild-type RNA after 14 h of development. comC cDNA was cloned by 5′ RACE from wild-type RNA after 0–2 h of development. Sequences were assembled from the Dictyostelium Genome Project database and verified by sequencing.

The lagDEcoRI disruption vector: lagD DNA was PCR-amplified from cDNA clone CMP11_H01 (Van Driessche et al., 2002) using M13 primers, digested with EcoRI, cir
cularized, digested with BclI, and ligated to BamHI-linearized pBSRI (Shaulsky et al., 1996). Vector DNA was linearized with EcoRI and transformed to generate a lagD− strain by homologous recombination. A lagDCplasmid rescue product from the resulting Dictyostelium strain was used for subsequent disruptions.

The comEC EcoRI disruption vector: a PCR product was amplified from an EcoRI plasmid rescue of the IS277 allele with the primers: GCGGATCCAAATGTGTGTAGCCTG and GCGGATCCAGTTAGTTGGCCT (BamHI sites underlined), digested with BamHI, and ligated into pBSRI (Shaulsky et al., 1996). This vector deletes 3.8 kb of sequence 5’ to IS277 and was used for subsequent disruptions.

32P-labeled lagC, lagD (Fig. 1), cotB (Fosnaugh and Loomis, 1989), emcA (Jermyn et al., 1987), and cprD (Souza et al., 1998) probes were prepared as described (Shaulsky and Loomis, 1993). The lagC probe was digested from the pBluescriptlagC plasmid described below using HindIII.

**Dark-field microscopy**

Cells were developed for 4–6 h on agar with 1 mM caffeine (Sigma), illuminated from below with an opaque ring placed above the light source to generate a dark-field effect, and photographed every 30 s as an integrated sum of 6 frames/s for 30 min. Images were stacked, stacks were sliced to generate a new 2D image with the x-axis representing distance and the y-axis representing time. Pixel intensity over time was plotted. Variations in pixel intensity for at least 60 points were determined over time. Each slice was normalized to remove drift using a nonparametric regression and a single wavelength cosine function was fit through each slice by nonlinear least squares. Parameters from each fit were subjected to analysis of variance (ANOVA) using the genotype information.

**RNA assays**

RNA preparation and Northern blots were performed as described (Kibler et al., 2003). RNase preparation was performed with the RPAIII kit (Ambion) according to the manufacturer’s protocol. The 121-nucleotide comC-specific probe was PCR amplified from the 277EcoRI plasmid with a T7 oligonucleotide and the oligonucleotide TGGATGATG GGTAGTGATGC. The probe was labeled by in vitro transcription with 32P-UTP. Protected products were resolved on 5% polyacrylamide, 8 M urea gels. Gels were fixed in 40% methanol, 10% acetic acid, dried, and autoradiographed.

Expression arrays and data analysis were performed as described (Good et al., 2003; Kibler et al., 2003; Van Driessche et al., 2002). Briefly, we collected RNA samples at 2-h intervals from developing cells, measured the expression levels of each gene using an array of nearly 8,000 genes, and normalized them by division by the average expression of that gene throughout development and extracting the log2 of that ratio. To plot the similarity between a mutant and the wild type, we calculated the correlation between the expression levels of all the genes at each time point (x-axis) to the expression of the respective genes in the wild-type sample and summarized the results across all 8,000 genes. The most similar wild-type time point was plotted on the y-axis.

In situ RNA hybridization was performed as described (Escalante and Loomis, 1995; Shaulsky et al., 1996) with minor modifications (Kibler et al., 2003). Riboprobes are described in Fig. 1.

**β-Galactosidase reporters**

**lagC lacZ**

A 2066-bp fragment of the lagC promoter was PCR-amplified from genomic DNA with the primers: GCTCTA GAAATTGCAAAACAGACCCAGA (XhoI underlined) and GTCAAAGCTTGAGTGATATTATTTATTTTCCAT (HindIII underlined) and replaced the XhoI-HindIII actin15 promoter of pA15Gal (Shaulsky and Loomis, 1993).

**lagD lacZ**

A 710-bp fragment of the lagD promoter was PCR-amplified from genomic DNA with the primers: CAGTCT AGACAGTGTCTTTTCCCAGTG (XhoI underlined) and GTCAAAGCTTGATACATATTATTTATTTTCCAT (HindIII underlined) and replaced the actin15 promoter of pA15Gal as above.

**β-Galactosidase staining**

Cells were developed on filters, fixed, permeabilized, stained with X-gal, and counterstained with eosin Y (Shaulsky et al., 1995).

**Gene expression constructs**

act15 lagC

pBluescriptSK− was digested with HindIII and XhoI and ligated to linker 1, which provided an NcoI and a BglII site. Linker 1 was annealed from oligonucleotides: (1) AGCTTC CATGCGATGGGATCTGATTACAAAAAGATGATGAT GATAAAC; (2) TCGAGTTTATCATCATCATTTGTTTG. The plasmid was digested with NcoI and BglII and ligated to the 2.7-kb lagC coding sequence amplified from genomic DNA with primers: (1) CATGGCCATGGAAGAAAATAATATTACTG (NcoI underlined); (2) GAAGATCTAATTATTTATTTATTTTCCATGCA TGGGA (BglII underlined), generating pBluescriptlagC. The lagC coding sequence was excised with XhoI and partial HindIII and replaced the HindIII-XhoI lacZ sequence of pA15Gal.
act15/lagD

lagD DNA (2.8 kb) was PCR-amplified from a cDNA clone using primers: (1) CCCAAGCTTATATGGATTCTAATAATGTATTTTTTTTAATAC (HindIII underlined); (2) GAAGATCTATTGAATGCTTTTTAACCAGG (BgIII underlined), digested with HindIII-BgIII and ligated to a HindIII-XhoI digested pA15Gal, using a BgIII-XhoI linker.

lagD/lagD

A 1.2-kb Clal-HincII fragment containing the lagD promoter and the 5’ end of the coding sequence was digested from the lagDClaI plasmid. The 3’ end of the coding sequence was HincII-XhoI digested from the act15/lagD plasmid. Fragments were subcloned into a Clal-XhoI-digested pDXA3H (Manstein et al., 1995).

act15/comC

comC coding DNA was amplified from genomic DNA with 2 primer pairs across a common XbaI site. Product A was amplified with primers: (A1) CAGGGATCCATGATTAAAAATATTTATTTTTTATATTTC (BamHI underlined); (A2) CACATACACACAAACCCACAG and digested with BamHI-XbaI. Product B was amplified with primers: (B1) CTTGTAAATGTAATGTTTCCTAGC; (B2) CATCTCGAGATGATTGTGTATTACCACCATCAG (XhoI underlined) and digested with XhoI-XbaI. Products were ligated with BamHI-XhoI-digested pDXA3H.

Cell-cell adhesion

Assays were performed as described (Geltosky et al., 1979) with minor modifications in triplicate. Cells were developed for various times, resuspended in PBM (20 mM KH2PO4, 10 μM CaCl2, 1 mM MgCl2, pH 6.1) and 5 mM EDTA, and disaggregated by repeat pipetting. Cells were counted (initial count), shaker at 200 rpm, 22°C for 0, 20, 30, 40, and 60 min, and counted. The number of unaggregated cells was the sum of single cells and two-cell aggregates (final count). Cell-cell adhesion was defined as: (initial count – final count)/(initial count).

Cell-substrate adhesion

Cells were developed on filters for various times, harvested, and disaggregated. Approximately 500 cells in 50 μl were deposited on a microscope slide and incubated at room temperature for 15 min. Excess liquid was removed and cells were photographed and counted (initial number). Slides were dipped 20 times in PBM, and adhered cells photographed and counted (final number). Cell-substrate adhesion was defined as: (final number)/(initial number). Assays were repeated four times.

Cell motility

Motility was measured as described (Yuen et al., 1995) with minor modifications. Cells were developed for various times, harvested, and disaggregated as above. Cells were deposited on a microscope slide and allowed to settle for 10 min. Thirty cells were tracked for 30 min at room temperature by photographing at 15-s intervals. After every minute, the location of each cell was determined and the data analyzed to determine directional change, instantaneous velocity, and total distance traveled. Assays were performed in duplicate for each strain and time point.

cAMP chemotaxis

Vegetative cells were harvested and washed in K/K2, pelleted, and spotted (origin) on K/K2-buffered 1% Noble agar with or without 10 μM cAMP (Browning et al., 1995). Cell migration away from the origin was measured after 20 h of incubation at 22°C. Assays were performed in duplicate.

Results

comC, lagD, and lagD are communication genes

Two alleles of lagC were isolated in a screen for mutants that sporulate in chimerae with wild-type cells but not in pure populations (Kibler et al., 2003). The developmental morphology, sporulation, and the ability to synergize were identical to the previously described lagC– strain, AK127 (Dynes et al., 1994), which was used for subsequent analyses.

lagD encodes an 888-amino acid (aa) protein, with an N-terminal signal peptide and a C-terminal transmembrane domain (Dynes et al., 1994). Comparison of LagC to the sequence databases revealed high similarity to lagD (BAA84094). We renamed the gene lagD, in keeping with the Demerec nomenclature (Demerec et al., 1966) and the Dictyostelium gene naming convention. lagD encodes a predicted 895-aa protein, with an N-terminal signal peptide and a C-terminal transmembrane domain (Fig. 1A). LagC possesses two and LagD three potential IPT sequences, immunoglobulin-like folds found in the mammalian plexin and in the MET-receptor proteins (Coates and Harwood, 2001). One of the IPT domains is common to both proteins (Fig. 1B) and contains the predicted cell-cell adhesion domain of LagC (Siu, C.H., personal communication). LagC and LagD are 69% similar, 55% identical (Fig. 1B). We disrupted lagD at nucleotide 1356 (Fig. 1A).

comC (AA50843) encodes a predicted 1501-aa protein with an N-terminal signal peptide, a C-terminal transmembrane domain, and 14 EGF domains (Fig. 1C). It is most similar to the epidermal growth factor-related protein 1 (UEGF-1) from sea urchin, but since the similarity is mostly
located in the EGF repeat region, a functional similarity is not proposed. The original mutation (IS277, Fig. 1C) resulted from an insertion 4244 bp downstream of the translational start site. To create a null allele, we generated an insertion 710 nucleotides downstream of the start site with a deletion of 3.8 kb of coding sequence (ISCOMC, Fig. 1C).

The predicted signal peptides and transmembrane domains in the three proteins suggest membrane association. The EGF domains of ComC and the IPT domains of LagC and LagD implicate them in protein-protein interactions and in juxtacrine signaling.

Sporulation in chimerae

Isolating lagC in our screen was surprising because previous work showed that lagC− cells do not sporulate in chimerae with wild-type cells (Table 1).

comC−, lagC−, and lagD− cells are unable to sporulate in pure populations but sporulate fairly well in chimerae with wild-type cells (Table 1). In contrast, they do not sporulate in any pairwise combination, whereas most other strains isolated in our screen do synergize with each other and with these three mutants (Kibler et al., 2003; Table 1, and data not shown). It is also important to notice that the presence of mutant cells in the chimera had no measurable effect on the sporulation efficiency of the wild-type cells. If two strains synergize with the wild type but not with each other, they may be defective in elements of one pathway (Kibler et al., 2003; Sussman, 1954). We propose that comC, lagC, and lagD are elements of one pathway.

Aggregation

The aggregation morphologies of comC−, lagC−, and lagD− reveal remarkable differences between the strains (Fig. 2). After 10 h of starvation, wild-type cells exhibit a branched streaming pattern toward central aggregation points (Fig. 2, WT, 10 h). lagC− cells form wide streams that frequently break and form secondary centers before reaching the primary center (Fig. 2, lagC−, 10 h and movies in supplement). lagD− cells completely fail to stream (Fig. 2, lagD−, 10 h). comC− streams exhibit an elaborate branching pattern and are thinner than the wild-type streams (Fig. 2, comC−, 10 h). Thus, the three strains exhibit distinct morphologies 10 h after starvation and lagC− and comC− exhibit somewhat opposite phenotypes.

Later, lagC− and lagD− aggregates fail to rotate whereas comC− aggregates do rotate (see movies in supplement). Rotational movement may reflect cAMP relay (Dormann and Weijer, 2001; Siegert and Weijer, 1995), suggesting that comC− cells are capable of cAMP relay, whereas lagC− and lagD− cells display cAMP relay defects.

The terminal morphologies of lagC−, lagD−, and comC− are similar but distinct from the wild type. At 16–22 h, wild-type structures progress from fingers to culminants, but the mutant strains aggregate at 16 h and then disaggregate at 24 h (Fig. 2). The lagC− and comC− cells form loose aggregates before disaggregating. The lagD− cells aggregate and disperse repeatedly (Fig. 2, and movies in supplement). These observations are consistent with previous findings that lagC− cells cannot maintain a dominant aggregation center (Dynes et al., 1994; Sukumaran et al., 1998; Wang et al., 2000) and suggest that comC− and lagD− also fail to maintain aggregation centers. This indicates that the three mutants are defective in one signaling pathway that regulates aggregation.

Adhesion and motility

The streaming and aggregation-disaggregation phenotypes of the mutants may result from aberrant cell motility, cell-substrate adhesion, or cell-cell adhesion. We therefore measured these properties in the three strains. Other than a transient increase in cell-substrate adhesion in comC− cells, we did not detect differences between the mutants and the wild type (data not shown). Regarding the cell-cell adhesion assay, we did not test the mutations in a csaA− background, which was used to demonstrate the adhesion defect of lagC− (Wang et al., 2000).

cAMP wave propagation

To test whether the streaming defects result from defective cAMP responses, we recorded the dark-field optical density wave propagation of the three strains (Siegert and Weijer, 1989). Dark-field waves correspond to cAMP waves; the lighter the band, the higher the cAMP concentrations. After 20 h, all the strains migrated away at the origin and then migrate away, toward higher cAMP concentrations. Statistical analysis of the data shows that the wave period in lagC− cells is shorter compared to the wild type and faster than the wild-type waves and lagD− cells fail to generate waves altogether (Fig. 3B; movies in supplement). Statistical analysis of the data shows that the wave period in lagC− and comC− cells is short compared to the wild type (Fig. 3C).

The inability of lagD− cells to generate dark-field waves may be due to an inability to generate, to respond, or to propagate cAMP signals. To assess their response, we tested cAMP-chemotaxis on agar. In this assay, cells are spotted (origin) and starved on agar containing cAMP. As the cells develop, they generate a local gradient by degrading cAMP at the origin and then migrate away, toward higher cAMP concentrations. After 20 h, all the strains migrated away from the origin an average distance of 3.5 mm in the absence of cAMP and 6.5 mm in the presence of cAMP. There was no significant difference between the wild type
and the mutants (data not shown). Because lagD− cells chemotax normally toward cAMP, we suspect that their defect is either in generating or propagating the cAMP signals.

Global gene expression patterns

Microarrays detect gene expression on a genome scale and can be used as detailed phenotypes (Good et al., 2003; Hughes et al., 2000; Kibler et al., 2003; Van Driessche et al., 2002). To compare wild-type development to that of the three mutant strains, global gene expression at each mutant time point (x-axis) was compared to all of the wild-type

<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable spore production</th>
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<tr>
<td>lagC−</td>
<td>4.4 × 10^5 ± 2.0 × 10^5</td>
</tr>
<tr>
<td>lagD−</td>
<td>3.0 × 10^5 ± 4.3 × 10^4</td>
</tr>
<tr>
<td>comC−</td>
<td>2.1 × 10^6 ± 1.6 × 10^6</td>
</tr>
</tbody>
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Table 1
Synergy of mutant strains

*Viable spores produced by 5 × 10^7 cells in 1:1 mixes. Results are given as average and standard deviation of three replications.

*Less than 1 spore in 10^8 cells.
time points (y-axis) and the most similar time point was plotted. Comparing $lagC^-$ to the wild type we observed that the mutant developed with normal timing for the first 4 h, slowed down until 6 h, accelerated to normal timing by 8 h, regressed to the 4-h pattern at 10 h, accelerated to normal timing by 12 h, and regressed back to the 4-h pattern for the remainder of development (Fig. 4, $lagC^-$). $lagD^-$ cells exhibited a sinusoidal behavior; they developed to the 4-h pattern of wild-type gene expression and regressed to the vegetative pattern thrice before progressing to the 6-h pattern at the end of the experiment (Fig. 4, $lagD^-$). $comC^-$ cells developed with accelerated timing for the first 4 h, where their transcriptional pattern remained unchanged until 18 h. At that time they briefly regressed to the 4-h wild-type pattern and then progressed to the 8-h wild-type pattern (Fig. 4, $comC^-$). These results, indicating that the three strains experience waves of developmental progression and regression, are consistent with the observation of aggregation and disaggregation shown in Fig. 2. Analysis of specific marker genes serves to sharpen these observations.

**Developmental initiation**

To test whether the mutant cells enter development with proper timing, we analyzed the expression of the vegetative cysteine protease gene $cprD$ (Souza et al., 1998). Growing
wild-type cells express \( \text{cprD} \), and expression is greatly reduced by 8 h of development (Fig. 5A). Overexposure of the blot revealed an upregulation of \( \text{cprD} \) to about 10% of its vegetative level after 14–20 h and a subsequent decay thereafter (Fig. 5B). \( \text{comC}^-/\text{H11002}, \text{lagC}^-/\text{H11002}, \text{and lagD}^-/\text{H11002} \) cells express \( \text{cprD} \) during growth and exhibit a downregulation after 8 h (Fig. 5C–E). Therefore, they properly transition between growth and development. Unlike wild type, however, the mutants express high levels of \( \text{cprD} \) after 14 h, equal or higher than the intensity of the vegetative expression (Fig. 5C–E). This finding is consistent with the aggregation-disaggregation observation and with the global gene expression data (Figs. 2 and 4, respectively). It confirms the published results on \( \text{lagC}^- \), suggests that \( \text{lagD}^- \) and \( \text{comC}^- \) cells have a similar defect, and indicates that the mutants experience waves of differentiation and dedifferentiation.

**Cell type differentiation**

In wild-type cells, the prespore gene \( \text{cotB} \) is induced at 8 h and the prestalk gene \( \text{ecmA} \) at 16 h (Fig. 5A). \( \text{lagD}^- \) fails to express either gene, indicating a failure to undergo cell-type differentiation (Fig. 5D). \( \text{lagC}^- \) and \( \text{comC}^- \) fail to express \( \text{ecmA} \), but express very low levels of \( \text{cotB} \) at 10–14 h (Fig. 5C and E). These findings were confirmed by X-gal staining of \( \text{cotB}/\text{lacZ} \) and \( \text{ecmA}/\text{lacZ} \)-marked mutants (data...
not shown). They are consistent with previous work, which showed that lagC− cells are unable to express ecmA (Dynes et al., 1994), but show that the cells differentiate as prespore cells, albeit in a limited way. The defects in expressing markers of cell-type differentiation indicate that comC−, lagC−, and lagD− mutants share defects in cell-type specification, supporting the hypothesis that they participate in one pathway.

Although pure populations of comC−, lagC−, and lagD− cells are unable to differentiate spores, they sporulate in chimerae with wild-type cells (Table 1). We tested whether chimeric development also rescues the stalk defects. Fig. 6A shows that in chimerae with wild-type cells, lacZ-marked lagD− cells also occupy the lower sorus in chimerae with wild-type cells (Fig. 6B). These data are consistent with the notion of multiple prespore compartments (Kibler et al., 2003), although they may not reflect a defect in signaling but rather in cell-cell adhesion. The shared cell-autonomous, anterior prespore-zone defect of lagC− and lagD− cells suggests additional functional similarities between the genes.

**Spatial expression of lagC and lagD**

We followed the expression of lagC and lagD during development by in situ hybridization and by using β-galactosidase reporters (comC is not expressed during late development). The in situ results represent the mRNA steady-state levels, and the stable β-galactosidase reporter reveals the developmental history of the cells (Fig. 7). lagC is expressed as a collar below the tip of the finger (PST-O) at 16 h and in the top (funnel) of the stalk tube during culmination (Fig. 7A). The lagC/lacZ expression patterns support these in situ results. At 14 h, staining is found throughout the prestalk region (Fig. 7B, 8 h). The tight aggregates exhibit homogenous staining (12 h), and at 16–24 h (Fig. 7B) the staining is enriched in the PST-O region, consistent with the in situ data. lagD expression is enriched in all prestalk cells at 16 h and in the PST-O region at culmination (Fig. 7C). The lagD/lacZ expression patterns support these in situ results. At 14 h, staining is found throughout the prestalk region (Fig. 7D, 14–16 h). During terminal differentiation, lagD becomes enriched in...
In conclusion, lagC and lagD expression become prestalk enriched after aggregation with partially overlapping distributions. These findings suggest that the proteins have the potential to perform a common function, but also the possibility that they may participate in distinct processes.

Regulatory relationships

A possible explanation for the lack of synergy between the mutants is that one gene may be necessary for the expression of the others. To test that, we measured the expression of each gene in the absence of the others. Our results support a network in which comC inhibits lagC expression and induces lagD expression and lagC and lagD are mutually inductive (Fig. 8A). The following data support this model.

**comC expression**

comC mRNA was measured by an RNase protection assay (Fig. 8B). The transcript is evident in samples from wild-type cells at 0–2 h of development and greatly reduced thereafter (Fig. 8B, WT). comC− cells lack this transcript,
Fig. 7. Cell type specificity of lagC and lagD. (A) In situ RNA hybridization with an antisense probe against lagC. Staining is evident in the PST-O region (16 h) and in the funnel (20 h) (arrows). (B) Wild-type cells expressing lagC/lacZ were developed in pure populations. Whole mounts were stained with X-gal (blue). Arrows indicate the PST-O zone. (C) In situ RNA hybridization with an antisense probe against lagD (Fig. 1). Staining is evident throughout the prestalk region (16 h) and at the PST-O zone (20 h) (arrows). (D) Wild-type cells expressing lagD/lacZ were developed on filters in pure populations and stained with X-gal (blue). Arrows indicate increased staining in the prestalk zone (16 h) and in the upper cup (UC), lower cup (LC), and stalk tube (ST) (20–24 h).
with maximal expression at 8–12 h, no expression at 14–20 h, and moderate expression at 22–24 h (Fig. 8C, lagD–), indicating that lagD is a positive regulator of lagC (Fig. 8A, iii). comC– cells express higher than wild-type levels of lagC. They also begin to express lagC earlier than wild-type cells, with levels detectable as early as 2 h and sustained up to 16 h, peaking at 8–12 h (Fig. 8C, comC–). These results suggest that comC negatively regulates lagC (Fig. 8A, ii).

**lagD expression**

lagD mRNA is detectable in wild-type cells between 10 and 24 h of development, peaking at 14–16 h (Fig. 8C, WT). All three mutants fail to express lagD (Fig. 8C), indicating that comC and lagC are required for lagD expression (Fig. 8A, i, iv) and that the lagD– mutant is a null. The finding that lagD regulates the expression of lagC is somewhat surprising because lagC is induced before lagD. We suspect that lagD and perhaps lagC are expressed at earlier stages as well, but their level of expression is below the limit of detection.

**lagD activates lagC**

The model (Fig. 8A, iii) predicts that overexpression of lagD should upregulate lagC. This prediction is fulfilled as lagD–[lagD/lagD] cells exhibit elevated levels of lagC (Fig. 8E).

**Functional relationships**

To further test our model, we examined the functional relationships between the genes. The lagC–lagD– double mutant cells are more similar to lagC– than to lagD– by two criteria. First, lagC–lagD– cells stream and form aggregates (Fig. 9A). The streams are similar to those of lagC–, whereas lagD– cells do not stream at all (Fig. 2). Second, lagC–lagD– cells generate optical density waves (Fig. 9B), lagD– cells do not generate optical density waves, whereas lagC– cells do (Fig. 3). If two mutations confer different phenotypes, and the double mutant takes the phenotype of one of them, that one is considered epistatic (Avery and Wasserman, 1992; Zupan et al., 2003). Accordingly, lagC is epistatic to (acts downstream of) lagD. Therefore, we place lagC as the terminal node between the pathway and the developmental phenotype (Fig. 8A, v).

The relationships between lagC and lagD were confirmed by cross-complementation. We predicted that overexpression of one gene in the absence of the other would reveal the downstream gene, if the resulting strain were a phenotypic rescue of the host defect. Ectopic expression of lagC in a lagD– background rescues the developmental morphology because lagD–[act15/lagC] cells generate fruiting bodies and viable spores (Fig. 9C). The reverse experiment tested the possibility that the genes perform

![Image](50x55)
partially overlapping functions. Because ectopic lagD expression in a lagC− strain fails to rescue the lagC− phenotype (Fig. 9D), we conclude that lagD expression cannot compensate for the lack of lagC expression, arguing against a functional overlap between the two genes and in favor of the epistasis model (Fig. 8A, iii). To test whether the lagD/ lagD construct was effective, we expressed it in lagD/ H11002 cells and found that it rescues the lagD− phenotype. The lagD− [lagD/lagD] cells stream, aggregate, and form normal fruiting bodies (Fig. 9E), indicating that the lagD expression construct is effective. We also found that the lagD− [lagD/ lagD] express the transgene during growth, when lagC is not expressed, indicating that the high-copy number lagD/ lagD vector overcomes the lagC-dependence of the lagD promoter (data not shown). These data support the hypothesis that lagC and lagD positively regulate each other’s expression (Fig. 8A, iii, iv) and that lagC is the known terminal element in the pathway.

Fig. 9. Functional relationships between comC, lagC, and lagD. (A) Top view of lagC− lagD− cells after 10 h of agar development. Bar = 2 mm. (B) Optical density waves of lagC− lagD− cells (see Fig. 3B for detail). (C–E) Side view of lagD− [act15/lagC] (C), lagC− [lagD/lagD] (D), and lagD− [lagD/lagD] (E) cells after 24 h of filter development. Bar = 1 mm. (F–I) Top view of comC− lagC− (F), comC− (G), lagC− [lagC/lagC] (H), and lagD− [act15/lagC] (I) cells after 10 h of agar development. Bar = 2 mm.
We tested the model’s predictions regarding the regulatory role of \textit{comC} on lagC and lagD by generating a \textit{comC\textsuperscript{−}lagC\textsuperscript{−}} double mutant. The strain generates streams that are similar to the \textit{lagC\textsuperscript{−}} and different from the \textit{comC\textsuperscript{−}} streams (Fig. 9F, Fig. 2, and Fig. 9G, respectively), indicating that \textit{lagC} is epistatic to \textit{comC} (Fig. 8A, ii).

Finally, the expression data in Fig. 8C suggested that \textit{lagC} overexpression should mimic the \textit{comC\textsuperscript{−}} phenotype because \textit{comC\textsuperscript{−}} cells overexpress \textit{lagC}. This prediction is also fulfilled. The streaming phenotypes of \textit{comC\textsuperscript{−}} cells, \textit{lagC\textsuperscript{−}[lagC/lagC]}, \textit{lagD\textsuperscript{−}[lagD/lagD]}, and \textit{lagD\textsuperscript{−}[act15/lagC]} are similar (Fig. 9G, H, and I, and data not shown, respectively), each exhibiting elaborate streams with intricate branching patterns. These data support the hypotheses that \textit{comC} downregulates \textit{lagC} and that \textit{lagD} upregulates \textit{lagC} (Fig. 8A ii and iii, respectively).

**Discussion**

Several lines of evidence indicate that \textit{comC}, \textit{lagC}, and \textit{lagD} act in a common signaling pathway. Development of \textit{comC\textsuperscript{−}}, \textit{lagC\textsuperscript{−}}, and \textit{lagD\textsuperscript{−}} mutants arrests at the loose aggregate stage and is followed by disaggregation. The cells fail to sporulate in pure populations or in chimerae with each other, but sporulate fairly well when codeveloped with wild-type cells. In our model that describes the interactions between the three genes, \textit{comC} functions early, inhibits \textit{lagC}, and activates \textit{lagD}. \textit{lagC} and \textit{lagD} are mutually inductive. \textit{lagC} is the terminal node, epistatic to both \textit{comC} and \textit{lagD}, because all of the \textit{comC} and \textit{lagD} phenotypes are accounted for by altered expression of \textit{lagC}.

Formally, the relationship between the genes may be due to a “dependent sequence” effect. In a dependent sequence, completion of an early developmental stage, such as aggregation, may be essential for the manifestation of a later stage, such as spore formation, but early expressed genes do not necessarily regulate the activity of late expressed genes (Loomis et al., 1976). The microarray data would support that argument because inactivation of either one of the genes leads to transcriptional changes on a genomic scale, so the transcriptional dependence alone is insufficient to support the model. However, two functional experiments provide strong support for the model. First, the lack of synergy between the null mutants indicates that the genes are members of one pathway. Second, the epistasis data provide compelling support for the model and for the ordering of gene action. These data show that despite the vast changes in gene expression caused by the alteration of one gene (e.g., inactivation of \textit{lagD}), alteration of only one additional gene (e.g., activation of \textit{lagC}) can restore the wild-type phenotype. All of the relationships in the model are supported by expression and by epistasis data, leading us to propose that the model reflects functional relationships rather than a dependent sequence effect.

Although the genes control each other’s expression, they do not encode classical transcription factors, so they probably regulate each other through other molecules. One candidate is the G box-binding factor GBF, which is required for the expression of all postaggregative genes and implicated in \textit{lagC} signaling (Sukumaran et al., 1998). The early expression of \textit{comC} places it at the top of the network. Although \textit{comC} expression is detected during growth and early development, the \textit{comC\textsuperscript{−}} developmental defects are mostly evident at later stages, with no obvious growth defects. We propose that \textit{comC} functions during the transition from growth to development.

The sequence similarity between LagC and LagD suggests that the proteins perform similar functions, probably signaling through cell-cell adhesion. First, the morphological phenotype suggests that \textit{lagC} and \textit{lagD} function early in aggregation and during postaggregative development. Second, we propose that LagC and LagD play roles at multiple stages of development because their transcripts assume a prestalk-specific pattern at later stages. The early diffuse expression pattern followed by prestalk enrichment suggests that these proteins confer differential cell-type adhesion.

LagC mediates cell adhesion through heterophilic interactions, but its binding partner is unknown (Wang et al., 2000). The LagC and LagD protein sequences are similar around the predicted protein-protein binding domain, but the actual binding domains are different. This is consistent with a possible heterophilic interaction between the proteins. If LagC and LagD were binding partners, cells defective in one would bind to cells defective in the other and rescue each other’s development. Instead, \textit{lagC\textsuperscript{−}} and \textit{lagD\textsuperscript{−}} cells fail to rescue each other’s defects in chimeric mixtures, suggesting the opposite. However, this simple interpretation may be incorrect. The inability of the mutants to synergize may reflect the fact that \textit{lagC} and \textit{lagD} are required for each other’s expression, so each mutant fails to express both genes. Therefore, it is possible that LagC and LagD are heterophilic binding partners.

Our model proposes that LagD regulates \textit{lagC} expression yet \textit{lagC} mRNA is detectable before \textit{lagD} mRNA. In addition, the morphological defect of \textit{lagC\textsuperscript{−}} cells appears less severe than the defect of \textit{lagD\textsuperscript{−}} cells and both defects occur before the transcripts are first observed. The most likely explanation is that both genes are expressed early in development but their expression is below our limit of detection.

Earlier studies revealed that different cell types express LagC at different levels with a bimodal pattern of expression during terminal differentiation (Geltosky et al., 1979, 1980). This differential expression may facilitate cell sorting during morphogenesis (Braga and Harwood, 2001; Siu et al., 1988). The prestalk-enriched expression of \textit{lagC} and \textit{lagD} is supportive of that notion.

LagC is a cell-cell adhesion molecule, but it is dispensable for cell adhesion, as \textit{lagC\textsuperscript{−}} cells are adhesive due to the function of gp24 (\textit{cada}) and gp80 (\textit{csaa}) (Wang et al., 2000). Our studies stress the role of \textit{lagC} in communication and add to a growing body of evidence for the role of
adhesion molecules in communication (Juliano, 2002). In light of the communication roles of comC, lagC, and lagD, we propose that the adhesion function of LagC may be secondary to its communication function.

Perturbations in comC, lagC, and lagD result in strains that are unable to progress beyond the loose aggregate stage. While wild-type cells aggregate and form fruiting bodies, the mutant mounds disperse and sometimes reaggregate. The aggregation-disaggregation of lagC\textsuperscript{−}, lagD\textsuperscript{−}, and comC\textsuperscript{−} cells is also reflected in the gene expression data. As the mutants aggregate, cprD expression decreases; when they disperse, cprD gene expression increases. This is also reflected in the sinusoidal nature of the microarray phenotype. The dedifferentiation and redifferentiation of the mutant strains suggest an interesting plasticity in Dictyostelium development. In wild-type cells, a switch from differentiation to dedifferentiation and redifferentiation is made when cells are prevented from completing the entire developmental program. For instance, when portions of a developing slug are removed, the cells regulate, dedifferentiate, and redifferentiate, and regenerate the proper proportions of prestalk and prespore cells (MacWilliams and Bonner, 1979). It is possible that the aggregation-dispersion phenomenon observed in comC\textsuperscript{−}, lagC\textsuperscript{−}, and lagD\textsuperscript{−} cells results from a failure to progress beyond developmental check points, inducing these strains to backtrack along the developmental program.

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References