Disruption of Aldehyde Reductase Increases Group Size in Dictyostelium*

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Developing Dictyostelium cells form structures containing ~20,000 cells. The size regulation mechanism involves a secreted counting factor (CF) repressing cytosolic glucose levels. Glucose or a glucose metabolite affects cell-cell adhesion and motility; these in turn affect whether a group stays together, loses cells, or even breaks up. NADPH-coupled aldehyde reductase reduces a wide variety of aldehydes to the corresponding alcohols, including converting glucose to sorbitol. The levels of this enzyme previously appeared to be regulated by CF. We find that disrupting alrA, the gene encoding aldehyde reductase, results in the loss of alrA mRNA and AlrA protein and a decrease in the ability of cell lysates to reduce both glyceraldehyde and glucose in an NADPH-coupled reaction. Counterintuitively, alrA−/− cells grow normally and have decreased glucose levels compared with parental cells. The alrA−/− cells form long unbroken streams and huge groups. Expression of AlrA in alrA−/− cells causes cells to form normal fruiting bodies, indicating that AlrA affects group size. alrA−/− cells have normal adhesion but a reduced motility, and computer simulations suggest that this could indeed result in the formation of large groups. alrA−/− cells secrete low levels of countin and CF50, two components of CF, and this could partially account for why alrA−/− cells form large groups. alrA−/− cells are responsive to CF and are partially responsive to recombinant countin and CF50, suggesting that disrupting alrA inhibits but does not completely block the CF signal transduction pathway. Gas chromatography/mass spectroscopy indicates that the concentrations of several metabolites are altered in alrA−/− cells, suggesting that the Dictyostelium aldehyde reductase affects several metabolic pathways in addition to converting glucose to sorbitol. Together, our data suggest that disrupting alrA affects CF secretion, causes many effects on cellular metabolism, and has a major effect on group size.

A fascinating but poorly understood area of biology is how cells create tissues of a specific size (1–4). A simple model system for this phenomenon is the formation of fruiting bodies in the eukaryote Dictyostelium discoideum, where developing cells form groups of ~20,000 cells (see Refs. 5–10 for a review). Dictyostelium normally lives as individual cells that eat bacteria on soil surfaces. As the cells overgrow the bacteria, they starve. The cells then differentiate into either stalk or spore cells and cooperatively form fruiting bodies consisting of a thin, 1–2-mm-high stalk supporting a mass of spores, with the goal of allowing spores to be dispersed by the wind to new patches of soil and a new source of bacteria. For optimal spore dispersal, the fruiting body needs to be as large as possible, with an upper limit dictated by the ability of the stalk to support the spore mass without collapsing or the spore mass sliding down the stalk. Dictyostelium thus have evolved a mechanism to maintain an upper limit to the size of the group of cells that will form an individual fruiting body.

Much of the development of Dictyostelium appears to be regulated by secreted factors. When a cell begins starving, it signals to other cells that it is starving by secreting a glycoprotein called conditioned medium factor (11–13). As the relative density of starving cells increases, the conditioned medium factor concentration concomitantly increases (14). When the conditioned medium factor concentration passes a threshold concentration, indicating to the population that there is a high density of starving cells, the cells begin aggregating using delayed pulses of extracellular cAMP as a chemotactant (15–18).

The aggregating cells form streams that flow toward an aggregation center. If there are more than ~20,000 cells in an aggregation stream, the streams break into groups (19). To elucidate how cells sense the presence of too many cells in a stream and how the subsequent morphogenetic reformation occurs, we isolated a shotgun antisense transfectant called smlAas that formed very small fruiting bodies due to excessive stream breakup (20). This transfectant, as well as smlA−/− cells where the corresponding gene was disrupted by homologous recombination, appeared to be oversecreting a factor that when added to starving wild-type cells caused them to form small groups (21). Using this as a bioassay, we partially purified the factor and found that it was a 450-kDa complex of polypeptides we named counting factor (CF)1 (21). Disruption of countin, a gene encoding one of the subunits of CF, resulted in cells that formed large fruiting bodies due to streams staying intact and coalescing into large groups (22). Diffusion calculations based on the observed accumulation rate of CF indicated that in general the concentration of a secreted factor such as CF could

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The abbreviations used are: CF, counting factor; contig, group of overlapping clones; CM, conditioned starvation medium; MES, 2-[N-morpholinol]ethanesulfonic acid.

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be used to indicate to cells the number of cells in a stream or group (22, 23). Computer simulations of a stream of cells indicated that decreasing cell-cell adhesion and/or increasing random cell motility would cause a stream to dissipate and subsequently fragment (24). If the adhesion then increased or the random motility decreased, the simulations predicted that the dissipated cells would coalesce into a series of groups rather than a single stream. The simulations also predicted that if the adhesion and/or motility were regulated by a secreted factor such as CF, the resulting feedback would allow a very precise control of group size.

We found that CF decreases cell-cell adhesion and increases cell motility (24, 25). Decreasing adhesion causes the formation of smaller groups, whereas increasing adhesion or decreasing motility causes the formation of larger groups (24–27). Together, the observations suggested that, as predicted by the computer simulations, CF regulates group size by regulating adhesion and motility so as to cause a large stream or group to dissipate.

The expression of adhesion molecules and cell motility are regulated by the CAMP pulses that mediate chemotaxis (28–32). CF potentiates the CAMP-stimulated CAMP pulse and represses a CAMP-induced cGMP pulse (33). The signal transduction pathway that allows CF to regulate CAMP signaling, adhesion, and motility is unknown. High levels of glucose cause cells to form large fruiting bodies (34). CF appears to decrease intracellular glucose levels (35). Increasing glucose levels by adding exogenous glucose negates the effect of CF on group size and mimics the effect of decreasing CF on the CAMP-stimulated CAMP pulse, adhesion, and motility. This suggested that CF might regulate glucose levels to regulate group size.

Two-dimensional gels of aggregating cells showed a prominent spot that seemed to be most intense in *contiu* cells and least intense in smIA* cells (25). Two amino acid sequences obtained from tryptic peptides were used to identify sequence fragments from the *Dictyostelium* sequencing project. When these fragments were assembled, an open reading frame was identified that coded for a predicted protein with strong similarity to aldehyde reductase. The closely related protein aldose reductase reduces a wide variety of aldehydes, and their exact functions within cells are still unknown. Aldose reductase is thought to be responsible for some of the complications of diabetes such as neuropathy, cataracts, and retinopathy (37, 38). The high levels of glucose in diabetics cause the production of high levels of sorbitol and the conversion of glucose and NADPH to sorbitol and NADP⁺ (36). Both aldose reductase and aldehyde reductase convert glucose and NADPH to sorbitol and NADP⁺ (36). The left arm primers were TCGCGCACTGCTGGTTCCTGATTTAC, which added a SacII restriction site and the last 19 nucleotides of the coding region and the termination codon of *alrA* in pAlrAOE was then sequenced to verify that the new vector was created.

**MATERIALS AND METHODS**

Sequence Assembly—Preliminary sequence data were obtained from the *Dictyostelium* BLAST site (available on the World Wide Web at dictydb.wustl.edu) using the raw reads and contigs provided by the Baylor Sequencing Center and the Institute of Biochemistry (Cologne, Germany) together with the Institute of Molecular Biotechnology (Jena, Germany) and the EUDICT consortium. Sequences were assembled and analyzed using software from the Genetics Computer Group (Madison, WI).

*Genomic DNA Extraction—* Two million *Dictyostelium* vegetative cells were collected by centrifugation and resuspended in 0.5 ml of GL buffer (120 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5% SDS). 5 μl of an RNAse mixture (50 units/ml RNAse A and 100 units/ml RNAse T1) (Roche Applied Science) was added to the cells and incubated for 20 min at 55°C. 50 μl of 10 mg/ml proteinase K (Roche Applied Science) was added, and the mixture was incubated for an additional 2 h at 55°C. An equal volume of 1 ml Tris (pH 8.0)-buffered phenol was added, and the mixture was gently vortexed. After centrifugation at 10,000 × g for 5 min, the aqueous phase was similarly treated with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The aqueous phase was mixed with 0.2 volumes of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol alcohol. After centrifugation at 13,000 × g for 15 min, the pellet was washed with 70% ethanol, dried, and resuspended in 50 μl of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0).

Disruption of alrA by Homologous Recombination—PCR was done using Ax4 genomic DNA as a template to create DNA fragments flanking the aldehyde reductase gene. The left arm primer was GGGGGCCTCAGGCGAATTCAAAAGTTCGAACAC, adding a HincII site, and the right arm primer was GGTACCAGGGGCGAGCTTGTAACAGGGG, adding an Apal restriction site. This generated a 1.2-kb fragment. The backbone of the construct used to generate the aldehyde reductase knockout was pBB, pBlueScript SK (+) (Strategene, La Jolla, CA) containing a 1.4-kb bacterial resistance cassette inserted into the XbaI-HindIII sites (39, 40). The left arm fragment generated by the above PCR was digested with SacII and Eagl and the right arm fragment was digested with HincII and Apal. The fragments were then ligated into the corresponding sites of pBB to generate pARKO. The SacII-Apal fragment of pARKO was then used to transform Ax4 cells, and asparagine-resistant cells were selected on 10 μg/ml G418 and then checked to verify that disrupted *alrA* was introduced. Expression of AlrA in *alrA* cells—To express AlrA in *Dictyostelium* cells, the expression vector pDXA3-C (42) was altered by Dr. William Deere to remove the ATG upstream of the DNA insertion site. The sequence of the altered vector from the HindIII site (underlined) to the KpnI site was 5′-AAGCTTGAAGTTGCTCTCGAT-3′, and this vector was designated pDXA3-D. A SMART RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) was used to generate cDNA from *Ax4* RNA. This cDNA was then used to obtain cDNA encoding the aldehyde reductase protein using the forward primer 5′-GGGTTTACCCATGACACCATCT-3′ (containing a KpnI restriction site and the first 23 nucleotides of the coding region of *alrA*) and the reverse primer 5′-CTGGGAACTTTAGGAACTTACCT-3′ (containing an Xhol restriction site and the last 19 nucleotides of the coding region and the termination codon of *alrA*). This cDNA was digested with KpnI and Xhol and ligated into the similarly digested vector. The cDNA in the resultant vector pAlrAOE was then sequenced to verify that there were no errors. *alrA* cells were then transformed with pAlrAOE and the helper plasmid pREP following Manstein et al. (42), and transformants were selected with 2.5 μg/ml G418 and then checked to verify that disrupted *alrA* was introduced.
The amino acid sequence of the putative aldehyde reductase. Underlines indicate the sequences of the two tryptic peptides obtained from the CF-regulated spot on two-dimensional gels of 6-h starved cells. A gray box indicates the peptide used as an antigen for antibody production. Nucleotide sequence data are available in the Third Party Annotation section of the DDBJ/EMBL/GenBankTM data bases under the accession number TPA: BK001032.

There are four identified Dictyostelium genes encoding putative proteins with similarities to AlrA

The sequence identification from the Dictyostelium sequencing project and the tentative identification are listed, along with the percentage identity to AlrA and the percentage identity to the conserved amino acids in the NCBI conserved domain consensus aldo/keto reductase sequence (pfam00248).

| Sequence no.     | Identification                  | Percentage identity |
|------------------|---------------------------------|---------------------|
|                  | To AlrA | To consensus |
| AlrA             | 100     | 99.6       |
| aa-gener10543    | 51      | 77.2       |
| aa-gener10691    | 41      | 100        |
| aa-gener10680    | 37      | 97.9       |
| aa-numgt979      | 36      | 83.3       |

GCTATCAT were used to amplify a 217-bp sequence from sslA (accession number AAM34288). RNA isolation from vegetative cells and Northern blots was performed following Brock et al. (40). The 780-bp alrA fragment described above was used as a probe.

Anti-aldehyde Reductase Western Blots and Cell Fractionation—A total of 1 × 10⁶ cells were collected by centrifugation and resuspended to 50 μl in Laemmli sample buffer. Following the ECL protocol (Amerham Biosciences), the blot was then stained with a 1:2,000 dilution of an affinity-purified antibody made against the aldehyde reductase-specific peptide CWNTFHKKEHVRPALER (Bethyl Laboratories Inc., Montgomery, TX). For cell fractionation, Ax4 cells were collected by centrifugation and either used immediately or starved in shaking culture in PBM (20 mM KH₂PO₄/K₂HPO₄, 1 mM EDTA, 0.25 M sucrose) for 3 h as described above. 1 × 10⁹ cells were collected and resuspended to 1 × 10⁸ cells/ml in ice-cold MES buffer (20 mM MES, pH 6.5, 1 mM EDTA, and 0.25 M sucrose). Cells were lysed by a 5.0-μm cameo 25N-syringe filter (Osmonis/MSI, Westborough, MA). Cell fractionation was done using centrifugation following Brock et al. (21).

Aldehyde Reductase Assay—A 5.0-μm syringe filter was washed with 5 ml of PBM, and then 3 ml of cells at 5 × 10⁸ cells/ml were lysed by a single passage of these cells through the filter. For some assays, the lysate was clarified by centrifugation at 19,000 × g for 1 min. The aldehyde reductase activity was determined by the decrease of NADPH absorption at 340 nm (relative to the background absorption at 400 nm) at room temperature using dl-glyceraldehyde, water, or glucose as substrates for the enzyme (45). 500 μl of 0.135 μM Na₂HPO₄/KH₂PO₄, pH 6.2, 200 μl of water, 100 μl of 0.73 mM NADPH tetrasodium salt (Sigma), and 100 μl of 50 mM mercaptoethanol were added to 100 μl of cell lysate. This was placed in a cuvette, and then either 25 μl of 0.04 M dl-glyceraldehyde, 4 mM glucose, or water was added. The enzymatic activity over the course of 2 min was determined for each substrate by Z = (Å₂₆₂ – Å₂₆₂ – Å₂₆₂ – Å₂₆₂ – Å₂₆₂) (Å₂₆₂ – Å₂₆₂ – Å₂₆₂ – Å₂₆₂ – Å₂₆₂). Following Gabbay and Kinoshita (45), 1 unit of activity was defined as the ability to oxidize 1 μmol of NADPH/hour. Given an extinction coefficient for NADPH of 6.22 OD/cm/mM, the units of activity were 4,823 mol of NADPH/hour. The values for water were then subtracted from those of glyceraldehyde and glucose. Protein concentrations were determined with a Bio-Rad protein assay in comparison with a bovine serum albumin standard dilution curve.

Adhesion and Motility Assays—Ax4 and alrA cells were grown and assayed for adhesion following the protocol of Desbarats et al. (46) as modified by Roisin-Bouffay et al. (24), allowing the disaggregated cells 2 min to aggregate before scoring for single cells. To measure motility, midlog phase cells growing in HL5 were collected by centrifugation, resuspended and washed in PBS, and resuspended to 1 × 10⁷ cells/ml. 200 μl of cells were placed on a cover glass in a well of an 8-well slide. For 0-h motilities, midlog cells in HL5 were diluted to 2 × 10⁷ cells/ml with HL5, and 200 μl of cells was placed in a well of an 8-well slide. For all of the time points, cells were allowed to settle for 15 min, and motility was then measured by videotaping the cells following Yuen et al. (16). The approximate distance moved by a cell was measured in 1-min increments over 10 min.

Computer Simulations—The JAVA computer simulations used the program described in Roisin-Bouffay et al. (24) with the following modifications. The aggregation stream at the beginning of the simulation was 2000 cells in length and 17–22 cells in width. For the distribution of cell motilities, we used the actual distribution of motilities observed with either wild-type or alrA cells. The cell-cell adhesions were set as the cell-cell adhesions observed with wild-type or alrA cells.

Glucose and Osmolality Assays—Glucose levels were measured as described by Jang et al. (35). To measure osmolality, ~2 × 10⁵ vegeta-
tive cells or cells starved at 5 \times 10^6 \text{cells/ml} for 6 \text{h in PBM in shaking culture were collected by centrifugation. The pellets were briefly recentrifuged, the remaining supernatant was removed, and the pellets were frozen at } -80^\circ \text{C. The pellets were thawed, and the osmolality of } 50 \mu\text{l of a 1:1 mixture of the lysed cells and distilled water was measured with a model 5004 Micro Osmometer (Precision Systems, Natick, MA). A standard curve was constructed using distilled water and dilutions of a 100-mosmol/kg H_2O standard solution (Precision Systems), and the osmolality of the original cell lysate was calculated. Protein concentrations were measured using a Bio-Rad protein assay and bovine serum albumin for a calibration curve.}

**Metabolite Analysis**—For vegetative cell samples, cells were grown to 1–2 \times 10^6 \text{cells/ml in HL5, and for development cells were starved at } 5 \times 10^6 \text{cells/ml in PBM in shaking culture. A total of } 1 \times 10^6 \text{cells were collected by centrifugation at } 1,500 \times g \text{ for 5 min. The pellets were resuspended in } ~5 \text{ ml of the remaining supernatant, and this was recentrifuged. The supernatants were carefully aspirated, and the pellets were frozen at } -80^\circ \text{C. After 1–4 days, the pellets were thawed, and } 2 \text{ ml of water was added. This mixture was vortexed and then clarified by centrifugation at } 23,000 \times g \text{ for } 10 \text{ min at } 4^\circ \text{C. 1.2 ml of the slightly cloudy supernatant was then frozen on dry ice. After thawing, acetone was added to } 50\%, \text{ and the samples were processed for azetropic dehydration and trimethylsilylation of compounds as described in Shoemaker and Elliot (47) starting at the acetone addition step. Gas chromatography/mass spectrometry of the processed samples was performed as described by Shoemaker and Elliot (47).}

**RESULTS**

**Disrupting Aldehyde Reductase Results in Huge Groups**—On a two-dimensional gel of whole cells, there was a major protein whose staining intensity seemed to vary as a function of the extracellular CF concentration (25). After digesting the protein from the spot with trypsin, we obtained the amino acid sequence of two of the tryptic peptides. A search of the *Dictyostelium* genomic DNA sequence identified an open reading frame encoding both of the peptides (see Ref. 25 and Fig. 1). An NCBI protein-protein BLAST search (available on the World Wide Web at www.ncbi.nlm.nih.gov/blast/) using the deduced amino acid sequence of the open reading frame found a 45% identity to porcine alcohol dehydrogenase [NADP^+]-catalyzed reductase (48, 49) and a 44% identity to human (50) and mouse (51) aldehyde reductase and to a putative *Arabidopsis* mannos-6-phosphate reductase (NADPH-dependent) protein (gi: 15226502). The NCBI conserved domain search performed in conjunction with the BLAST search found the *Dictyostelium* protein identified above to be 99.6% aligned with the *araA* aldehyde reductase and to a putative *Arabidopsis* mannose-6-phosphate reductase (NADPH-dependent) protein (gi: 15226502). The NCBI conserved domain search performed in conjunction with the BLAST search found the *Dictyostelium* protein identified above to be 99.6% aligned with the *araA* aldehyde reductase family and 95.7% aligned with the *araA* aldehyde reductase-diketogulonate reductase family signatures. The *araA* reductase superfamily is made up of a number of related NADPH-dependent oxidoreductases with wide substrate specificities for carbonyl compounds (50). One of the major members of this family is aldehyde reductase, which uses NADPH as a cofactor to reduce a wide variety of aldehydes to the corresponding alcohols (52). The predicted protein identified above, designated AlrA for aldehyde reductase A, is 33.6 kDa with a pI of 5.95. The spot we observed on two-dimensional gels had an apparent molecular mass of ~24 kDa and a pI of ~6.0. Thus, although the predicted molecular mass is higher than what we observed, the predicted pI is close to the observed pI. The lower molecular mass either may be due to proteolytic

**FIG. 3.** Disruption of alrA causes a decrease in the activity of NADPH-coupled reduction of glyceraldehyde and glucose. A, clarified cell lysates of Ax4 parental (WT) or alrA cells were prepared at the indicated times of development and incubated with NADPH and either glyceraldehyde or water. The decrease in NADPH concentration was measured as the decrease in absorption at 340 nm. For each experiment, the value for water was subtracted from the glyceraldehyde value. The activity is shown in units of \mu\text{g of NADPH oxidized/}\mu\text{g of protein (in the clarified lysate) added to the reaction. B, activities were measured as in A with the exception that glucose was used instead of glyceraldehyde. For both A and B, values are means \pm S.E. from three independent experiments.
A colony of cells was photographed. Growth, the region from the edge of the plate to the growing edge of the inoculated at a point near the edge of the plate. After several days of growth, the region from the edge of the plate to the growing edge of the colony of cells was photographed. A, parental Ax4 cells; B, alrA− cells. Bar (B), 1 cm. Side views of wild-type (C) and alrA− (D) cells show the relatively huge groups formed by alrA− cells. Bar (D), 1 mm.

**Fig. 4. Disruption of alrA results in long streams and huge groups.** SM/5 agar plates were spread with bacteria, and cells were inoculated at a point near the edge of the plate. After several days of growth, the region from the edge of the plate to the growing edge of the colony of cells was photographed. A, parental Ax4 cells; B, alrA− cells. Bar (B), 1 cm. Side views of wild-type (C) and alrA− (D) cells show the relatively huge groups formed by alrA− cells. Bar (D), 1 mm.

**Fig. 5. Expression of AlrA in alrA− cells causes these cells to form normal fruiting bodies.** The indicated cell types were grown on SM/5 agar plates spread with K. aerogenes bacteria as a food source, and representative aggregates that formed behind the growth front were photographed. Bar, 1 mm. WT, wild type.

processing of AlrA may or may be an artifact of the SDS-polyacrylamide gel, since disparities between the predicted mass and the apparent mass on an SDS-polyacrylamide gel have been observed for other proteins (53–56). There are no predicted transmembrane domains and no strongly charged regions. A BLAST search of the preliminary directory of Dictyostelium genes using the data base of all predicted proteins plus GenBank™ Dictyostelium sequences found strong matches of AlrA to four putative gene products (Table I). A search of the same Dictyostelium sequences using the aldo-keto family consensus sequence found matches to the same gene products (Table I). Together, the data suggest that there are at least five putative protein sequences in the Dictyostelium genome that resemble aldo-keto reductases and that, of these, AlrA has the highest identity with the NCBI consensus sequence for aldo-keto reductases.

**Fig. 6. Aldehyde reductase is present in growing and developing cells.** Wild-type cells were starved on filters, and samples were harvested at the indicated times (h) after starvation. V, vegetative cells. A Western blot of the samples was stained with anti-aldehyde reductase antibodies; the heavy band is at 33 kDa.

**Fig. 7. Subcellular distribution of AlrA protein.** Vegetative (Veg) and 6-h starved (Dev) Ax4 cells were lysed and then fractionated by centrifugation. Nuclei and unbroken cells were pelleted in the low speed spin. The supernatant was then centrifuged at a medium 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 a SDS-polyacrylamide gel. A Western blot of the gel was then stained with anti-AlrA antibodies. The heavy band is at 33 kDa.

**Fig. 8. alrA− cells accumulate low levels of extracellular CF50 and countin.** 20-h CM was prepared by starving the indicated cell types at $5 \times 10^6$ cells/ml in 5 ml of PBM buffer. 20 μl of the CM was mixed with 5 μl of 5× Laemmli sample buffer, and 18 μl of this mixture was electrophoresed in a lane of an SDS-polyacrylamide gel. Western blots of duplicate gels were stained with antibodies against CF50 (top panel) or antibodies against countin (lower panel).

**Table I**

| Addition                  | Percentage increase in group number |
|---------------------------|-----------------------------------|
| Ax4 (wild-type parental)  |                                   |
| alrA                      |                                   |
| 15% smlA− cells           | 184 ± 33                          | 193 ± 20                       |
| 200 ng/ml recombinant countin | 163 ± 18                          | 120 ± 2                       |
| 6.3 ng/ml recombinant CF50 | 169 ± 28                          | 114 ± 3                       |

To determine whether this putative aldehyde reductase functions to regulate group size, we used homologous recombination to replace a region of the associated gene (starting 295 bp upstream of the A in the ATG encoding the first methionine in the open reading frame and ending 105 bp downstream of the T in the TAA stop codon) with a blasticidin resistance cassette. We identified a transformant designated alrA− where PCR indicated that the alrA gene had been disrupted, and reverse transcriptase-PCR indicated that the alrA mRNA was absent.
A Northern blot of RNA from vegetative cells probed with a fragment of the alrA cDNA indicated that wild-type cells contain a ~0.9-kb alrA mRNA, whereas no band was detected in alrA cells (Fig. 2A). A standard assay for aldehyde reductase activity is to measure the ability of a cell lysate to oxidize NADPH to NADP in response to an aldehyde such as glyceraldehyde. As shown in Fig. 3A, vegetative (0-h) wild-type cells have a low level of aldehyde reductase activity; this increases at 2 h of starvation and then decreases. Compared with the activity of wild-type cells, the aldehyde reductase activity of alrA cells was somewhat less at 0 h and considerably less at 2, 4, and 6 h. The alrA cells also had a lower activity than wild-type cells using the aldose glucose as a substrate (Fig. 3B).

Together, the data indicate that the alrA cells lack the alrA mRNA and the AlrA protein and have reduced NADPH-coupled aldehyde reductase and aldose reductase activities.

Growing alrA cells appeared grossly normal by light microscopy and grew as fast as parental cells. Compared with wild-type parental cells (Fig. 4A), developing alrA cells formed huge unbroken streams and huge groups (Fig. 4B). Closer examination of the groups showed that whereas wild-type cells formed fruiting bodies (Fig. 4C), the alrA cells formed huge mounds of cells (Fig. 4D) that generally did not appear to progress to the formation of fruiting bodies, although occasionally smaller groups of alrA cells did form large fruiting bodies.

The large groups formed by the alrA cells could be due to the presence of a second mutation or to an effect of the disruption on a nearby gene. To check this, the coding region of the alrA cDNA was expressed under the control of an actin promoter with this construct on a Dictyostelium plasmid. This plasmid was then transformed into alrA cells. The resulting alr/actin15::alrA cells formed normal sized and even some small fruiting bodies (Fig. 5). This suggests that the absence of AlrA from cells causes them to form large groups.

To determine when AlrA is present during development, a Western blot of vegetative and developing wild-type cells was stained with anti-AlrA antibodies. AlrA is present in vegetative cells; the levels decrease slightly upon starvation and remain roughly constant until 10 h of development and then decline until at 25 h there is very little detectable protein (Fig. 6).

Multiple attempts with a wide variety of fixatives failed to yield cells that could be stained with the anti-AlrA antibodies by immunofluorescence (data not shown). As shown in Fig. 7, fractionation of both vegetative and 6-h developing cells by differential centrifugation indicated that AlrA was not detectable in the low speed pellet (containing nuclei and cytoskeletons) and the medium speed pellet (containing large organelles such as mitochondria and lysosomes). There is also very little AlrA in the high speed pellet fraction (containing microsomes and small vesicles),

![Figure 10](image-url). Computer simulations of streams of wild-type and alrA- cells. The simulations used the observed cell-cell adhesion and motility of the two cell lines. The values are the means ± S.E. values of the sizes of the groups produced in four independent simulations.

![Figure 9](image-url). Disruption of alrA does not significantly affect cell-cell adhesion but decreases cell motility. A, cells were starved on filter pads, and at the indicated times the cells were dissociated, and cell-cell adhesion was measured. Values are means ± S.E. (n = 5). B, cells were harvested from shaking culture (0 h) or starved for 6, 8, 10, or 12 h on filter pads and dissociated, and videomicroscopy was used to examine their motilities. Values are means ± S.E. from 30 cells/time point. C, cells were starved for 6 h on filter pads and dissociated, and videomicroscopy was used to examine their motilities. The motilities were binned in 1-μm/min intervals, and the percentage of the 55 cells examined with motilities in each range was plotted. WT, wild type.
and ribosomes (67, 68)). AlrA was apparently confined to the high speed supernatant fraction, which contains cytosolic proteins (66). Together, the data suggest that AlrA is present as a cytosolic protein in vegetative and early developing cells.

**alr**A/H11002 Cells Accumulate Low Extracellular Levels of countin and CF50—We previously consistently observed a spot on two-dimensional gels that was strongest in countin/H11002 cells and weakest in smlA/H11002 cells; the addition of recombinant countin to starving cells decreased the intensity of this spot, whereas the addition of anti-countin antibodies to starving cells increased it (25). After digestion of this spot with trypsin, only two tryptic peptides eluted from the gel. This suggested that there could be other tryptic peptides present in the spot, and for that matter there could be other proteins present in the spot. To determine whether the levels of AlrA in smlA−, wild-type, and countin− cells were variable; for instance, sometimes smlA− cells had more and other times smlA− cells had less AlrA than parental cells. This suggests that there may be an unknown protein with a pI and a molecular mass similar to those of AlrA and that CF represses the levels of this unknown protein.

One possible reason the alr**A**− cells form large groups is that they have a reduced accumulation of extracellular countin and CF50. To check this, Western blots of conditioned starvation medium (CM) were stained for countin and CF50. Compared with the CM from parental wild-type cells, the CM from alr**A**− cells had less countin and CF50 (Fig. 8).

**alr**A− Cells Are Sensitive to CF—To determine whether AlrA is part of the CF signal transduction pathway, we examined the number of groups formed by cells in the presence or absence of smlA− cells. When wild-type cells are mixed with smlA− cells, the high levels of CF secreted by the smlA− cells cause the

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**Fig. 11. alr**A− cells have a reduced osmolality. Vegetative cells and cells starved for 6 h were collected by centrifugation and lysed, and osmolality was measured by freezing point depression. A, osmolality of lysed cells; values are means ± S.E. from three independent experiments. A t test indicated that there is a significant difference between parental and alr**A**− cells for both vegetative (veg; p < 0.005) and starving cells (p < 0.005). WT, wild type. B, the protein concentration of each sample was measured in duplicate, and the ratio of osmolality to average protein concentration for each sample was calculated; values are means ± S.E. of the ratios from three independent experiments. A t test indicated that there is a significant difference between parental and alr**A**− cells for both vegetative (p < 0.01) and starving cells (p < 0.025). The average protein concentration ± S.E. in units of mg/ml for the lysed pellets is shown at the bottom of the chart.
we previously published (24). However, whether looking at percentage of cells in aggregates are different from the values mized is different from that previously used, the values for the parently seasonal changes in the behavior of cells and because during development (Fig. 9) (24, 46, 69 previously observed, the adhesion of wild-type cells increases. As suggested that increasing cell-cell adhesion and/or decreasing cell motility would cause group size to increase (24, 25). As with mixing with smlA cells, adding the recombinant proteins to alrA cells increased the number of mounds that formed fruiting bodies. Together, the data suggest that alrA cells are sensitive to CF, recombinant countin, and recombinant CF50, although the responses to recombinant countin and recombinant CF50 appear to be less than what is observed in parental cells. This in turn suggests that alrA cells have a functional although slightly impaired CF signal transduction pathway.

alrA Cells Have Lower Motilities—Computer simulations suggested that increasing cell-cell adhesion and/or decreasing cell motility would cause group size to increase (24, 25). As previously observed, the adhesion of wild-type cells increases during development (Fig. 9A) (24, 46, 69–71). Because of apparently seasonal changes in the behavior of cells and because the reaggregation time in the adhesion assay we have optimized is different from that previously used, the values for the percentage of cells in aggregates are different from the values we previously published (24). However, whether looking at vegetative cells or cells starved for 2, 4, or 6 h, the adhesion of alrA cells was roughly similar to that of parental cells.

Besides adhesion, motility can affect group size. There was no significant difference in the motility of vegetative alrA and parental cells (Fig. 9B). At 6 and 8 h of development (when streams are forming and breaking up), the alrA cells had decreased motilities compared with parental wild-type cells, and then at 10 and 12 h (when the streams have broken and mounds are forming) the motility of alrA was again comparable with that of parental cells. At 6 h of development, the distribution of alrA cell motilities was skewed toward lower values compared with wild-type cells, but in the alrA population there were still some cells with motilities greater than 10 μm/min (Fig. 9C). A computer simulation of the behavior of cells in a stream, when the cells were modeled as having the observed adhesion and distribution of cell speeds of either wild-type or alrA cells at 6 h, predicted that the alrA cells would form groups approximately twice the size of the groups formed by wild-type cells (Fig. 10). This suggested that although the actual cell-cell adhesion decreased slightly in alrA cells compared with parental cells, the decrease in motility of alrA cells compared with parental cells is enough to increase the group size. Together, the data suggest that disruption of alrA does not have a significant effect on cell-cell adhesion but does significantly decrease motility when the cells are forming and breaking streams and that this decreased motility is predicted to increase group size.

Disruption of Aldehyde Reductase Affects Several Metabolic Pathways—Since aldo-keto reductase is thought to produce the osmolyte sorbitol, we examined the osmolality of alrA cells. As shown in Fig. 11A, alrA− cells have a significantly lower osmolality than parental cells both at the vegetative stage and at 6 h of development. When the protein concentrations of the cell lysates were measured and used to normalize the osmolalities, the alrA cells still had a significantly lower osmolality (Fig. 11B). There was no significant difference in the protein concentrations of parental and alrA− cells, although for both cell lines the protein concentrations increased somewhat at 6 h compared with the vegetative cells. Together, the data indicate that disruption of alrA does not significantly affect the protein concentration in cells but does decrease osmolality.

If the main function of aldehyde reductase was to produce sorbitol from glucose, one might expect that disruption of aldehyde reductase would cause glucose levels to increase. To test this hypothesis, we measured the level of glucose in parental wild-type and alrA− cells. As shown in Fig. 12, the level of glucose in the vegetative (0 h of starvation) and 6-h starved wild-type cells was similar to what we previously observed for Ax4 cells (35) and somewhat lower than what we previously observed for 2-h starved cells. Compared with the parental cells, the alrA− cells had lower glucose levels, with a statistically significant difference at 4 h. These results indicate that disruption of alrA causes cells to contain lower rather than higher levels of glucose.

To determine whether the levels of other compounds are affected by disrupting alrA, cells were ruptured by freeze-thawing, and the soluble components were isolated by centrifugation. After trimethylsilyl derivitization, gas chromatography/mass spectrometry was performed. For vegetative cells, the gas chromatography spectra of the parental and alrA− extracts were roughly similar (Fig. 13, A and B), although the sizes of some peaks were visibly changed (Fig. 13, A and B, and Table III). For the vegetative cells, the differences were observed as increases in the levels of compounds in the alrA− extracts. At 6 h of development, the differences between wild-type and alrA− were mostly observed as increases in the levels of compounds in the alrA− cells, although alrA− cells had decreases in the levels of glucopyranose, lactose, and trehalose (Fig. 13, C and D, and Table III). Together, the data suggest that disruption of alrA affects the levels of several metabolites in Dictyostelium.

DISCUSSION

The aldo-keto reductases form a large superfamily of enzymes that reduce C=O to C–OH in a broad range of aldehydes and ketones (50, 72). There are 6 members of the family in bacteria, 14 in yeast (73), and possibly 5 in Dictyostelium. Disruption of alrA in Dictyostelium abolishes the majority of the NADPH-coupled reduction of glyceraldehyde and glucose, suggesting that AlrA is responsible for a large fraction of the
aldehyde reductase activity in Dictyostelium cells, at least with respect to these substrates.

Gas chromatography/mass spectroscopy indicated that, compared with parental vegetative cells, vegetative alrA⁻ cells had increased levels of six identified compounds, all of which contain a carboxylic acid. Given that many organic acids are converted to the corresponding aldehydes and that aldehyde reductase then converts these to the corresponding alcohols, a possible explanation for the increased levels of the organic acids in alrA⁻ cells is that the conversion to the alcohol is at least partially disrupted and that the levels of the aldehydes increase. This could in turn cause an increase in the levels of the organic acids simply by affecting the equilibrium of an enzymatic reaction or by nonenzymatic oxidation of the aldehydes to the corresponding acids. We did not observe decreased levels of alcohols or any other compound in the gas chromatography runs due to slight temperature differences, are as follows: lactic acid (60), alanine (103), glycine (124), β-aminobutyric acid (184), valine (247), norleucine (337), unknown (343), isoleucine (373), glycine (388), succinic acid (406), 2,4-dihydroxyprymidine (440), piperic acid (477), serine (490), threonine (535), unknown (574), β-alanine (597), succinic acid (719), 3-amino-2-piperidone (725), methionine (755), γ-aminobutyric acid (775), creatinine (798), 2-phenylethylamine (809), unknown (858), phenylalanine (910), glutamic acid (917), suberylglycine (923), spermine (928), 2-thienylcarbonylglycine (937), arabinose (970), asparagine (980), 4H-furo 3,2-C pyran-2-6H-one (1032), putrescine (1049), unknown (1058), phosphorimidic acid (1106), glutamine (1110), 9H,6-hydroxypurine (1133), fructose (1153), galactose (1193), formiminoglutamic acid (1212), 4H-furo 3,2-C pyran-2-one (1228), 2-thienylcarbonylglycine (1228), ribulose (1259), lysine (1269), tyrosine (1287), unknown (1314), glucopyranose (1341), dodecenedioic acid (1351), xanthine (1369), palmotoleic acid (1377), palmitic acid (1399), D-erythro-2-pentulose (1408), benzenoacetic acid (1414), L-aspartic acid (1420), mucoinositol (1430), dodecenedioic acid (1440), fructose (1499), linoleic acid (1515), oleic acid (1534), unknown (1545), anhydroaltr-2-heptulose (1568), xanthine (1582), pseudouridine (1596), melibiose (1616), D-ribosuranose (1624), orotidine (1631), α-ketoadipic acid (1658), unknown (1672), inosine (1710), adenine (1728), lactose (1754), L-fructose (1763), trehalose (1773), D-fructose (1792), L-arabinohepxonic acid (1855). The presence of a compound in two different peaks is due to the creation of different trimethylsilyl derivatives.

The physiological function of aldo-keto reductases is unclear, and disruption studies suggest that some individual aldo reductases are not necessary for viability. In yeast, disruption of genes encoding specific aldo reductases tends to affect some but not all of the aldose reductase activity, suggesting that there are overlapping functions and specificities (73). Disruption of the aldo reductase gene in mice has no effect on the viability, gross appearance, or organ morphology (74, 75). These mice had slightly elevated serum levels of Ca²⁺ and Mg²⁺, increased urine volume and urinary Ca²⁺, and decreased urine osmolality. Disruption of alrA in Dictyostelium had no effect on the growth rate of cells. This is very surprising, since alrA cells lack a majority of aldehyde reductase activity and have altered levels of several metabolites. The viability of alrA cells could be due to redundancy in the metabolic pathways affected by AlrA, as evidenced by the residual amount of pentose, which could be due to the lack of reduction of a C=O in the pentose, as well as higher levels of adenosine. Adenosine is not a ketone or an aldehyde, so it is unclear why there are higher levels of this compound in alrA⁻ cells. The 6-h starved alrA⁻ cells had lower levels of three different sugars, which could be due to their precursors being aldehydes or ketones.

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aldehyde reductase observed in the alrA− cells and by the finding that of the metabolites we could identify by gas chromatography/mass spectrometry, none were completely missing in alrA− cells.

Disrupting alrA has a profound effect on development, causing the formation of large groups. Expression of the alrA cDNA in the alrA− cells rescued the group size phenotype, showing that the effect on group size of disrupting alrA was not due to an effect on a nearby gene or a second mutation. However, disruption of alrA does not have an obvious effect on cell growth. One possible explanation for this is that although there is redundancy in the Dictyostelium aldehyde/aldose reductases, the levels of some metabolite affected by the loss of AlrA may not be of significance to growth, as long as there is some of the metabolite present, but this altered level does affect development. Another possible explanation is that AlrA is required for the production or degradation of a compound that is required for or inhibits normal development but that is not required for or does not affect growth.

alrA− cells form smaller groups when mixed with smlA− cells and when starved in the presence of recombinant countin or recombinant CF50. This suggests that alrA− cells have some response to CF and that AlrA is not a key component of the CF signal transduction pathway. Reducing the extracellular levels of either countin or CF50 causes the formation of large groups (22, 43), and we found that alrA− cells have a reduced extracellular accumulation of both proteins. This suggests that one reason alrA− cells form large groups is that they have a defect in the synthesis, secretion, or processing of extracellular CF. However, the response of alrA− cells to recombinant countin and recombinant CF50 in terms of reducing group size and increasing group number is not as great as the response of wild-type cells to these two proteins. This suggests that AlrA is required for a complete response of cells to the CF components and that another reason alrA− cells form large groups is that they have a somewhat attenuated response to CF.

Cells lacking either countin or CF50 have high cell-cell adhesion and reduced motilities (22, 43). The alrA− cells have reduced motilities at 6 and 8 h of development, as would be expected from the observation that they have low levels of extracellular countin and CF50. However, the alrA− cells have roughly normal cell-cell adhesion. This suggests that some effect of disrupting alrA counteracts the effect of low levels of extracellular countin and CF50.

The alrA− cells have a significantly reduced osmolality compared with parental cells, suggesting that, as in other systems, aldose reductase is involved in regulating Dictyostelium cellular osmolality. Dictyostelium cells respond to an increase in extracellular osmotic strength by increasing levels of cGMP, which causes myosin to be phosphorylated and to redistribute itself to the cell cortex, presumably to strengthen the cell membrane (76). Dictyostelium cells thus appear to use several different mechanisms to regulate and respond to osmotic pressure.

We previously found that CF represses cytosolic glucose levels and that increasing cytosolic glucose levels increases group size (35). This suggested that glucose or some metabolite of glucose might affect group size. The observation that aldose reductase cells form huge groups but do not have abnormally high glucose levels suggests that a metabolite of glucose rather than glucose per se might be a key element of the CF signal transduction pathway.

In rat vascular smooth muscle cells, aldose reductase inhibitors or aldose reductase antisense oligonucleotides block the activation of protein kinase C and the activation of the transcription factor NF-kB by some but not all signals, suggesting that aldose reductase activity is necessary for the function of some but not all signal transduction pathways (77). The observation that disruption of alrA in Dictyostelium does not appear to affect growth or adhesion but does affect CF secretion, motility, and group size during development suggests that much remains to be understood about this enigmatic enzyme.

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