ABC transporters required for endocytosis and endosomal pH regulation in *Dictyostelium*

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SUMMARY

In *Dictyostelium*, the RtoA protein links both initial cell-type choice and physiological state to cell-cycle phase. *rtoA*$^-$ cells (containing a disruption of the *rtoA* gene) generally do not develop past the mound stage, and have an abnormal ratio of prestalk and prespore cells. RtoA is also involved in fusion of endocytic/exocytic vesicles. Cells lacking RtoA, although having a normal endocytosis rate, have a decreased exocytosis rate and endosomes with abnormally low pHs. RtoA levels vary during the cell cycle, causing a cell-cycle-dependent modulation of parameters such as cytosolic pH (Brazill et al., 2000). To uncover other genes involved in the RtoA-mediated differentiation, we identified genetic suppressors of *rtoA*. One of these suppressors disrupted two genes, *mdrA1* and *mdrA2*, a tandem duplication encoding two members of the ATP binding cassette (ABC) transporter superfamily. Disruption of *mdrA1/mdrA2* results in release from the developmental block and suppression of the defect in initial cell type choice caused by loss of the *rtoA* gene. However, this is not accomplished by re-establishing the link between cell type choice and cell cycle phase. MdrA1 protein is localized to the endosome. *mdrA1*$^-$*/mdrA2*$^-$ cells (containing a disruption of these genes) have an endocytosis rate roughly 70% that of wild-type or *rtoA*$^-$ cells, whereas *mdrA1*$^-$*/mdrA2*$^-$*/rtoA*$^-$ cells have an endocytosis rate roughly 20% that of wild-type. The exocytosis rates of *mdrA1*$^-$*/mdrA2*$^-$ and *mdrA1*$^-$*/mdrA2*$^-$*/rtoA*$^-$ are roughly that of wild-type. *mdrA1*$^-$*/mdrA2*$^-$ endosomes have an unusually high pH, whereas *mdrA1*$^-$*/mdrA2*$^-$*/rtoA*$^-$ endosomes have an almost normal pH. The ability of *mdrA1/mdrA2* disruption to rescue the cell-type proportion, developmental defects, and endosomal pH defects caused by *rtoA* disruption, and the ability of *rtoA* disruption to exacerbate the endocytosis defects caused by *mdrA1/mdrA2* disruption, suggest a genetic interaction between *rtoA*, *mdrA1* and *mdrA2*.

Key words: ABC; Multidrug; pH; Cell-type choice; Cell fate, Musical chairs

INTRODUCTION

Little is known about how a set of undifferentiated cells can break symmetry and differentiate into distinct cell types. One of the simplest organisms that exhibits this behavior is the eukaryote *Dictyostelium discoideum*. *Dictyostelium*, which eats bacteria and increases in number by fission, lives as a single cell on soil surfaces. When the cells overgrow an area and starve, they form an aggregate of 2×10^4-10^5 cells, which develops into a fruiting body consisting of two cell types: a column of stalk cells supporting a mass of spore cells.

It has been suggested that the pH of intracellular vesicles, in part, regulates initial cell-type choice (Gross et al., 1988). In support of this hypothesis, we had previously recovered a mutant of *Dictyostelium* that has an altered endosomal pH and accordingly an altered ratio of initial cell types (Wood et al., 1996; Brazill et al., 2000). The gene disrupted in this strain, *rtoA*, was originally identified using shotgun antisense transformation (Spann et al., 1996) in a screen designed to identify mutants with altered cell-type differentiation. Transformants having any observable developmental abnormality were screened by assaying for changes in the percentages of cells differentiating into prestalk and prespore cells. The *rtoA*$^-$ mutant had an abnormally high percentage of prestalk cells (Wood et al., 1996) (R. R. Ammann and R.H.G., unpublished). The *rtoA*$^-$ gene disruption transformant had the same phenotype as the original antisense mutant, including a high prestalk/prespore ratio, and a developmental arrest at the mound stage.

In addition to pH, it has been shown that the initial differentiation of *Dictyostelium* cells occurs by a combination of asymmetric cell division and a simple 'musical chairs' mechanism based on the cell cycle (Araki et al., 1997; Araki et al., 1994; Gomer and Firtel, 1987; Krefft and Weijer, 1989; McDonald and Durston, 1984; Ohmori and Maeda, 1987; Weijer et al., 1984a; Zimmerman and Weijer, 1993). The initial cell-type decision is made at the initiation of starvation. For each pair of sister cells in S or early G2 phase, one sister differentiates into a prestalk cell whereas the other sister becomes a null cell (a cell type that initially expresses neither prespore nor prestalk markers, but which can later differentiate into either cell type). For each pair of sister cells in late G2 or M, one sister differentiates into a prespore cell and the other sister becomes a null cell (*Dictyostelium* grown under standard laboratory conditions has an undetectable G1 phase (Weijer et al., 1984b)). This mechanism regulates only initial

differentiation. The eventual fate of the cell is still plastic, and a variety of factors such as adenosine, ammonia, a chlorinated hydrocarbon called DIF and oxygen can change the final fate (Brookman et al., 1987; Gross et al., 1983; Kay et al., 1989; Kwong and Weeks, 1989; Schaap and Wang, 1986; Sternfeld, 1988; Williams et al., 1987; Xie et al., 1991).

Cell-cycle analysis of *rtoA*$^{-}$ cells demonstrated that the increase in the percentage of prestalk cells was not due to changes in S, G2 or M phases. However, prestalk and prespore cells from *rtoA* disruptants originate from cells in any phase of the cell cycle at starvation. As in wild-type cells, the sisters of the differentiated cells are all null in the *rtoA*$^{-}$ mutant. These results suggest that RtoA is not involved in the asymmetric cell-division mechanism or cell-cycle progression per se, but rather is involved in some process that varies during the cell cycle, and which can be measured at the time of starvation to select initial cell type (Wood et al., 1996).

Examination of the amount of RtoA protein per cell uncovered a marked heterogeneity in the amount of RtoA in individual vegetative cells (Brazill et al., 2000). The cells with high RtoA are all in the 'prespore' phase of the cell cycle, whereas the sisters of these cells have low levels of RtoA. The cells in the 'prestalk' phase of the cell cycle have intermediate levels of RtoA, suggesting that relative levels of RtoA protein may determine initial cell type choice. Sixty per cent of the RtoA amino acid sequence is ten tandem repeats of an 11 amino acid serine-rich motif, which we found to have a random coil structure. A recombinant protein containing this domain was able to catalyze the fusion of phospholipid vesicles, suggesting a role for RtoA in vesicle fusion. Light and electron microscopy confirmed that *rtoA*$^{-}$ cells have a defect in the fusion of endocytic vesicles. In wild-type cells, sister cells tend to have different vesicle processing rates, whereas in *rtoA*$^{-}$ cells the processing rates are generally the same for all cells. *rtoA*$^{-}$ cells also have a decreased exocytosis rate and a decreased pH of endocytic/exocytic vesicles.

The products of many genes regulate the pH of cells and organelles. One class of proteins that does this is the ATP binding cassette (ABC) transport proteins. These proteins are found in a number of organisms, but are most noted for their involvement in cystic fibrosis (Riordan et al., 1989), multidrug resistance in human cancers (Gros et al., 1986; Gros et al., 1988; Riordan et al., 1985) and chloroquine resistance in the malaria-causing paramecium *Plasmodium falciparum* (Krohstad et al., 1987; Reed et al., 2000). Proteins belonging to this family use the energy from hydrolysis of ATP to transport molecules from one side of a membrane to another. These transported molecules can range in size and complexity from single ions to peptides (Gilson et al., 1988; Higgins, 1992). In *Dictyostelium*, there exist ABC transporters that are involved in the release of signals from cells (Good and Kuspa, 2000; Shaulsky et al., 1995). The *P. falciparum* ABC transporter, Pgh1, which is involved in chloroquine resistance, is believed to control the pH of the food vacuole, the site of chloroquine activity (Cowman et al., 1991; van Es et al., 1994a). When Pgh1 is mutated, the food vacuole is not properly acidified. Chloroquine, being a weak base, is unable to enter the food vacuole due to a decreased pH gradient. Pgh1 expressed in mammalian cells is localized to the intercellular vesicles. In addition, lysosomal acidification and increased accumulation of drug were observed, supporting the role of Pgh1 in pH regulation (van Es et al., 1994a; van Es et al., 1994b).

To identify other genes involved in the RtoA-regulated mechanism for choice of cell type, we initiated a second-site suppressor screen of the *rtoA*$^{-}$ mutant. Second-site suppressor analysis is a genetic tool that can be used to identify components of genetic pathways (Shaulsky et al., 1996). Usually, genetic suppressors rescue the defect of the original mutation by modifying a downstream or interacting component in the faulty pathway. Using restriction-enzyme-mediated integration (REMI), we created random insertional mutations in *Dictyostelium* cells lacking RtoA. Selecting for mutations that allowed *rtoA*$^{-}$ cells to develop past their original block at the mound stage, we identified *mdrA1* and *mdrA2*, an apparent tandem duplication that encodes proteins with high homology to ABC transporter proteins.

MATERIALS AND METHODS

Cell culture

DH1 wild-type cells were grown in shaking culture in HL5 medium supplemented with 20 μg/l biotin, 5 μg/l vitamin B12, 200 μg/l folic acid, 400 μg/l lipoic acid, 500 μg/l riboflavin and 600 μg/l thiamine supplemented with uracil as described by Gomer et al. (Gomer et al., 1991). A mixture of 0.3 g/l streptomycin sulfate and 0.1 g/l ampicillin were used as antibiotics. *mdrA1*$^{-}$/*mdrA2*$^{-}$ and *rtoA*$^{-}$/*mdrA1*$^{-}$/*mdrA2*$^{-}$ cells were grown in submerged stationary culture in the above medium, or on bacterial plates. The procedure of Wood et al. (Wood et al., 1996) was used to determine the percentages of prestalk and prespore cells at low cell density. For development, cells at mid-log phase (2×10^6 cells/ml) were washed in buffer and plated on filter pads following Jain et al. (Jain et al., 1992).

REMI mutagenesis and suppressor screen

Isolation of second site suppressors followed Shaulsky et al. (Shaulsky et al., 1996) with the following modifications. *rtoA*$^{-}$ cells were grown to 4×10^6 cells per ml in liquid HL5 medium as above. Cells were cooled on ice for 15 minutes, harvested by centrifugation and resuspended at 1×10^7 cells per ml in ice-cold electroporation buffer (10 mM Na_2HPO_4, 10 mM KH_2PO_4 and 50 mM sucrose, pH 6.1). Five ml of cells were mixed with 10 μg/ml of *Bam*HI-linearized pUCBsrΔBam and 30 units/ml of DpnII restriction endonuclease (New England Biolabs, Beverly, MA) immediately before electroporation. Aliquots of 0.8 ml were placed into 0.4 cm gap cuvettes (Bio-Rad, Pinole, CA) and electroporated at 1.0 kV and 3 μF with a Bio-Rad Gene Pulser (Bio-Rad). The cells were transferred immediately into HL5. The cells were plated at 1×10^6 cells per 10 cm plate and incubated for 15 hours. Blasticidin S (Calbiochem, San Diego, CA) was added to each plate to a concentration of 4 μg/ml and the cells were incubated for another 6 days. Drug-resistant colonies were collected and plated at approximately 200 cells per 10 cm plate on SM/5 agar with *Klebsiella aerogenes*. Plates were screened for mutants that showed more advanced development than the original *rtoA*$^{-}$ mutant. Individual plaques were isolated and plated on SM/5 agar with *K. aerogenes*.

Nucleic acid manipulation

Cloning of the DNA sequences flanking the insertion site and recapitulation of the mutant by homologous recombination were performed using the method of Kuspa and Loomis (Kuspa and Loomis, 1992). Sequencing of this clone was performed as previously described (Wood et al., 1996). Additional sequence was obtained from the *Dictyostelium* Genome Sequencing Project at the Jena Genome Sequencing Center (Jena, Germany) and by PCR of genomic DNA

and cDNA. Northern and Southern blot analyses were performed following Wood et al. (Wood et al., 1996) using a DNA probe to a ~500 bp region near the middle of *mdrA2* obtained by RT-PCR (Perkin Elmer, Branchburg, NJ) from mRNA isolated with an Oligotex Direct mRNA Isolation Kit (Qiagen, San Clarita, CA) with the primers 5′-ATCGTAAAGATCCAGTTGG-3′ and 5′-TCTCTAACCGTTGACACTGG-3′.

Preparation of a recombinant MdrA1 fragment and immunofluorescence

The reverse transcriptase polymerase chain reaction (RT-PCR) was used to generate a DNA fragment for fusion protein production. Poly(A+) RNA was isolated from cells starved for 5 hours using an Oligotex Direct mRNA Isolation Kit (Qiagen, San Clarita, CA). cDNA was created by performing RT-PCR for 1 cycle (90°C for 5 minutes, 23°C for 10 minutes, 42°C for 30 minutes and 95°C for 10 minutes) with MuLV reverse transcriptase following the manufacturer's directions (Perkin Elmer, Branchburg, NJ). To generate cDNA for the second ATP binding region (amino acids 745-973) of MdrA1, the primer 5′-CGGAATTCAAATGAAATCGGC-TGG-3′ was used. Using this cDNA as a primer, PCR was carried out for three cycles (95°C for 30 seconds, 37°C for 30 seconds, 72°C for 1 minute) followed by 27 cycles (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute) with Bio-X-act polymerase following the manufacturer's directions (Intermountain Scientific Corp., Salt Lake City, UT). The above primer as well as 5′-GCGGATCCGGTTATGT-TAAACCGGG-3′ were used to amplify the DNA. The primers created an *Eco*RI site at the 3′ end and a *Bam*HI site at the 5′ end of the PCR product. The purified product was then digested with *Eco*RI and *Bam*HI and ligated into the *Bam*HI/*Eco*RI digested expression vector pGEX3 (Amersham, Piscattaway, NJ) after the enzymes had been heat inactivated. Fusion protein was induced and purified using the BPER system and a glutathione-agarose column (Pierce, Rockford, IL) following the manufacturer's direction. The protein was eluted in three fractions, which were then pooled. These fractions consisted of >85% fusion protein. The fusion protein was used to immunize a rabbit as described in Jain et al. (Jain et al., 1997). Serum was collected and was purified using an E-Z-Sep kit (Pharmacia). For affinity purification of the above sera, the fusion protein was attached to a beaded agarose support using an AminoLink immobilization kit (Pierce, Rockford, IL). The antibody was purified using their suggested protocol. Immunofluorescence was performed with this antibody using the method of Zhu and Clarke (Zhu and Clarke, 1992), without the agarose overlay.

Antibody production and western blots

Because we found that the above antibody gave a high background when used for western blots, the synthetic peptide NYFNNKHNKKQNDDSD from MdrA2 was conjugated to keyhole limpet hemocyanin and this was then used to immunize a rabbit at Biosynthesis Inc. (Lewisville, TX). Serum was collected two weeks after the third injection and was purified using an E-Z-Sep kit (Pharmacia). The antibody was purified as described above using the synthetic peptide attached to agarose beads. 1×10^5 cells were collected at different stages of development and boiled for 3 minutes in SDS PAGE sample buffer in a final volume of 100 μl. A 20 μl aliquot was loaded onto a 7.5% PAGE gel and western blots were done following Jain and Gomer (Jain and Gomer, 1994).

Light microscopy

To monitor pinocytosis, 200 μl of cells at a density of 1×10^5 cells/ml in HL5 were placed onto an 8-well coverglass bottom slide (Type 136439, Nunc, Naperville, IL) and allowed to settle for 10 minutes. The medium was then gently changed to 2 mg/ml rhodamine isothiocyanide dextran (RD) (Sigma) in HL5 for 10 minutes then replaced with HL5. After 80 minutes, lysosomes were stained by adding Lysosensor Green DND-189 (Molecular Probes) to 1 mM. After 10 minutes, the medium was changed to HL5 for an additional 10 minutes. Cells were then fixed for 3 minutes in HL5 containing 1% formaldehyde, after which the medium was changed to HL5. This procedure was also performed without chasing the RD for 80 minutes with HL5. Images were taken with a Nikon Diaphot inverted microscope using a ×60 1.4 NA phase/fluorescence objective lens. Phase illumination was provided by a 12V/100W lamp operating at 4 V, and the epifluorescence illumination was provided by a 12V/100W lamp operating at 11.5 V. Both lamps used external power supplies to reduce heating of the microscope. A heat-blocking filter was in the phase illumination beam. A Photometrics cooled CCD camera operating at an exposure of 16× and gain of 24 db supplied a video image to a computer and direct monitor. To ensure that signals were not saturating, an oscilloscope was used to monitor the video signal. Nuclei were stained with DAPI following Wood et al. (Wood et al., 1996). Staining of calmodulin in fixed and permeabilized cells was done following Zhu and Clarke (Zhu and Clarke, 1992), omitting the agarose overlay step.

Endocytosis, exocytosis and pH assays

Endocytosis was assayed as previously described (Buczynski et al., 1997a). Fluid phase exocytosis was measured using FITC-dextran (FD) (Sigma) as previously described (Bush et al., 1996) with slight modifications. Cells grown to 2×10^6 cells/ml were harvested and resuspended in HL5 containing FD at 2 mg/ml. The cells were allowed to internalize FD for 3 hours and then were collected and resuspended in fresh HL5. At the indicated times, 1 ml of cells was collected by centrifugation and washed once in HL5 and twice in 5 mM glycine-NaOH (pH 8.5) containing 100 mM sucrose. The cells were then lysed in 1 ml of the same buffer containing 0.2% Triton X-100. The fluorescence of the lysate was measured in an Aminco Bowman Series 2 Luminescence Spectrometer. Excitation and emission wavelengths for FD were 495 nm and 520 nm, respectively. Endocytic pH was measured following Aubry et al. (Aubry et al., 1993) as described by Brazill et al. (Brazill et al., 2000). BCECF staining to measure cytosolic pH was done as described previously (Brazill et al., 2000).

RESULTS

Mutagenesis and selection of suppressors

Wild-type *Dictyostelium* cells, when starved, enter into a 24 hour developmental program culminating in the formation of a fruiting body consisting of a mass of spore cells supported by a column of stalk cells (Fig. 1). However, strains carrying a mutation in the *rtoA* gene are not able to progress fully through development and are arrested at the mound stage (Fig. 1). This developmental phenotype allowed us to design a suppressor screen to identify other genes that may be involved in the RtoA pathway. By mutating the *rtoA*$^-$ mutant strain and selecting for clones that proceed past the mound stage, we could potentially isolate genes that are involved in initial cell type choice that act downstream of RtoA. We generated approximately 10,000 independent transformants of the *rtoA*$^-$ strain using a plasmid carrying the blasticidin resistance cassette. These transformants were plated on agar with bacteria to generate clones for each individual transformant. After a week of growth, the clones were screened for development past the mound stage, the point at which the *rtoA*$^-$ strain arrests. Thirteen such clones were identified and isolated. These clones actually represented 11 separate suppressors, as two of the suppressors were isolated twice. Genomic DNA fragments flanking the plasmid insertion site along with the intervening

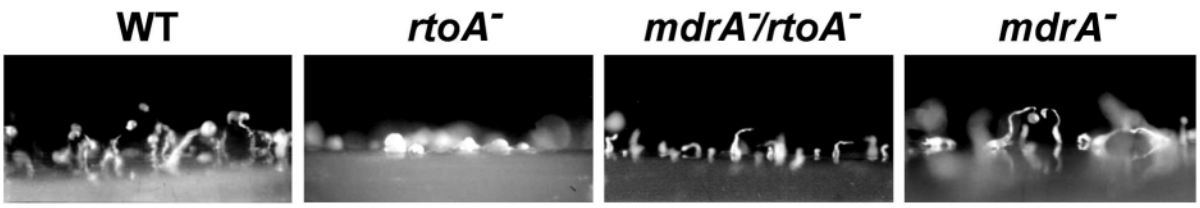

Fig. 1. Disruption of *mdrA1* and *mdrA2* results in a partial rescue of the developmental defect exhibited by mutation of *rtoA*. Wild-type DH1 cells, *rtoA*$^-$ cells, *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells and *mdrA1*$^-$/*mdrA2*$^-$ cells were plated with bacteria on agar. As the bacteria were consumed, development was triggered and after 24 hours fruiting structures formed. A side view of these resulting developmental structures is shown. The width of each frame is 3.5 mm.

plasmid were isolated and cloned. One of the clones contained DNA from two novel *Dictyostelium* genes, *mdrA1* and *mdrA2*, which encode proteins with similarity to ABC transporter/multidrug resistance proteins (see below).

This REMI mutant actually was an insertion/deletion mutant where the 3′ end of *mdrA1* and the 5′ end of *mdrA2* had been replaced with the REMI plasmid, causing a deletion of the last 89 amino acids of MdrA1 and the first 534 amino acids of MdrA2. The plasmid containing *mdrA1/mdrA2* DNA was linearized and transformed into the *rtoA*$^-$ strain. The resulting gene disruption by homologous recombination successfully recapitulated the rescue of the developmental arrest, indicating that the isolated genes were responsible for suppression. Proper insertion was verified by Southern blots (data not shown). The *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells did not grow in suspension culture and grew slowly in liquid medium while attached to plastic plates, much like the phosphatidylinositide 3-kinase mutant *Δddpik1/Δddpik2*, which has a defect in pinocytosis (Buczynski et al., 1997b). Cells from the original *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$, as well as the recapitulation strain, were able to progress through development and produce a fruiting body. However, this fruiting body was abnormal in that it appeared to have a thickened stalk and basal disk (Fig. 1). In addition, we introduced the insertional clone into wild-type cells to determine whether disruption of just *mdrA1* and *mdrA2* caused a development phenotype. Like the *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells, the *mdrA1*$^-$/*mdrA2*$^-$ cells were unable to grow in suspension culture but did grow (albeit slowly) in liquid medium while attached to plastic plates. As seen in Fig. 1, *mdrA1*$^-$/*mdrA2*$^-$ cells are able to proceed through development, although *mdrA1*$^-$/*mdrA2*$^-$ fruiting bodies also exhibit thickened stalks and basal disks. *mdrA1*$^-$/*mdrA2*$^-$ cells also tend to be rounder than wild-type cells when placed in liquid culture (data not shown).

Sequence similarity of MdrA1 and MdrA2 to ABC transporters

Genomic and cDNA clones were sequenced and the deduced amino acid sequences were compared with various protein databases using BLAST. The insert had affected two tandemly oriented highly similar genes, *mdrA1* and *mdrA2*, both of which are members of the ABC transporter family. Comparison of the nucleotide sequence also showed a similarity in how these genes are coded. Each is made up of two coding regions at the 5′ end of the gene, with the first being ~430 and ~470 bp, respectively, and the second being ~270 bp in both genes; a third coding region containing ~3300 and 3700 bp, respectively, is situated at the 3′ end. In the two genes the first intron is 92 and 140 bp and the second intron is 104 and 135 bp, respectively. The predicted sizes of MdrA1 and MdrA2 are 149 kDa and 166 kDa, respectively. The amino acid sequences of the two proteins have a 50.2% identity over their entire length, with an 84% identity in the middle 276 amino acids. The main differences between the two proteins are three repetitive regions with a length of 10-25 aa and a ~50 aa arginine-rich region found in MdrA2 but not in MdrA1.

As is found in other members of the ABC transporter family, MdrA1 and MdrA2 contain two copies each of the ABC transporter sequence motif that constitutes a nucleotide-binding fold. The sequence is found in similar locations on each gene, from amino acids 85-255 and 728-916 in MdrA1 and 98-288 and 841-1029 in MdrA2. Both contain the two characteristic six transmembrane-spanning regions and Walker A and B motifs found in ABC transporter proteins (Higgins et al., 1986; Higgins et al., 1985; Walker et al., 1982). ABC transporters can have several topologies based on the placement of the nucleotide-binding domain (NBD) and the transmembrane domain (TMD) (Van Veen and Konings, 1998). MdrA1 and MdrA2 fall into the group with the structure $\{NBD\text{-}TMD\}_2$, which places them with PDR5 from *Saccharomyces cerevisiae* (Balzi et al., 1994) and WHITE from *Drosophila melanogaster* (O'Hare et al., 1984) (SWISS-PROT accession no. P10090). Other structures include $\{TMD\text{-}NBD\}_2$, two separate peptides $\{TMD\}_2$ $\{NBD\}_2$ and 'half-size' transporters {TMD-NBD} and {NBD-TMD} (Van Veen and Konings, 1997). Fig. 2 aligns the NH_2-terminal and COOH-terminal ABC cassettes of MdrA1 and MdrA2 with the consensus ABC transporter cassette. Given their proximity to each other and their high sequence identity, we believe that these two genes represent a tandem duplication. Tandem duplication events have been seen in a number of *Dictyostelium* genes including the adhesion protein gp24 and the discoidin I gene family (Loomis and Fuller, 1990; Welker, 1988). Sequence comparisons with other proteins show a high similarity to yeast proteins Pdr5 (29% and 27% identity for MdrA1 and MdrA2, respectively) and Bfr1 (29% and 26% identities). PDR5 is a *S. cerevisiae* gene cloned for its ability to confer resistance to cycloheximide and sulfometuron methyl when in multicopy (Leppert et al., 1990). $bfr1^+$ is a *Schizosaccharomyces pombe* gene cloned by its ability to confer resistance to Brefeldin A (SWISS-PROT accession no. P41820) when in multicopy (Nagao et al., 1995a). The *Drosophila* proteins Scarlet (EMBL/GenBank/DDBJ accession no. P45843) and White show 29% and 30% identity to MdrA1, and 28% and 29% to MdrA2, respectively.

Expression pattern of *mdrA2*

The developmental expression of *mdrA2* was examined by

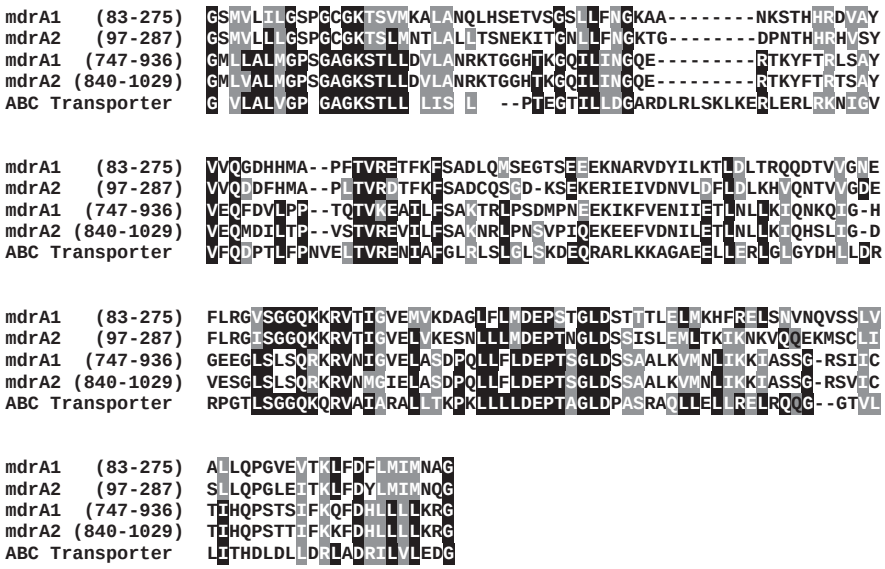

Fig. 2. MdrA1 and MdrA2 have sequence similarity to known ABC transporter proteins. The complete nucleotide and derived amino acid sequence of *mdrA1* and *mdrA2* are available in GenBank as AF246689. The N-terminal and C-terminal ABC transporter domains of MdrA1 and MdrA2 are aligned with the consensus ABC transporter domain from the Pfam protein families database (Bateman et al., 2000). The alignment was done using the program CLUSTAL W (Thompson et al., 1994). Black boxes indicate identical amino acids whereas shaded boxes indicate conservatively substituted residues.

probing a northern blot of RNA from developing cells with a cDNA probe of *mdrA2*. The 4.2 kb *mdrA2* mRNA exhibited a low level of expression in vegetative cells with an increase and slight peak after 5 hours of development. The transcript then dropped back to previous levels until 20 hours, at which point it continued to increase until the end of development at 25 hours (Fig. 3A). To visualize the production of MdrA2 protein, we raised antibodies against a peptide of MdrA2. These antibodies were then affinity purified, and used to stain western blots. To ensure that the antibodies were specific for MdrA2, we used them to stain a western blot of lysates from *mdrA1*$^-$/*mdrA2*$^-$ cells, wild-type DH1 cells, *rtoA*$^-$ cells and *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells (Fig. 3B). Both wild-type and *rtoA*$^-$ cells show a cross-reactive band at approximately 160 kDa, which is absent in the *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ and the *mdrA1*$^-$/*mdrA2*$^-$ cells. This suggests that the antibodies recognize MdrA2. Similar results were obtained with the antibody against MdrA1 (data not shown). To examine the developmental production of MdrA2, we stained a western blot of cell lysates from developing cells (Fig. 3C). As seen with the mRNA, a small amount of protein is present in vegetative cells, and the protein levels peak at roughly 5 hours and decrease by 10 hours. We observed another peak of production at 12.5 hours, which does not correlate with expression of mRNA. Finally, we observed a slight increase in protein levels beginning at 20 hours, which parallels the mRNA expression.

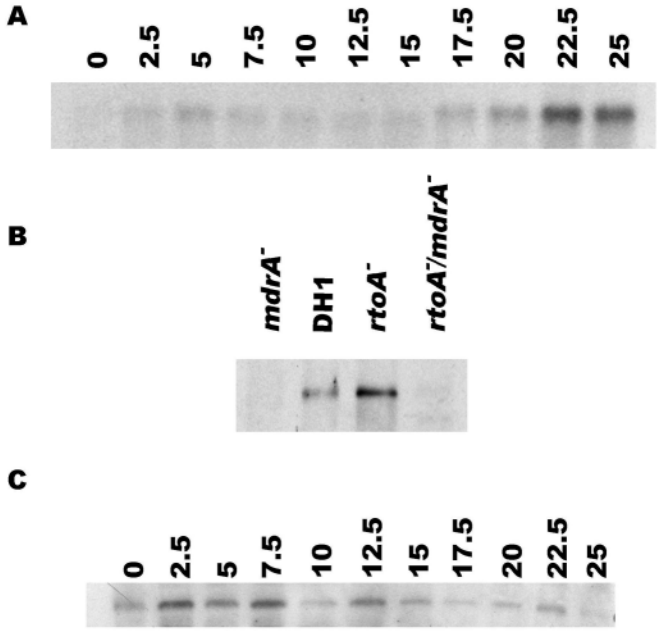

Fig. 3. The expression of *mdrA2* is developmentally regulated. A northern blot of total cellular RNA isolated from DH1 cells at 2.5 hour intervals after starvation on filter pads was probed with a radiolabelled fragment of the *mdrA2* gene (A). A western blot of lysates from 1×10^5 cells of *mdrA1*$^-$/*mdrA2*$^-$, DH1, *rtoA*$^-$ and *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ was probed with affinity-purified anti-MdrA2 peptide antibodies (B). A similar western was performed using 1×10^5 cells of DH1 at 2.5 hour intervals after starvation on filter pads (C). *mdrA*$^-$, *mdrA1*$^-$/*mdrA2*$^-$; *rtoA*$^-$/*mdrA*$^-$, *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$.

Suppression of altered prestalk/prespore ratio

We previously observed that *rtoA*$^-$ cells have an increased percentage of CP2-positive prestalk cells (Wood et al., 1996). To determine if disruption of *mdrA1/mdrA2* affects this increased ratio, we examined differentiation in cells developing on filter pads, as well as in cells starved at low cell density,

Table 1. Cell-type differentiation during normal development

Cell type	Prestalk (%)	Prespore (%)
DH1 parental	9.9±0.1	28.8±0.6
rtoA$^-$	16.2±0.5	33.3±0.4
rtoA$^-$/*mdrA1*$^-$/*mdrA2*$^-$	9.9±0.1	29.5±0.2
mdrA1$^-$/*mdrA2*$^-$	10.3±0.4	29.5±0.1

Cells were allowed to develop for 18 hours on filter pads. The aggregates were then dissociated, and the cells were fixed and stained for the prestalk marker CP2 or the prespore marker SP70. For each experiment, several fields of cells were examined and the total number of cells and the number of positive cells were counted. At least 200 total cells were examined. Values are averages of four independent determinations±s.e.m.

Table 2. Cell-type differentiation during development in submerged culture at low cell density

Cell type	Prestalk (%)	Prespore (%)
DH1 parental	10.7±0.6	32.2±2.7
rtoA$^-$	13.1±0.3	32.4±2.6
rtoA$^-$*/mdrA1*$^-$*/mdrA2*$^-$	10.5±0.6	32.2±4.1
mdrA1$^-$*/mdrA2*$^-$	11.2±1.0	31.3±4.4

Cells were starved in the presence of conditioned medium, and 6 hours after starvation cAMP was added to induce marker expression. At 18 hours after starvation, the cells were fixed and stained for the prestalk marker CP2 or the prespore marker SP70, and the number of positive cells in a well of 2×10^3 cells was counted. Values are averages of four independent determinations±s.d.

where cell-cell communication is minimized. In both cases, disruption of *mdrA1/mdrA2* in a *rtoA*$^-$ background reduced the percentage of CP2-positive prestalk cells to approximately wild-type levels (Table 1; Table 2). The percentage of CP2-positive prestalk cells was also approximately that of wild-type in *mdrA1*$^-$*/mdrA2*$^-$ cells. We also previously observed that disruption of *rtoA* caused a slight increase in the percentage of cells becoming SP70-positive prespore cells (Wood et al., 1996). However, *rtoA*$^-$*/mdrA1*$^-$*/mdrA2*$^-$ as well as *mdrA1*$^-$*/mdrA2*$^-$ cells have apparently normal percentages of cells becoming SP70-positive prespore (Table 1; Table 2).

Wild-type *Dictyostelium* cells use a musical chairs mechanism to choose their initial cell type. For a pair of sister cells in S or early G2 phase, one sister becomes prestalk and the other becomes null; sister cells in late G2 or M become prespore and null (Gomer and Firtel, 1986). This mechanism is disrupted in *rtoA*$^-$ cells, where cells at any phase of the cell cycle can become either prestalk or prespore (Wood et al., 1996). To determine whether disruption of *mdrA1/mdrA2* rescues this defect in *rtoA*$^-$ cells, we videotaped fields of growing cells and then starved them to initiate differentiation and development, as we did previously to obtain the above results. We observed that *rtoA*$^-$*/mdrA1*$^-$*/mdrA2*$^-$ cells had an apparently random pattern of differentiation (Fig. 4), suggesting that disruption of *mdrA1/mdrA2* rescues development and cell-type choice without rescuing the musical chairs mechanism of initial cell-type choice. For several cells, one sister became prestalk with the other sister becoming prespore; in other cases both sisters became prespore.

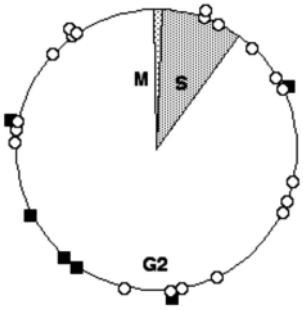

Fig. 4. Differentiation of *rtoA*$^-$*/mdrA1*$^-$*/mdrA2*$^-$ cells. Cells were grown in low-density monolayer culture overnight, with a field of the cells being videotaped. The medium was then gently changed to conditioned starvation buffer, and 6 hours later cAMP was added to induce the expression of cell-type-specific markers. Eighteen hours after starvation the cells were fixed, the videotape was turned off, and the cells were stained for the prestalk marker CP2 and the prespore marker SP70. The time of cytokinesis and the fate of the sister cell for each positive cell were then determined by examining the videotape. Open circles mark the phase that SP70-positive prespore cells happened to be in at the time of starvation, whereas filled squares mark where CP2-positive prestalk cells happened to be.

Subcellular location of MdrA1 protein

To determine the subcellular localization of the MdrA1 protein, cells were stained for MdrA1 by immunofluorescence. In wild-type vegetative cells, the antibodies gave two patterns of localization. One population of cells localized the protein to two or three large vesicles. In the other population, the cells localized the protein to tiny vesicles (Fig. 5B). However, the *rtoA*$^-$ cells only showed the tiny vesicle pattern of staining (Fig. 5F). This pattern of localization is similar to what we

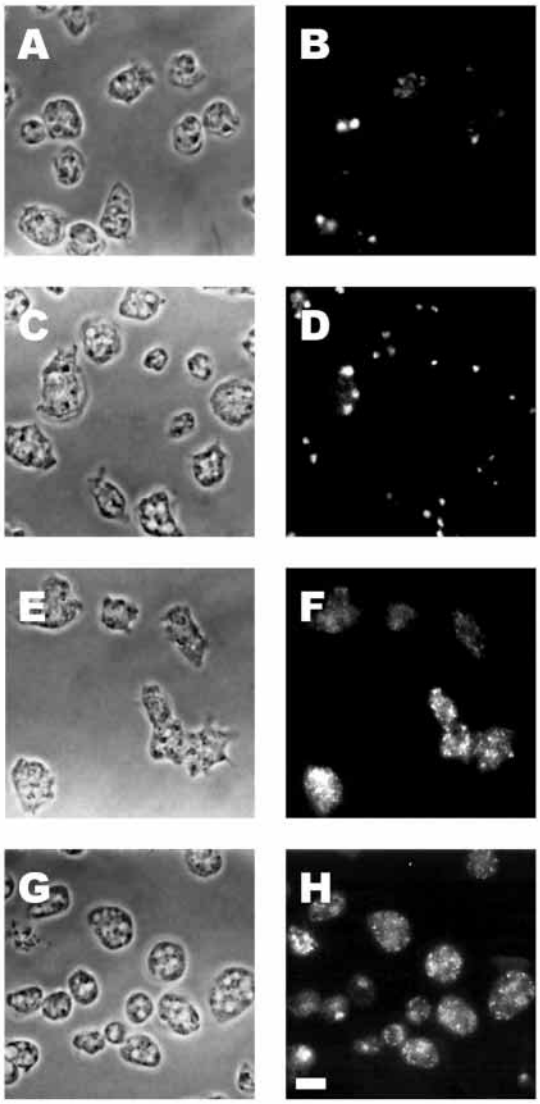

Fig. 5. Immunolocalization of MdrA1. Wild-type DH1 (A-D) and *rtoA*$^-$ (E-H) cells were fixed and stained with anti-MdrA1 antibody raised against the GST-MdrA1 fusion protein (B, D, F, H). The corresponding phase images are shown in A, C, E and G. The cells were either taken directly from growth media (A, B, E, F) or osmotically stressed (C, D, G, H) before fixation. Bar, 10 μm.

previously observed for the distribution of pulse-labeled endosomes approximately 1 hour after ingesting FITC- or RITC-labeled dextran in wild-type and *rtoA*$^-$ cells (Brazill et al., 2000). To determine whether MdrA1 is also localized to the contractile vacuole, colocalization studies were performed with antibodies to calmodulin and MdrA1. The two proteins did not colocalize, suggesting that MdrA1 is not present in the contractile vacuole system (data not shown). To confirm that MdrA1 is in the endosome/lysosome system, we took advantage of the fact that this system will condense into large vesicles when the cells are subjected to osmotic stress (Zhu and Clarke, 1992). We osmotically stressed cells by placing them into 0.1 M sorbitol and examined the localization of MdrA1. In wild-type cells, the MdrA1 protein became localized to large vesicles (Fig. 5B), suggesting that it is associated with the endosome/lysosome system. It is also interesting to note that in *rtoA*$^-$ cells, this condensation did not occur (Fig. 5H).

MdrA1/mdrA2 affects endocytosis

Because MdrA1 appears to be associated with the endosome/lysosome and has sequence similarity to ABC transporters, it could be involved in endosome/lysosome function. To test this possibility, we measured endocytosis by observing the uptake of FITC-dextran over the course of 90 minutes. The *mdrA1*$^-$/*mdrA2*$^-$ cells had an uptake rate roughly 70% that of wild-type. We previously observed that *rtoA*$^-$ cells have a rate of uptake similar to that of wild-type cells (Brazill et al., 2000). *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells exhibited very little internalization of FITC-dextran as compared to wild-type cells (Fig. 6A). We also examined exocytosis by loading cells with FITC-dextran and then observing its release. After 3 hours, the *mdrA1*$^-$/*mdrA2*$^-$ cells had taken up less FITC-dextran than the wild-type cells. Like the wild-type cells, the amount of FITC-dextran inside the cells decreased once the cells were transferred to fresh medium (Fig. 6B). A plot of the percent of the initially ingested material released as a function of time showed that both the *mdrA1*$^-$/*mdrA2*$^-$ and the *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells had exocytosis rates roughly similar to that of wild-type cells. Together, the data indicate that disruption of *mdrA1/mdrA2* somewhat inhibits endocytosis but does not seem to affect exocytosis, and when *mdrA1/mdrA2* is disrupted in *rtoA*$^-$ cells, there is an inhibition of endocytosis greater than that seen in *mdrA1*$^-$/*mdrA2*$^-$ cells alone.

As vesicles progress from endosomes to lysosomes to post-lysosomes, they undergo distinctive pH changes. During the first 20 minutes, the pH of wild-type endosomes drops from that of the medium to 5.0-5.2, and then rises over the next 40 minutes to plateau at a value of 5.8-6.2 (Aubry et al., 1993; Brazill et al., 2000). We previously observed that *rtoA*$^-$ vesicles are acidified much more quickly and acidified to a lower pH than wild-type (Brazill et al., 2000). Similar experiments with *mdrA1*$^-$/*mdrA2*$^-$ cells showed that *mdrA1*$^-$/*mdrA2*$^-$ endosomes have a significantly higher pH than wild-type (Fig. 7). Interestingly, *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells had endosomes with roughly wild-type pHs (Fig. 7). During the first 15 minutes, *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ endosomes had a slightly higher pH

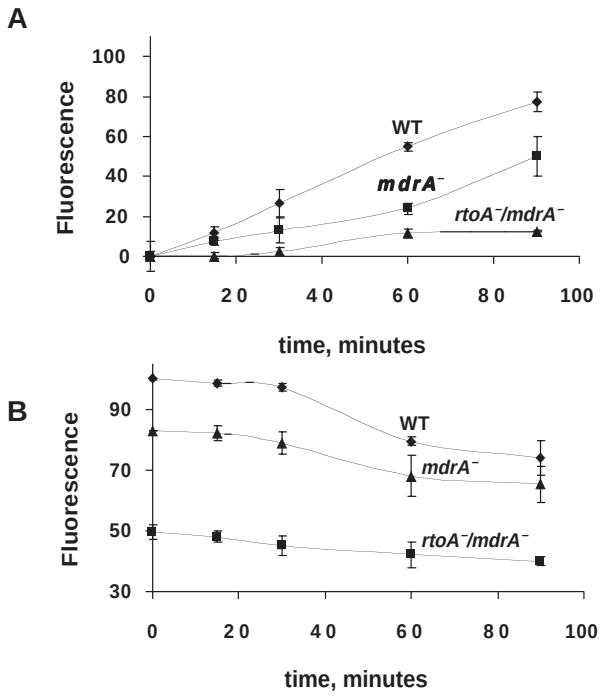

Fig. 6. (A) Endocytosis rates in wild-type, *mdrA1*$^-$/*mdrA2*$^-$ and *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells. Cells were collected and resuspended in HL5 containing FITC-dextran. At the times indicated by the symbols, cells were removed, washed twice in HL5 and once with wash buffer. They were then lysed in wash buffer containing Triton X-100 and the fluorescence measured as described in Materials and Methods. (B) Exocytosis rates in wild-type, *mdrA1*$^-$/*mdrA2*$^-$ and *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells. Cells were allowed to internalize FITC dextran for 3 hours; they were then washed and resuspended in fresh HL5. At the times indicated by the symbols, cells were collected by centrifugation, washed once and lysed in wash buffer containing Triton X-100. The fluorescence was then measured as discussed in Materials and Methods. Values are mean±s.e.m. *mdrA*$^-$, *mdrA1*$^-$/*mdrA2*$^-$; *rtoA*$^-$/*mdrA*, *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$.

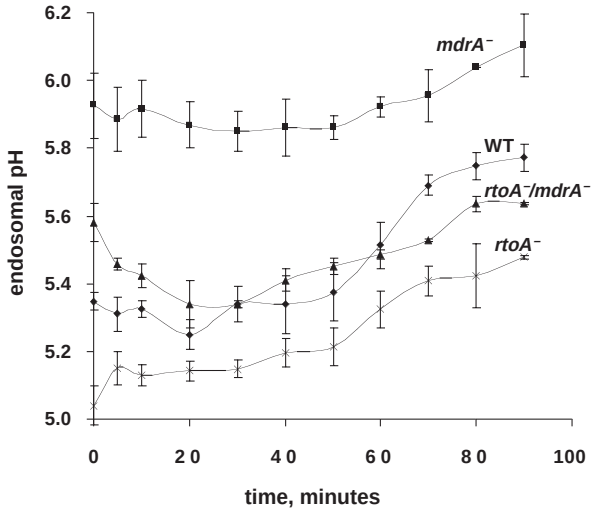

Fig. 7. Endosomal pH in wild-type, *rtoA*$^-$, *mdrA1*$^-$/*mdrA2*$^-$ and *rtoA*$^-$/*mdrA1*$^-$/*mdrA2*$^-$ cells. Cells were fed FITC-dextran for 10 minutes, collected, and resuspended in buffer. At the indicated times, aliquots of cells were removed, centrifuged, and resuspended in assay buffer, and fluorescence was measured using excitation at 450 and 495 nm. The pH values were obtained from a standard curve of the FITC dextran fluorescence ratio as a function of pH. Values are mean±s.e.m.

than wild-type, whereas at 70, 80 and 90 minutes the *rtoA*$^{-}$/*mdrA1*$^{-}$/*mdrA2*$^{-}$ endosomes had a slightly lower pH than wild-type. We previously observed that *rtoA*$^{-}$ cells tend to have higher cytosolic pHs than wild-type cells (Brazill et al., 2000). Similar measurements on *mdrA1*$^{-}$/*mdrA2*$^{-}$ and *rtoA*$^{-}$/*mdrA1*$^{-}$/*mdrA2*$^{-}$ showed that these cells also have a distribution of cytosolic pHs, with averages higher than that of wild-type cells (data not shown). The above data indicate that MdrA1 and MdrA2 help to acidify endosomes, and that disruption of either *rtoA* or *mdrA1/mdrA2* causes cytosolic pH to increase.

DISCUSSION

ABC transporters have been identified in a variety of organisms ranging from bacteria to man. Interestingly, multidrug transport proteins in both eukaryotes and prokaryotes are very similar in structure and function. ABC transporters are commonly divided into two clusters based on amino acid sequence of the NBD, protein topology and drug specificity (Van Veen and Konings, 1997). Members of the multidrug resistance protein (MRP) cluster are identified by the existence of five putative transmembrane segments N-terminal to the characteristic ABC TMDs and nucleotide binding sites. Members of the second group, the Pgp cluster, are important in microbial resistance to neutral or positively charged amphiphilic drugs. These proteins are found in a number of human pathogens including *P. falciparum* (pfMDRA) (Wilson et al., 1989), *Entamoeba histolytica* (ehPgp) (Descoteaux et al., 1992) and *Leishmania donovani* (ldMDRA) (Henderson et al., 1992), as well as in the non-pathogenic yeast *S. cerevisiae* (Pdr5p and Snq2p) (Balzi et al., 1994; Decottignies et al., 1995). *Dictyostelium* MdrA1 and MdrA2 appear to belong to this second cluster. Most ABC transporters regulate traffic across the plasma membrane. However, there are a number that are localized to intracellular membranes. One such protein is a major component of the peroxisome membrane and may be involved in protein import into this organelle (Gartner et al., 1992; Gartner and Valle, 1993; Kamijo et al., 1992). Another is PfMDRA from *Plasmodium*, which is localized to its digestive vacuole and is believed to be involved in pH maintenance (Cowman et al., 1991; van Es et al., 1994b). Expression of Pgh1 causes the lysosomes to acidify (van Es et al., 1994b). *Dictyostelium* MdrA1 is localized to endosomes (Fig. 5), and appears to perform a similar function, as disruption of *mdrA1/mdrA2* in either a wild-type or a *rtoA*$^{-}$ background causes endosomal pH to increase. The normal function of MdrA1 and MdrA2 thus appears to help keep the endosome properly acidified.

mdrA1/mdrA2 was selected as a suppressor of the *rtoA* developmental defect, suggesting some aspect of MdrA1 and MdrA2 function counteracts normal RtoA function. One defect associated with the *rtoA* mutation is improper endosomal/lysosomal function, including poor exocytosis and an inability to condense the endosome/lysosome in response to osmotic stress (Brazill et al., 2000). Thus, it is reasonable to expect that a mutation that suppresses the *rtoA* phenotype may be involved in the functioning of this system. Indeed, MdrA1 protein is localized to the endosome/lysosome. In addition, *mdrA1*$^{-}$/*mdrA2*$^{-}$ cells are partially defective in pinocytosis, an endosomal function. When the *mdrA1/mdrA2* mutation is placed in the *rtoA*$^{-}$ background, this defect is accentuated, suggesting that these two proteins operate in the same system. However, as RtoA is localized to the cytoplasm and possibly small vesicles (Brazill et al., 2000), and MdrA1 is associated with endosomes, these proteins probably do not form a stable complex, and their interaction is strictly genetic. Taken together, these facts argue strongly for a role of MdrA1 and MdrA2 in proper functioning of the endosome/lysosome and it is the disruption of this function that allows the *mdrA1/mdrA2* mutation to suppress *rtoA*$^{-}$. Of course, we have not ruled out the possibility that MdrA1 and MdrA2 may have other functions during development, and it is the loss of these functions that suppress the *rtoA* phenotype, especially the arrest at the mound stage. However, the experiments presented here support the role of the *mdrA1/mdrA2* mutation in suppressing the *rtoA* endosomal defects.

pH and cell-type choice

An important aspect of the *rtoA* phenotype is the inability of this strain to properly proportion its cells into prestalk and prespore cells at the initiation of development (Wood et al., 1996). This defect stems from a disruption of the musical chairs mechanism, which *Dictyostelium* uses to regulate initial cell-type choice. Disruption of *mdrA1/mdrA2* is able to suppress the gross inability of *rtoA*$^{-}$ cells to develop past the mound stage, and also causes *rtoA*$^{-}$ cells to initially differentiate into the correct percentages of prestalk and prespore cells (Table 1; Table 2). Although it has not been shown that the alteration of the initial percentages of prestalk and prespore cells is directly responsible for the arrest seen at the mound stage, it is none the less significant that loss of *mdrA1* and *mdrA2* can correct both phenotypes. However, this is not accomplished by repairing the musical chairs mechanism (Fig. 4). Strains carrying mutations in *mdrA1/mdrA2* and *rtoA* still recruit prestalk cells from throughout the cell cycle instead of from cells in S and early G2 phases. These strains are also unable to restrict prespore cells from initially coming from only late G2 and M phases. Thus, mutating *mdrA1* and *mdrA2* is able to suppress the altered prestalk/prespore phenotype of *rtoA*$^{-}$ in a manner that bypasses the cell-cycle-dependent cell-type-choice mechanism.

It has been theorized that pH plays a vital role in controlling differentiation in *Dictyostelium* (Furukawa et al., 1988). Acidification favors prestalk development, whereas alkalization promotes prespore differentiation. However, this regulation is thought to be based not on the pH of the cytoplasm, but on the pH of a specific class of vesicles (Gross et al., 1988). In prestalk cells, these vesicles would have a lower pH, whereas the same vesicles in prespore cells would have a higher pH. Mutants with defective vacuolar H^{+}-ATPases have abnormal endosomal pHs but normal cytosolic pHs, suggesting that there is not an absolute connection between the pH of a vesicle and cytosolic pH (Aubry et al., 1993; Davies et al., 1996). Our data support the idea that cytosolic pH does not determine initial cell-type choice, as *rtoA*$^{-}$ shows a high cytosolic pH and a high prestalk/prespore ratio, whereas *rtoA*$^{-}$/*mdrA1*$^{-}$/*mdrA2*$^{-}$ cells also have a high cytosolic pH but have normal prestalk/prespore ratios. *rtoA*$^{-}$ cells have endosomes with a lower pH than wild-type cells (Brazill et al., 2000). In addition, *rtoA*$^{-}$ strains have a higher percentage of prestalk cells than wild-type cells. Because *mdrA1*$^{-}$/*mdrA2*$^{-}$

cells have endosomes with higher pHs than wild-type, but initially differentiate into essentially normal numbers of prestalk and prespore cells (Table 1; Table 2), our data indicate that endosomal pH is not the primary determinant of the initial cell-type choice. This suggests either that vesicles that are not part of the endosome/lysosome system are responsible for a pH regulation of differentiation, or that something such as Ca^{2+} levels may regulate the initial cell-type choice (Azhar et al., 1998; Azhar et al., 1996; Saran et al., 1994).

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