rZIP, a RING-leucine zipper protein that regulates cell fate determination during *Dictyostelium* development

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SUMMARY

rZIP is an approx. 32 kDa, multi-domain protein of *Dictyostelium discoideum* whose structural motifs include a RING (zinc-binding) domain, a leucine zipper, a glutamine repeat, an SH3-binding region and a consensus phosphorylation site for MAP kinase. In vitro, rZIP forms homodimers and interacts specifically with the SH3 domain(s) of the Nck adaptor protein. rZIP is expressed maximally during cell differentiation at approximately equivalent levels in all cells. Disruption of the rZIP gene *rzp*A results in altered cellular aggregation, impaired slug migration, and aberrant patterning of prespore and prestalk cells, the major progenitor classes. In rzpA⁻ strains, prespore-specific genes are overexpressed and prestalk expression

zones are reduced. Conversely, constitutive overexpression of *rzp*A markedly decreases prespore-specific gene expression and significantly increases the expression of prestalk-specific genes. Further, induced transdifferentiation of prespore cells into prestalk cells is inhibited in rzpA⁻ slugs. In light of these patterning defects, we suggest that the RING/zipper protein rZIP plays an important role in early cell fate decisions in *Dictyostelium*, acting as a positive regulator of prestalk differentiation and an inhibitor of prespore differentiation.

Key words: zinc fingers, cAMP, SH3 domains, trinucleotide repeats, *Dictyostelium*

INTRODUCTION

In many developmental systems, cell fate is determined by cell-surface receptor mediated regulation of downstream pathways. The relative activity of various protein kinases is often central to this regulation, but it is increasingly evident that communication among components within a pathway is often dependent upon additional associations. Many specific homologous and heterologous interactions involve a variety of motifs that are common to proteins of diverse function. In some instances, individual proteins possess more than one interaction domain, allowing multimeric associations among three or more components. In addition, protein-protein binding is often regulated by site-specific phosphorylation. Protein-interaction motifs that are required for signal control of differentiation include SH3 domains, SH2 domains (Pawson and Schlessinger, 1993), leucine zippers (Alber, 1992), and PH domains (Bork, 1996). Other modular sequences, such as the zinc-binding RING (C₃HC₄) domain (Saurin et al., 1996) and the glutamine repeat (Jennings, 1995), may be similarly essential. We have now identified rZIP, a new protein in *Dictyostelium discoideum*, that contains several of these structural motifs and regulates cell determination during development, possibly through a receptor mediated intracellular signalling pathway.

Dictyostelium has a well characterized life cycle in which growth and development are effectively independent (Firtel,

1995; Loomis, 1982). Under appropriate conditions, cells grow and divide continuously. As nutrients become scarce, a developmental process is induced that is completed within approx. 24 hours. Individual cells secrete cAMP and recruit neighboring cells by stimulation of G protein-linked, cell-surface cAMP receptors that relay this signal and direct chemotactic movement. Many subsequent aspects of differentiation and pattern formation throughout development are also regulated by these receptors that are linked to distinct pathways of intracelluar signalling (Ginsburg et al., 1995; Kimmel and Firtel, 1991). Cells converge at the centers of cAMP synthesis, form loose aggregation mounds, and differentiate into prespore and prestalk cells. As the aggregates become more compact, extracellular cAMP levels rise and the differentiated cell types sort from one another. An extended tip of prestalk cells forms and the mound elongates into a pseudoplasmodium or slug. The prestalk cells constitute the anterior (approx. 20%) of the slug and the prespore cells compose the posterior (approx. 80%). In addition, a small population of prestalk cells, the anterior like cells (ALCs), are dispersed through the prespore region. Upon completion of morphogenesis the slug forms a mature fruiting body, consisting of a stalk of vacuolated cells that supports a spherical mass of single-celled spores. The non-terminally differentiated prestalk and prespore cells, respectively, give rise to the stalk and spore cells. The basal disc at the foot of the stalk and the upper and lower cups, groups of cells which cradle the spore head, derive from the ALCs.

Here we describe rZIP, a modular protein that plays a role in regulating differentiation and patterning during *Dictyostelium* development. rZIP forms homo- and hetero-dimers in vitro and represses prespore but activates prestalk differentiation in vivo. The multiple domains of rZIP include a RING motif, a leucine zipper, a glutamine repeat, an SH3 binding region and a consensus phosphorylation site for MAP kinase. We suggest that these domains contribute to the complex signalling pathways during *Dictyostelium* development that originates with the surface receptors for cAMP and regulates cell determination. rZIP further provides an excellent system to examine the interdependence of these protein motifs in pattern formation.

MATERIALS AND METHODS

Growth and development

Dictyostelium were grown axenically with 100 µg/ml thymidine and 20 µg/ml G418 as required. Cells were plated for development on nitrocellulose filters at 3×10^6 cells/cm^2 and on water-agar plates at 5.1×10^5 or 1.3×10^5 cells/cm^2. For transdifferentiation, slugs were allowed to develop on agar for 16-18 hours and cut using aluminum foil.

Construction of rzpA$^-$ and rzpA^{OE} strains and of the *rzpA* promoter/*lacZ* fusion

A *Bgl*II site was introduced into the leucine zipper region of *rzp*A and the *THY1* gene was inserted into this site. This entire fragment was transformed into the thy1$^-$ strain JH10 (Saxe III et al., 1993). rzpA$^-$ transformants were identified by PCR and Southern analyses. For *rzp*A over-expression, a full-length *rzp*A clone fused behind the actin 15 promoter was transformed into wild-type *Dictyostelium*.

To isolate the promoter for *rzp*A, we subcloned the 2 kb *Eco*RI/*Bam*HI fragment of the *rzp*A 5′ region from a genomic library. The *Bam*HI site is 6 bp upstream of the translation start site and approx. 225 bp 3′ to the transcriptional start site. The *Eco*RI site lies at least 800 bp upstream of the translational stop codon of the adjacent upstream open reading frame (Ginsburg and Kimmel, unpublished). This *Eco*RI/*Bam*HI fragment was fused to *lacZ* on a neomycin selectable vector.

β-galactosidase staining

Organisms on nitrocellulose were stained as described by Richardson et al. (1994). Vegetatively growing cells were allowed to adhere to a glass microscope slide for 30 minutes in a drop of axenic medium. The medium was then removed and the cells stained as before.

rZIP protein and antibody preparation and SH3 domain interaction

rZIP was expressed with a six-histidine amino-terminal tag using QIAexpressionist and the fusion protein purified by Ni^{2+} affinity chromotography (Qiagen). Rabbits immunized with the purified rZIP fusion produced polyclonal antibodies against rZIP. GST fusions with SH3 domains of Fyn, α-spectrin, phospholipase Cγ1, Eps8 and the three SH3 domains of Nck have been described previously (Rivero et al., 1994). Interaction studies between rZIP and these SH3 domains were conducted as described by Keane et al. (1995).

In vitro protein interaction

The leucine zipper/RING domain (see Fig. 1A) portion of *rzp*A was amplified by PCR and subcloned into pAR(ΔRI) (Blanar and Rutter, 1992). This construct directed expression of the leucine zipper/RING domain fragment fused to a FLAG recognition sequence (IBI) and a heart muscle kinase (HMK) phosphorylation site. The expressed

peptide was purified using the FLAG system. Labelling of the peptide and binding studies were carried out as previously described (Blanar and Rutter, 1992; Li et al., 1992).

RNA analyses

Total RNA was isolated, size separated and hybridized to blots as described by Kimmel (1987).

RESULTS

rZIP is a modular protein

*rzp*A was isolated as a partial length cDNA (SC1) that is a member of a family of *Dictyostelium* genes containing the trinucleotide repeat (AAC)$_n$ (Kimmel and Firtel, 1985). We have now isolated full length cDNA and genomic DNA fragments of *rzp*A and determined their entire sequences. *rzp*A is a single-copy gene in *Dictyostelium* (Kimmel and Firtel, 1985) and its cDNA and genomic sequences (GenBank accession number U67917) are colinear, indicating a lack of introns. The encoded protein, rZIP, is 271 amino acids (approx. 32 kDa) and contains several notable motifs, including a glutamine repeat, a RING (C$_3$HC$_4$) domain and a leucine zipper (Fig. 1A).

A perfect in-frame (CAA)$_{14}$ within the translated region encodes the glutamine repeat Q$_{14}$. Degenerate variations of (CAA)$_n$ give rise to runs of asparagines [(AAT)$_4$] and threonines [(AC^A/$_T$)$_{11}$]. Glutamine repeats are common to many developmentally regulated genes in *Dictyostelium* (Kimmel and Firtel, 1985), and other phyla (Bierkamp and Campos-Ortega, 1993; Duboule et al., 1987; Wharton et al., 1985).

The RING (C$_3$HC$_4$) domain, located near the carboxyl

A

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MDPLEEDFAFKYFTKGAPIKVESVLNFDLKEHLKSPYLWTTIVAGLGLAYYFLT

                                        <-- (CAA)₁₄ -->
SDDEIGIGPAGAFENNNAENGVDNFNQYNYINQDVIPPPQQQQQQQQQQQQQQN

            <- (ACᴬ/ᴛ)₁₁ ->        *
NHIHHNHNHSHNNNNHHNHVQQTTTTTTTTTTTTSSSFLPITPPPPSNVQHVKLK
                                  -------

        <------ ZIPPER ------>
NKNGEPLPLEEQNLLLLRENSNLRGENRDLRVVVGNQINNINDRMEKMQTDLER

    <----------------- RING ----------------->
EKFCHICEENEKQVCWRECGHRLCARCATLIKKTSHPQCSICRKLVSDFYLAWNT
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B

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        C C            C H C C           C C
rZIP    CHICEENEKQVCWRECGHRLCARCATLIKKTSHPQCSIC
        C IC EN+K V    CGH +C  C T  +++    C C
c-Cbl   CKICAENDKDVKIEPCGHLMCTSCLTSWQESEGQGCPFC
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Fig. 1. The rZIP protein. (A) Conceptual amino acid sequence of rZIP. The glutamine repeat Q$_{14}$ is encoded by the trinucleotide repeat (CAA)$_{14}$ and a threonine stretch is encoded by (AC^A/$_T$)$_{11}$. The proline-rich region is underlined, the leucine zipper and RING domain are highlighted. Bent arrows denote limits of the region encompassed in the HMK-zipper/RING fragment used for dimerization studies (see Fig. 2A). *Potential phosphorylation within the MAP kinase site PITP. (B) Sequence allignment of the RING domains of rZIP and c-Cbl. The conserved amino acids C$_3$HC$_4$ are identified. Identities and similarities (+) between the two RING domains are noted.

terminus of rZIP, is a well conserved zinc-binding motif defined by the consensus sequence:

$CX_2CX_{9-39}CX_{1-3}HX_{2-3}CX_2CX_{4-48}CX_2C$ (see Fig. 1B). RING domains are essential for the activity of various developmentally regulated genes and are suggested to mediate protein-protein interaction (Saurin et al., 1996).

Leucine zippers mediate protein dimerization (Alber, 1992). A leucine zipper motif, defined by four leucyl residues repeated every seventh amino acid, is evident near the central region of rZIP. Helical wheel analysis indicates that the intervening sequences can be modelled into an amphipathic helix with an incremental angle of 103º yielding an exact overlay of the four leucines.

When the entire rZIP protein sequence was compared by BLAST analyses (Altschul et al., 1990) of various databases,

most identities were aligned due to shared glutamine repeats or RING domains. The RING domains of c-Cbl, encoded by the mouse proto-oncogene *c-cbl* (Langdon et al., 1989) and the related human protein Cbl-b (Keane et al., 1995) have the greatest similarity to that of rZIP (Fig. 1B). These RINGs are approx. 40% identical (approx. 50% similar) in amino acid sequence with that of rZIP and share identical spacings of the critical C_3HC_4 sequences. Although rZIP differs greatly from the Cbl family outside of the RING motif, all three proteins have leucine zippers and proline-rich regions. In rZIP, the latter region forms part of the core for the consensus MAP kinase phosphorylation site PITP (see Fig. 1A).

rZIP will form homodimers in vitro
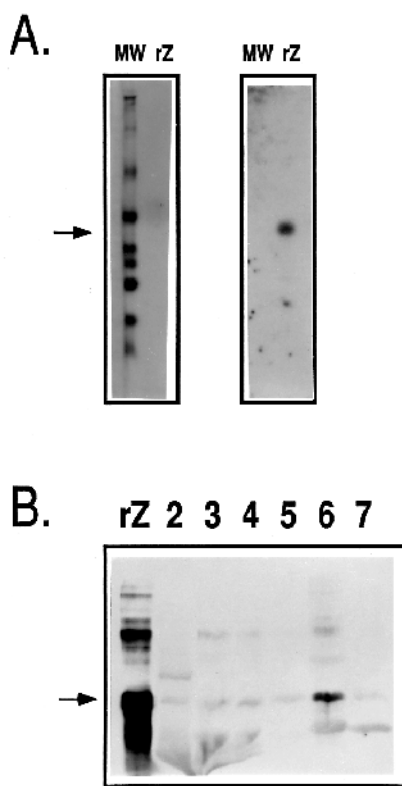
To analyze potential homologous interactions between rZIP

Fig. 2. Dimeric associations of rZIP. (A) In vitro homodimerization of rZIP. Purified H_6-rZIP fusion (rZ; <10 ng) was subjected to SDS gel electrophoresis, blotted to filters, and probed with the radiolabelled HMK-zipper/RING fragment of rZIP (see Fig. 1A). Left panel shows stained gel with size markers (MW). The right panel shows the autoradiograph of an equivalent blot. Arrow indicates the rZIP fusion. (B) In vitro association of rZIP with SH3 domains. Purified rZIP protein was incubated with various GST-SH3 domain fusions pre-bound to agarose-GSH. Following washing, the bound proteins were eluted, fractionated and blotted for western analysis with polyclonal anti-rZIP sera. The arrow indicates the rZIP band and the specific association of rZIP with the SH3 domains of Nck. Lane 1 contains 1 mg of purified H_6-rZIP fusion (rZ). Lanes 2-7 are proteins eluted from the specific agarose-GSH complexes. Lane 2 is a negative control with GST alone. The other lanes feature the GST fused to the SH3 domains of: Fyn (lane 3), phospholipase Cγ1 (lane 4), Eps8 (lane 5), the three SH3 domains of Nck (lane 6), and α-spectrin (lane 7). The bands in lane 1 above the rZIP band are multimers of rZIP.
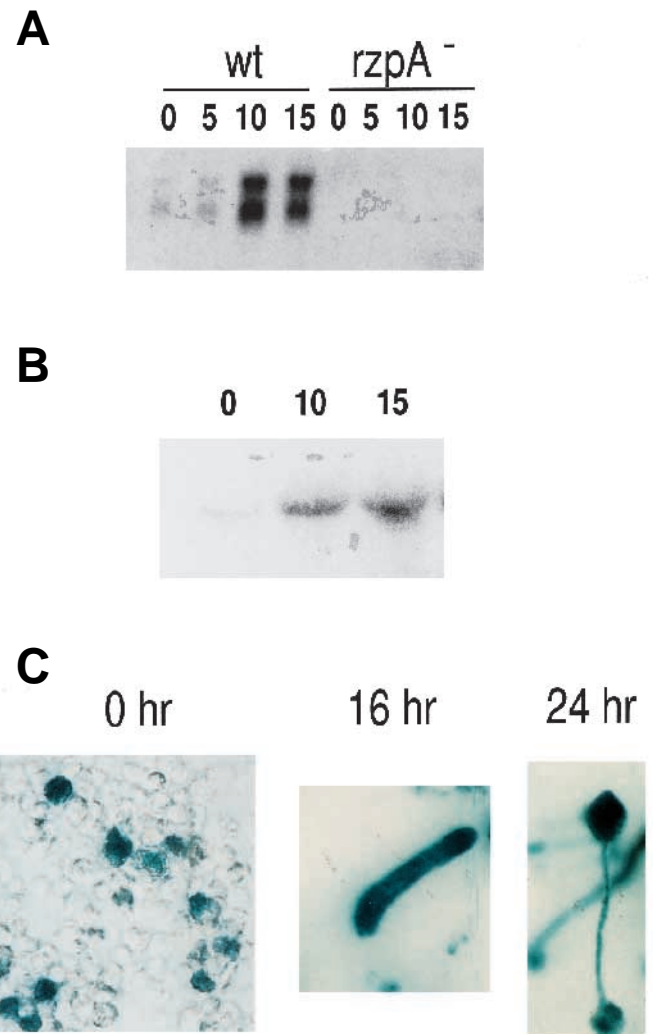

Fig. 3. *rzp*A expression. (A) Temporal expression of *rzp*A. Developmental northern blot of RNAs isolated from wild-type and rzpA⁻ strains during growth (0) or at hours of development (indicated at the top) and hybridized with an *rzp*A probe.
(B) Temporal expression of *rzp*A/*lac*Z. Developmental northern blot of RNAs isolated from *rzp*A/*lac*Z strain during growth (0) or at hours of development indicated and hybridized with a *lac*Z probe.
(C) Spatial expression of *rzp*A/*lac*Z. The *rzp*A/*lac*Z strain was stained for β-galactosidase activity in vegetative cells (0 hr), slugs (16 hr) and fruiting bodies (24 hr).

molecules, we used an in vitro protein blot assay. The *rzp*A sequence was truncated just upstream of the encoded leucine zipper and downstream of the RING domain (see Fig. 1A) and fused in frame at its new 5′ terminus to sequences encoding a FLAG-tag and a phosphorylation site for heart muscle kinase (HMK; Blanar and Rutter, 1992). This HMK-zipper/RING fusion was expressed in *E. coli*, purified by affinity to FLAG antisera, and radiolabelled in vitro with HMK and [γ^{32}P]ATP. In addition, full length rZIP was expressed in *E. coli* as an amino-terminal fusion with a six-histidine tag, purified by Ni^{2+} affinity chromatography, subjected to SDS gel electrophoresis and blotted to membrane filters. The bound protein was re-folded in situ and incubated with the radiolabelled HMK-zipper/RING fragment. A specific complex was observed with full-length rZIP (Fig. 2A). Binding to the size marker proteins (see Fig. 2A) or to whole cell protein extracts of *E. coli* or *Dictyostelium* was not detected (data not shown). Truncated forms of HMK-rZIP containing just the glutamine repeat, leucine zipper or RING domain were unable to form specific associations with full-length rZIP (data not shown).

rZIP binds specific SH3 domains in vitro

The Src homology 3 (SH3) domain is a approx. 50 amino acid sequence that interacts with specific proline-rich sequences (Cichetti et al., 1992; Mayer and Eck, 1995). Protein complexes stablized by these interactions are essential for many aspects of intracellular signalling (Pawson and Schlessinger, 1993). Various members of the SH3 family bind distinct proline-rich sequences preferentially. Cbl-b and c-Cbl will bind specific SH3 domains in vitro, presumably through interaction of their proline-rich regions (Keane et al., 1995; Rivero et al., 1994). We now demonstrate that rZIP is similarly capable of forming specific complexes with a subset of SH3 domains. In particular we tested rZIP binding to a variety of expressed SH3 domains from the tyrosine kinase Fyn, phospholipase Cγ 1 (PLC), cytoskeletal protein spectrin (Spc), receptor tyrosine kinase substrate Eps8, and the adaptor protein Nck. Purified rZIP fusion protein was incubated in vitro with mass equivalent quantities of various glutathione-S-transferase (GST) fusions to the various SH3 domains that had been pre-bound to agarose-glutathione (GSH). After extensive washing, the proteins that were retained on the agarose-GSH were eluted in SDS buffer, separated by SDS-PAGE, and blotted for western analysis against polyclonal antisera to purified rZIP. rZIP displayed specific interaction with SH3 domains of only one protein, Nck (Fig. 2B). rZIP did not interact significantly with GST fusions to the SH3 domains of Fyn, PLCγ, Eps8, or Spc, or with the control construct carrying only the GST domain.

Temporal and spatial expression of *rzp*A

The temporal expression pattern of *rzp*A was determined by developmental northern blot analysis (Fig. 3A). Vegetatively growing cells (0 hours) expressed low levels of *rzp*A mRNA but levels increased substantially as the differentiated cells appeared during the mound stage (approx. 10 hours). Expression levels remained unchanged through the rest of devel-

opment. Two different sized transcripts were detected at all developmental times examined, but since the gene does not contain any introns, they do not encode multiple forms of the rZIP protein.

To examine the cell-specific expression of *rzp*A we fused its full-length promoter to the reporter gene *lac*Z and monitored the expression and spatial patterning of the encoded β-galactoidase activity during *Dictyostelium* development. Northern analysis showed that a 2kb *rzp*A genomic fragment, including 225 bp of 5′-untranslated sequence and extending into the adjacent upstream open reading frame, directed fused *lac*Z expression in a pattern that is temporally similar to that of endogenous *rzp*A and consistent with the action of a full-length *rzp*A promoter (Fig. 3B). During growth (0 hr) when expression is very low, only approx. 5% of the cells had detectable β-galactosidase activity (Fig. 3C). When the strains were developed for various times, β-galactosidase staining was observed in all regions of the slug (16 hours) and the fruiting body (24 hours), indicating that *rzp*A is expressed in all cell-types throughout development (Fig. 3C).

rZIP is required for normal cellular motility during development

We created an rzpA$^-$ strain by homologous recombination within the endogenous gene of a wild-type organism. Three separate clonal isolates of rzpA$^-$ were confirmed by PCR and Southern and northern blot analyses (see Fig. 3A) and showed
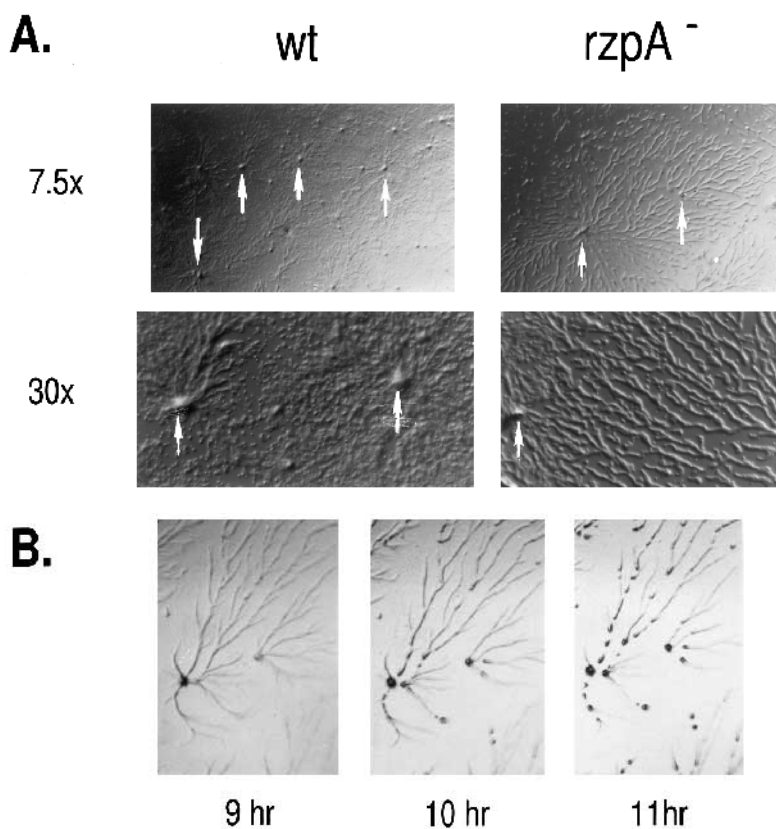
A.

7.5x

30x

B.

Fig. 4. Aggregation of developing wild-type and rzpA$^-$ strains. (A) Aggregation streams at low cell density (1.3×10^5 cells/cm^2) at magnifications indicated. Arrows indicate some aggregation centers. (B) Disruption of rzpA$^-$ streams into multiple centers of aggregation at times indicated.

identical developmental phenotypes. Although the rzpA⁻ strains were able to proceed through all the major stages of development, they displayed some prominent abnormalities. Their development was delayed by approx. 2 hours as compared with the wild-type strain, and during aggregation at a density of 1.3×10^5 cells/cm^2 the rzpA⁻ strains formed longer aggregation streams than did wild-type cells (Fig. 4A) and many fewer streaming centers (approx. 20% of wild-type; see Fig. 4A and Table 1). Rather than coalescing to a single center, individual rzpA⁻ streams often fractured along their length and formed subsidiary aggregates of variable size (Fig. 4B). At a higher cell density (5.1×10^5 cells/cm^2), where cell-cell communication was enhanced, the differences in aggregation patterns between the rzpA⁻ and wild-type strains were less apparent (Table 1).

Slugs of the rzpA⁻ strains were less elongate and much less motile than those of wild-type. When subjected to unidirectional light for 60 hours, wild-type slugs migrated an average distance of approximately 4 cm, whereas the rzpA⁻ slugs were
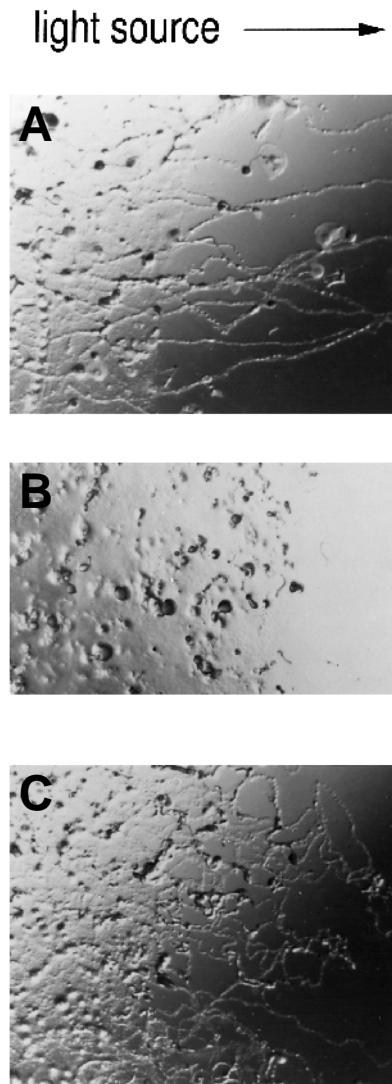
Table 1. Effect of cell density on aggregation of rzpA⁻ cells

| | Cell density (cells/cm^2) | |
Strain	1.3×10^5	5.1×10^5
Wild-type	68.8±18.9*†	35.2±28.6*
rzpA⁻	13.0±12.9*†	37.9±23.6*

*Aggregation sectors per 2.2 cm^2.
†Difference is significant to 1% level.

nearly immobile and often developed into fruiting bodies at the site of aggregation (Fig. 5).

Cell-type specific gene expression is perturbed in rzpA⁻ strains

Developmental nothern blots of RNA from wild-type and rzpA⁻ strains were hybridized with probes to prestalk and prespore cell-specific genes (Fig. 6). Expression of the prestalk-specific ecmA and ecmB mRNAs was slightly reduced in rzpA⁻ strains relative to that of wild type, while expression of prespore-specific cotB, pspA and pspB mRNAs (data not shown for pspB) was both precocious and significantly higher in rzpA⁻ cells.

The spatial patterns of cell-type specific gene expression in the rzpA⁻ strains were examined by use of the lacZ reporter gene fused to cell-type specific promoters. In wild-type slugs carrying prestalk-specific ecmA/lacZ, β-galactosidase activity was concentrated in the anterior, prestalk zone, with the weaker activity in anterior like cells (ALCs) scattered through the posterior, prespore region (Fig. 7A-1). In contrast, prestalk ecmA/lacZ expression in rzpA⁻ slugs was restricted to a smaller anterior zone and nearly absent in the ALCs. The lower cups and basal discs which derive from ALCs did not express β-galactosidase in rzpA⁻ fruiting bodies marked with ecmA/lacZ (Fig. 7A-2). Similarly, prestalk ecmB/lacZ expression in rzpA⁻ cells was not detected in ALCs distributed among the prespore cells, as observed with wild-type, but was restricted to the very rear of the slug (the rearguard zone) and to the central core of
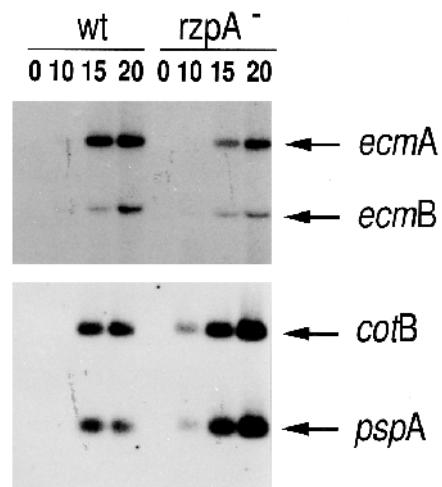

Fig. 5. Migration of (A) wild-type, (B) rzpA⁻ and (C) rzpA^{OE} strains. Cells developed for 60 hours in unidirectional light on agar plates. Arrow indicates direction of migration toward the light source.

Fig. 6. Cell-specific expression in rzpA⁻ cells. Northern blot of wild-type and rzpA⁻ RNA during growth (0) or at hours of development indicated, hybridized with probes of the prestalk-specific genes ecmA and ecmB and the prespore-specific genes cotB and pspA.

the anterior tip (Fig. 7B-1), and was reduced in the lower cups of the fruiting bodies (Fig. 7B-2).

In wild-type slugs carrying prespore *cot*B/*lac*Z, β-galactosidase was expressed primarily throughout the prespore zone, but additional weak blue staining was observed in some cells of the prestalk zone (Fig. 7C). It has been previously shown that this anterior staining resulted from the transdifferentiation of *lac*Z-expressing prespore cells into prestalk cells and their migration to the slug anterior (Detterbeck et al., 1994). Although the rzpA⁻ slugs carrying the *cot*B/*lac*Z construct also displayed very strong β-galactosidase activity in the prespore region, staining was absent from the anterior and the very posterior of the slug. There was near mutual exclusion of the prespore (*cot*B) and prestalk (*ecm*A) expression patterns in the rzpA⁻ slugs, suggesting that prespore to prestalk cell transdifferentiation was disturbed. The reduction in ALCs in the rzpA⁻ strain was consistent with their postulated role as intermediates between prespore cells and anterior prestalk cells in the transdifferentiation pathway (Abe et al., 1994; Sternfeld, 1992).

rzpA⁻ prespore cells are inhibited in transdifferentiation

The perturbations in spatial patterning of cell-specific expression in the rzpA⁻ strains suggested that their prespore to prestalk cell transdifferentiation was disrupted. To examine this more directly, we sliced wild-type and rzpA⁻ slugs transversely, just posterior to the prestalk/prespore boundary, and allowed the resultant 'halves' of each slug to resume development (Fig. 8). As previously shown, when the wild-type slugs were sectioned, each separate half formed a new slug with cell-type proportions that were similar to the original (Fig. 8; Raper, 1940). Since the back halves of slugs consisted almost entirely of prespore cells, a substantial transdifferentiation of prespore cells to prestalk cells was required to maintain constant prespore/prestalk proportions. Invariably, the front halves of the wild-type reformed slugs had completed development within 5 hours, approx. 5 hours earlier than did the rear halves. For Fig. 8, the front-half of the wild-type slug was removed from the microscope field.

The front halves of the rzpA⁻ slugs reformed slugs in a manner similar to the front halves of wild-type but, not surprisingly, these resulting rzpA⁻ slugs were unable to migrate (Fig. 8; see also Fig. 5).

Although mutant structures were delayed by approx. 1 hour as compared with the front-halves of wild-type slugs, they still completed development approx. 4 hours earlier than did the rear-halves of the wild-type strain. In contrast, the rear-halves of rzpA⁻ slugs remain arrested as mounds through the duration of the experiment (36 hours). If the rear halves of the rzpA⁻ organisms were kept in the dark, they sometimes elongated into slugs. However, rear slug formation of rzpA⁻ was delayed until
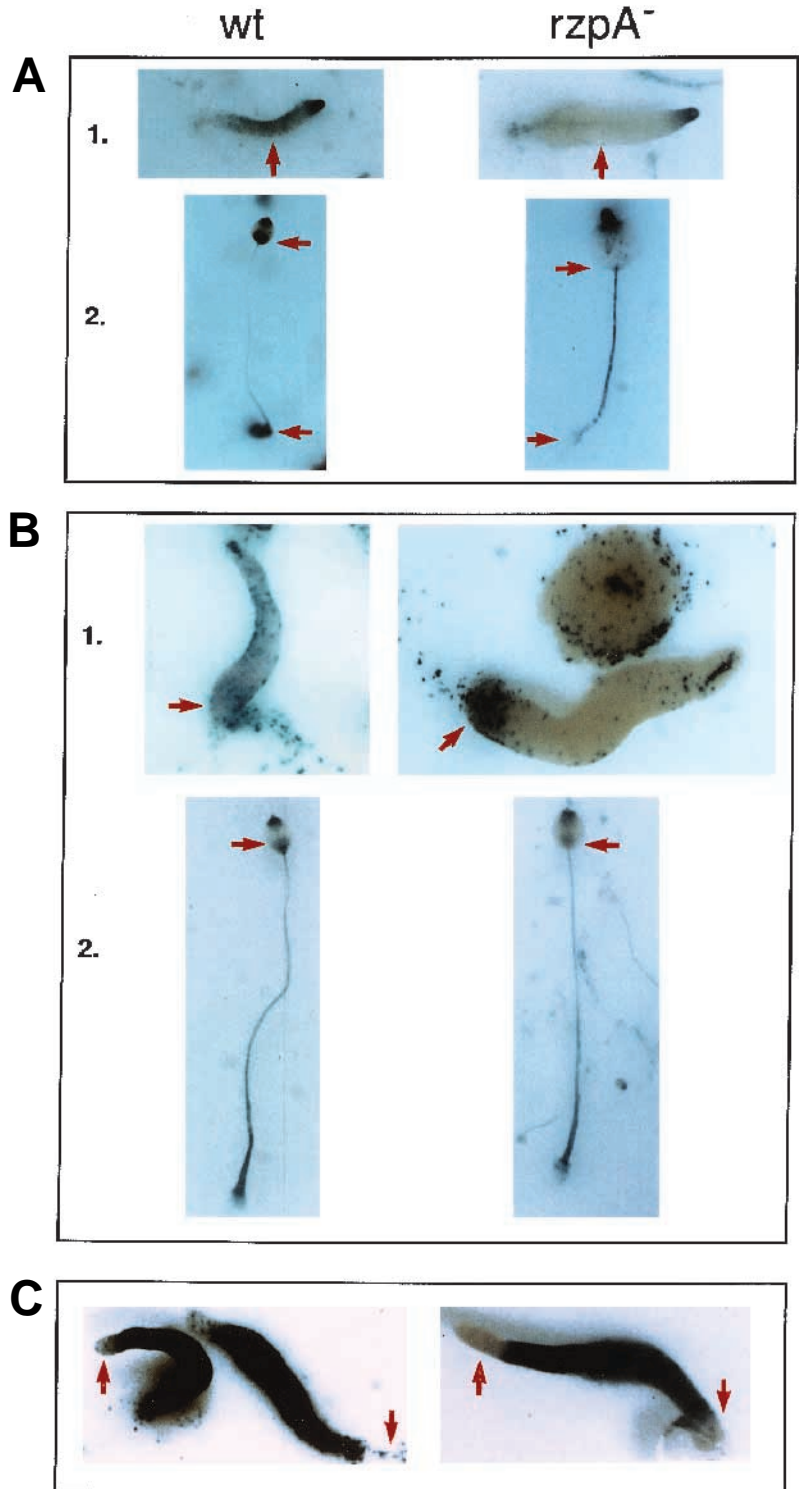

Fig. 7. Cell-specific expression patterns in rzpA⁻ strains. (A) Wild-type and rzpA⁻ strains carrying the prestalk *ecm*A/*lac*Z construct and stained for β-galactosidase. 1. Slug stage. 2. Fruiting body stage. Arrows indicate differences in expression patterns. (B) Wild-type and rzpA⁻ strains carrying the prestalk *ecm*B/*lac*Z construct and stained for β-galactosidase. 1, Slug. 2, Fruiting body. Arrows indicate differences in expression patterns. (C) Wild-type and rzpA⁻ slugs carrying the prespore *cot*B/*lac*Z construct and stained for β-galactosidase. Arrows indicate differences in expression patterns.

before   after cutting ⟶

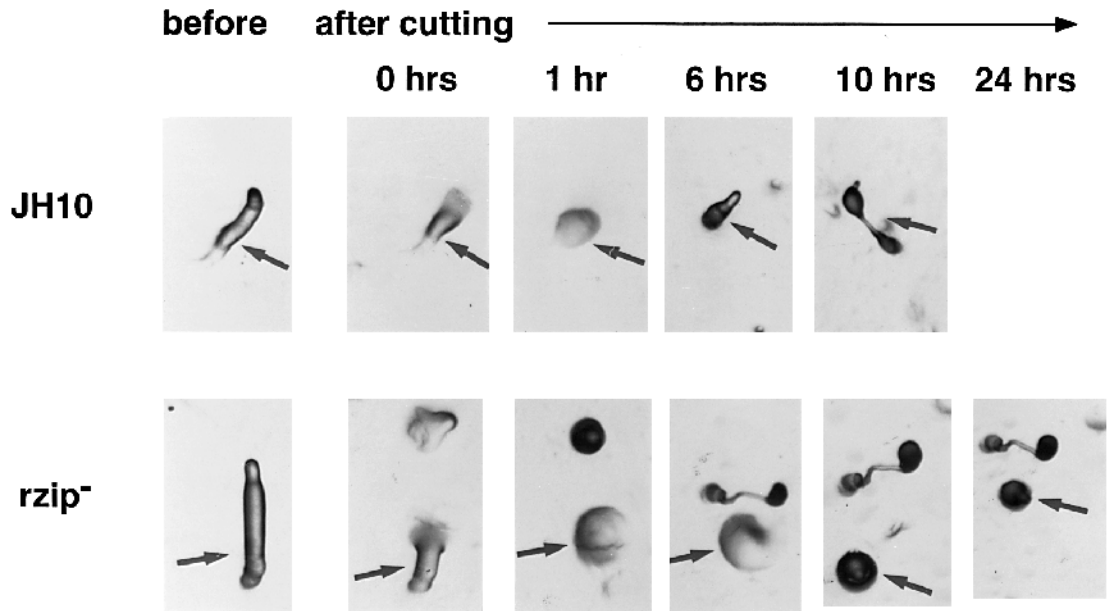| | 0 hrs | 1 hr | 6 hrs | 10 hrs | 24 hrs |

JH10

rzip⁻

Fig. 8. The redifferentiation of wild-type (JH10) and rzpA⁻ slugs after transverse dissection. Arrows indicate the rear halves of each slug. The front of the JH10 slug was removed from the microscope field.

10 hours after cutting, and never progressed to the fruiting body stage. These results strongly suggest that rzpA⁻ prespore cells are impaired in their ability to transdifferentiate from the prespore to prestalk state.

Overexpression of rZIP causes cell-specific effects of gene expression

The phenotypic effects of overexpression of rZIP in wild-type cells were investigated using the constitutive *act15* promoter (Knecht et al., 1986). Northern analyses showed that the resulting *act15/rzp*A (rzpA^OE) transformants overexpressed *rzp*A mRNA >100-fold (Fig. 9A). Western analyses similarly confirmed overexpression (>30-fold) of the rZIP protein (data not shown).

The rzpA^OE strains were developed for 60 hours in unidirectional light as an assay for slug migration (Fig. 5). These slugs exhibited haphazard migration and made frequent sharp turns away from the light source, suggestive of impaired phototaxis. This was distinct from the straight movement of wild-type slugs toward unidirectional side light (see Fig. 5). rzpA^OE strains remained as slugs for longer times than did the wild-type controls under all light conditions examined (darkness; unidirectional side light; overhead light), often not culminating until 10 hours after wild-type in overhead light.

Northern analyses showed dramatic effects of *rzp*A overexpression on cell-specific gene expression. The prespore-specific markers *cot*B and *psp*A (data not shown) were substantially underexpressed in rzpA^OE strains compared to controls, whereas the prestalk markers *ecm*A and *ecm*B were overexpressed (Fig. 9B). In wild-type, *ecm*B was not expressed until the tight aggregate stage (approx. 12 hours), but in the rzpA^OE strain, expression was even detected during vegetative growth.

Prestalk-specific *ecm*A/*lac*Z and *ecm*B/*lac*Z, and prespore-specific *psp*A/*lac*Z constructs were used to examine the regulation of cell-type patterning in the rzpA^OE strain. The anterior region of expression of *ecm*A/*lac*Z in rzpA^OE strains was expanded compared with the wild-type (Fig. 10A; see Fig. 7A-1). rzpA^OE strains carrying the prestalk *ecm*B/*lac*Z had more

intense β-galactosidase activity than did wild-type cells but it was concentrated toward the posterior of the slug (Fig. 10B), consistent with an expanded anterior prestalk A zone in this strain (see Figs 10A, 7A-1, and 7B-1). In contrast, very little β-galactosidase activity was detected with prespore *psp*A/*lac*Z
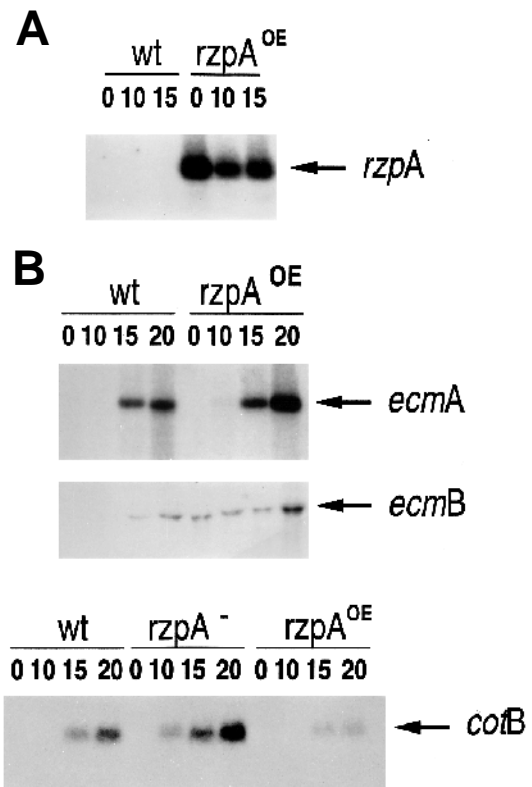

Fig. 9. Cell-specific expression in rzpA^OE cells. (A) Developmental northern blot of wild-type and rzpA^OE RNAs hybridized with a *rzp*A probe. (B) Developmental northern blots of wild-type, rzpA⁻ and rzpA^OE strains hybridized with probes to prestalk-specific *ecm*A and *ecm*B, and prespore-specific *cot*B.

in an rzpA^{OE} strain as compared with wild-type slugs (Fig. 10C), reflecting reduced expression of the endogenous *psp*A mRNA in rzpA^{OE} cells (see Fig. 9B).

DISCUSSION

The RING/zipper protein rZIP plays an important role in determining cell fate decisions during *Dictyostelium* development. This may involve regulatory pathways requiring complex interactions among various homologous and heterologous protein molecules through glutamine repeat, RING, zipper and SH3-binding motifs. rZIP function could be additionally modulated by protein kinases whose activities depend upon intracellular controls that originate with surface receptor activation.

The glutamine repeat is common to many developmentally regulated genes of most eukaryotes. In *Dictyostelium*, these genes may encode transmembrane (e.g. the cAMP receptor subtype 4; Louis et al., 1994), cytosolic (e.g. the PKA catalytic subunit; Mann and Firtel, 1991), or nuclear (e.g. the transcription factor GBF; Schnitzler et al., 1994) proteins. Although the precise molecular function of the glutamine repeat is not defined, it can serve as an activation domain for several transcription factors in yeast (Gerber et al., 1994). Glutamine repeats promote protein-protein dimerization in vitro (Stott et al., 1995), and inappropriate interactions due to an expanded glutamine repeat in the huntingtin protein are suggested to underly the neurodegenerative Huntington's disease (Burke et al., 1996; Li et al., 1995).

In addition to the glutamine repeat, the leucine zipper and RING domain may also regulate macromolecular interactions of rZIP. While two structurally similar leucine zippers are necessary and often sufficient for dimerization, some zipper proteins may require an additional domain (e.g. HLH; helix-loop-helix) for functional associations. Initial studies of rZIP suggest that both the leucine zipper and RING domain may contribute to its homodimer formation (Fig. 2A).

More than 80 proteins with extremely varied functions possess a zinc-binding RING (C$_3$HC$_4$) domain (Saurin et al., 1996). Unlike classical zinc fingers which are definitive DNA-binding moities, RING family members may be nuclear, cytosolic, or integral membrane proteins. Consistent with the length and sequence heterogeneity in the consensus motif defining the family (Fig. 1B), various members have different three-dimensional organizations (Borden et al., 1995) which may reflect their apparent functional diversity. It has been suggested that RING domains promote protein-protein associations, and perhaps different subfamilies have distinct structures and interactions.

The RING domains that are most similar to rZIP are on murine c-Cbl (Langdon et al., 1989), human Cbl-b (Keane et al., 1995), and SLI-1 (Yoon et al., 1995), a related *C. elegans* protein (Fig. 1B). SLI-1 has been implicated in a receptor tyrosine kinase mediated signalling pathway that regulates vulval development (Yoon et al., 1995) and the Cbl proteins, which like rZIP have a leucine zipper and SH3-binding domain (Keane et al., 1995; Rivero et al., 1994), are implicated in EGF receptor signalling (Galisteo et al., 1995). The *v-cbl* oncogene encodes a truncated version of c-Cbl that contains only the amino-terminal 40% of the wild-type cellular protein (Blake and Langdon, 1992). The deleted sequences include the RING domain, the leucine zipper, and the SH3 binding site(s). Correlated with this deletion is a subcellular redistribution of Cbl protein sequences from the cytoplasm to the nucleus and an acquisition to induce acute transformation (Langdon et al., 1992). By analogy we suggest that the RING, zipper and SH3-binding sites of rZIP are similarly important for control of cell-type determination through a receptor-regulated intracellular signalling cascade during *Dictyostelium* development.

Expression of *rzp*A in *Dictyostelium* may be limited to a subset of vegetatively growing cells, but later in development all of the major differentiated cell types appear to express *rzp*A (see Fig. 3). Consistent with this temporal expression pattern, rzpA$^−$ strains exhibit aberrant aggregation very early in development and altered patterning of prespore and prestalk cells during later stages. The prespore genes *psp*A and *cot*B are induced precociously and to abnormally high levels in rzpA$^−$ lines and are underexpressed in rzpA^{OE} lines which constitutively overexpress *rzp*A. In contrast, prestalk-specific gene expression in rzpA$^−$ lines is reduced relative to that of wild-type, while rzpA^{OE} strains overexpress the prestalk markers *ecm*A and *ecm*B. Further, *ecm*B expression is extremely precocious in rzpA^{OE} lines. Thus *rzp*A appears to be involved in a negative regulatory pathway for prespore differentiation but a stimulatory circuit for prestalk differentiation. These defective regulatory circuits for cell differentiation in rzpA$^−$ strains severely compromise the ability of prespore cells to transdifferentiate into prestalk cells. Perhaps rZIP plays a significant role in repression of prespore expression during this transdifferentiation.

As wild-type slugs migrate, they slough a population of prestalk cells into the slime trail. To maintain constant proportioning of their differentiated cell-types throughout development, there is a required redifferentiation of prespore cells into anterior-like cells (ALCs) and finally into anterior, prestalk cells (Abe et al., 1994; Sternfeld, 1992). Thus after prolonged migration, wild-type slugs that specifically express stable β-galactosidase in prespore cells exhibit staining in their anterior regions (Detterbeck et al., 1994; Harwood et al., 1991). In contrast, comparably marked rzpA$^−$ slugs have a sharp delineation of β-galactosidase staining at their prestalk/prespore boundaries (Fig. 7) and rzpA$^−$ strains which carry the prestalk-specific *ecm*A/*lac*Z or *ecm*B/*lac*Z show only very limited β-galactosidase staining in ALCs and ALC-derived structures as compared with an equivalent wild-type strain (Fig. 7A,B). However, the inability of rzpA$^−$ strains to undergo appropriate transdifferentiation is not solely due to poor slug migration. Unlike the wild-type strains, transected rzpA$^−$ slugs will not transdifferentiate a sufficient population of prespore cells into prestalk cells to permit continued development of posterior segments (Fig. 8). A dependent link may, therefore, exist among the events of transdifferentiation, cell movement within the slug, and migration of the slug. Thereby impaired transdifferentiation may abrogate slug migration.

The most posterior regions of *cot*B/*lac*Z marked rzpA$^−$ slugs stain poorly for β-galactosidase activity (Fig. 7C). The absence of rZIP may lead to the disruption of a morphogenic gradient within the prespore region of the slug, perhaps through alteration in production or perception of the cAMP signal. Further, the impaired phototaxis of rzpA^{OE} slugs (Fig. 5) is very similar to that of slugs which lack active cAMP-dependent protein kinase (PKA) in prestalk cells (Bonner and Williams, 1994).
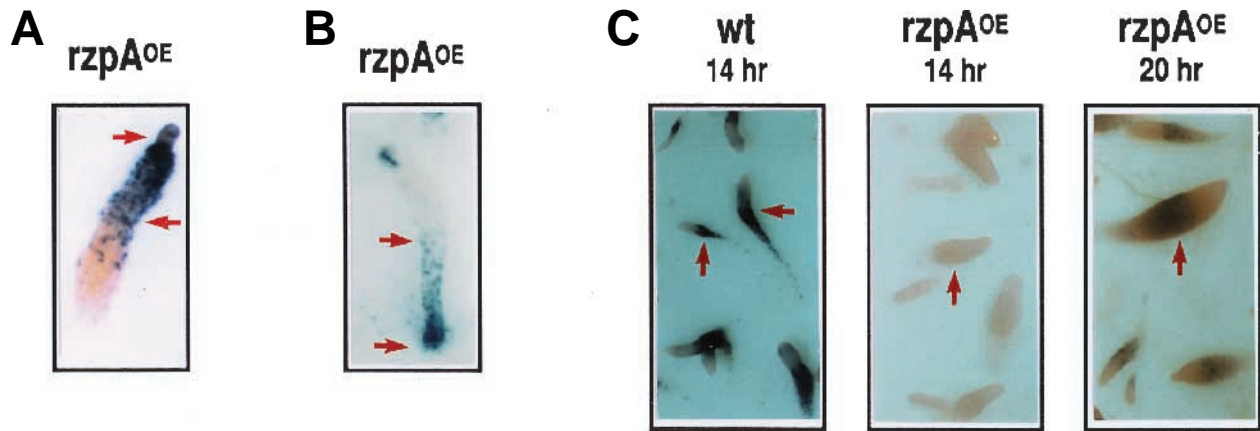
Fig. 10. Cell-specific markers in rzpA[OE] strains. (A) rzpA[OE] strains carrying the *ecm*A/*lac*Z construct and stained for β-galactosidase. Arrows indicate differences in expression pattern compared with wild-type (Fig. 7B). (B) rzpA[OE] strains carrying the prestalk *ecm*B/*lac*Z construct and stained for β-galactosidase activity. Arrows indicate differences in expression pattern compared with wild-type (Fig. 7B). (C) rzpA[OE] strains carrying the prespore *psp*A/*lac*Z construct and stained for β-galactosidase at developmental times indicated. Arrows indicate reduction of β-galactosidase in rzpA[OE] compared with wild-type.

Other data also support a defective cAMP signalling pathway in rzpA^{−} cells. During aggregation at a low cell density $(1.3 \times 10^5$ cells/cm$^2)$ they form fewer aggregation centers and much longer aggregation streams than do wild-type cells (Fig. 4, Table 1). Similar abnormal patterns of aggregation have been observed in mutants with defined defects in cAMP signal-relay (Gerisch, 1971; Riedel et al., 1973) and can be mimicked by specifically inhibiting cAMP signalling in wild-type cells by exogenous drug treatment (Newell and Ross, 1982). Furthermore, when rzpA^{−} cells are plated at a higher cell density $(5.1 \times 10^5$ cells/cm$^2)$ to enhance endogenous cAMP signalling, a more normal aggregation phenotype is observed.

The regulation of prestalk and prespore differentiation in *Dictyostelium* involves complex interactions among a variety of activation pathways and lateral inhibitions (Loomis 1993). Although the position of rZIP within these regulatory circuits is yet to be resolved, there are several characterized genes with which rZIP is likely to interact, either directly or indirectly. Strains which lack a functional cAMP receptor subtype 4 (CAR4) express higher prespore-specific and lower prestalk-specific mRNA levels than do wild-type strains (Louis et al., 1994). Thus, like rZIP, CAR4 inhibits prespore but stimulates prestalk differentiation. In addition, slugs of both car4[−] and rzpA[−] strains have reduced ALC expression and poor motility. These phenotypic similarities strongly suggest an involvement of rZIP in an intracellular signalling pathway that originates with cAMP activation of CAR4 at the cell surface. However, since *rzp*A transcripts are detectable in vegetative cells (see Fig. 3A), whereas *car4* mRNA is not expressed prior to tight mound formation (Louis et al., 1994), it is not surprising that the rzpA[−] and car4[−] strains are not precise phenocopies of one another. By analyzing gene expression and cellular patterning in strains in which both genes are either disrupted and/or misexpressed, we will be able to define their epistatic relationship more clearly.

At the intracellular level, essential functions for three protein kinases [glycogen synthase kinase 3 (GSK3), cAMP-dependent protein kinase (PKA), and MAP kinase 2 (ERK2)] have been described for prespore/prestalk differentiation of *Dictyostelium*. Strains that lack GSK3 overexpress prestalk *ecm*B and underexpress prespore cell-specific markers (Harwood et al., 1995). Preliminary data also suggest an upstream involvement of CAR4 in GSK3 regulation. Both CAR4 and rZIP could be antagonistic to GSK3 in prestalk/prespore determination pathways. A model outlining these possible interactions is shown in Figure 11.

PKA is required for both prestalk and prespore expression (Mann and Firtel, 1993). Specific inhibition of PKA in prestalk cells blocks continued prestalk differentiation (Zhukovskaya et al., 1996) and terminal differentiation of the stalk (Harwood et al., 1992). Similarly, prespore cells which express a dominant-negative regulatory subunit of PKA become arrested and do not progress towards spore cell differentiation (Hopper et al., 1993). Since expression of ERK2 in prespore cells is required for continued prespore-specific gene expression (Gaskins et al., 1996), and since rZIP will downregulate this expression pattern, these proteins may also participate in a linked regulatory pathway. Phosphorylation by ERK2 of the consensus MAP kinase site within the proline-rich region of rZIP may alter the interaction of rZIP with an endogenous SH3-domain
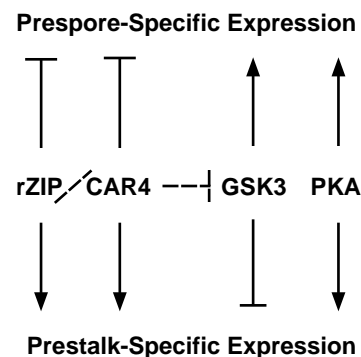

Fig. 11. A model showing the effects of several proteins on prestalk- and prespore-specific gene expression. rZIP and CAR4 inhibit prespore- and promote prestalk-specific gene expression. GSK3 has the opposite effects and PKA is required for the expression of both sets of developmentaly regulated genes. Potential interactions among these proteins are indicated by dashed lines; rZIP and/or CAR4 may inhibit the action of GSK3.

partner to potentiate prespore differentiation. Currently the extent of the SH3 population in *Dictyostelium* is unclear. The in vitro binding data of rZIP in conjunction with the more extensive studies of Cbl suggest that rZIP could function in a multimeric complex involving an Nck-like SH3-SH2 adaptor protein. Alternatively rZIP may be a novel adaptor. Its multiple protein interacting domains could underlie a new intracellular signalling mechanism regulated by protein phosphorylation.

The overall regulatory modes for prestalk and prespore determination in *Dictyostelium* are mirrored in many other developmental systems. GSK activity is critical for dorsal/ventral patterning in *Xenopus* (Pierce and Kimelman, 1995; Xi et al., 1995) and for establishing segment polarity in *Drosophila* (Perrimon, 1994). PKA and MAP kinases play essential roles in general aspects of cell growth and development. Central to the activation or inhibition of these pathways is a transmembrane signalling cascade. A similar linkage between the cAMP receptors and rZIP of *Dictyostelium* may recall or predict related interactions in other organisms. It is now essential to define the genetic heirarchies and physical associations by which rZIP regulates cell fate.

REFERENCES

Abe, T., Early, A., Siegert, F., Weijer, C. and Williams, J. (1994). Patterns of cell movement within the *Dictyostelium* slug revealed by cell type-specific, surface labeling of living cells. *Cell* **77**, 687-699.

Alber, T. (1992). Structure of the leucine zipper. *Curr. Opin. Genet. Dev.* **2**, 205-210.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Bierkamp, C. and Campos-Ortega, J. A. (1993). A zebrafish homologue of the *Drosophila* neurogenic gene *Notch* and its pattern of transcription during early embryogenesis. *Mech. Dev.* **43**, 87-100.

Blake, T. J. and Langdon, W. Y. (1992). A rearrangement of the *c-cbl* proto-oncogene in HUT78 T-lymphoma cells results in a truncated protein. *Oncogene* **7**, 757-762.

Blanar, M. A. and Rutter, W. J. (1992). Interaction cloning: Identification of a helix-loop-helix zipper protein that interacts with c-Fos. *Science* **256**, 1014-1018.

Bonner, J. and Williams, J. (1994). Inhibition of cAMP-dependent protein kinase in *Dictyostelium* prestalk cells impairs slug migration and phototaxis. *Dev. Biol.* **164**, 325-327.

Borden, K. L. B., Boddy, M. N., Lally, J., O'Reilly, J., Martin, S., Howe, K., Solomon, E. and Freemont, P. S. (1995). The solution of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. *EMBO J.* **14**, 1532-1541.

Bork, P. (1996). Pleckstrin's repeat perfomance: a novel domain in G-protein signalling? *Trends Biochem. Sci.* **21**, 245-246.

Burke, J. R., Enghild, J. J., Martin, M. E., Jou, Y.-S., Myers, R. M., Roses, A. D., Vance, J. M. and Strittmatter, W. J. (1996). Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH. *Nature Med.* **2**, 247-250.

Cichetti, P., Mayer, B. J., Thiel, G. and Baltimore, D. (1992). Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho. *Science* **257**, 803-806.

Detterbeck, S., Morandini, P., Wetterauer, B., Bachmair, A., Fischer, K. and Macwilliams, H. K. (1994). The 'prespore-like cells' of *Dictyostelium* have ceased to express a prespore gene: Analysis using short-lived beta-galactosidases as reporters. *Development* **120**, 2847-2855.

Duboule, D., Haenlin, M., Galliot B. and Mohier, E. (1987). DNA sequences homologous to the *Drosophila opa* repeat are present in murine mRNAs that are differentially expresse in fetuses and adult tissues. *Mol. Cell. Biol.* **7**, 2003-2006.

Firtel, R. A. (1995). Integration of signaling information in controlling cell-fate decisions in *Dictyostelium*. *Genes Dev.* **9**, 1427-1444.

Galisteo, M. L., Dikic, I., Batzer, A. G., Langdon, W. L., and Schlessinger, J. (1995). Tyrosine phosphorylation of the c-*cbl* proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. *J. Biol. Chem.* **270**, 20242-20245.

Gaskins, C., Clark, A. M., Aubry, L., Segall, J. E. and Firtel, R. A. (1996). The *Dictyostelium* MAP kinase ERK2 regulates multiple, independent developmental pathways. *Genes Dev.* **10**, 118-128.

Gerber, H. P., Seipel, K., Georgiev, O., Höfferer, M. Hug, M., Rusconi, S. and Schaffner, W. (1994). Transcriptional activation modulated by homopolymeric glutamine and proline stretches. *Science* **263**, 808-811.

Gerisch, G. (1971). Periodische signale steuern die musterbildung in zellverbanden. *Naturwissenschaften* **58**, 430-438.

Ginsburg, G. T., Gollop, R., Yu, Y. M., Louis, J. M., Saxe, C. L. and Kimmel, A. R. (1995). The regulation of *Dictyostelium* development by transmembrane signalling. *J. Euk. Microbiol.* **42**, 200-205.

Harwood, A. J., Early, A. E., Jermyn, K. A. and Williams, J. (1991). Unexpected localisation of cells expressing a prespore marker of *Dictyostelium discoideum*. *Differentiation* **46**, 7-13.

Harwood, A. J., Hopper, N. A., Simon, M. N., Driscoll, D. M., Veron, M. and Williams, J. G. (1992). Culmination in *Dictyostelium* is regulated by the cAMP-dependent protein kinase. *Cell* **69**, 615-624.

Harwood, A. J., Plyte, S. E., Woodgett, J., Strutt, H. and Kay, R. R. (1995). Glycogen synthase kinase 3 regulates cell fate in *Dictyostelium*. *Cell* **80**, 139-148.

Hopper, N. A., Harwood, A. J., Bouzid, S., Veron, M. and Williams, J. G. (1993). Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP-dependent protein kinase and evidence for its upstream regulation by ammonia. *EMBO J.* **12**, 2459-2466.

Jennings, C. (1995). How trinucleotide repeats may function. *Nature* **378**, 127.

Keane, M. M., Rivero, L. O., Mitchell, J. A., Robbins, K. C. and Lipkowitz, S. (1995). Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene. *Oncogene* **10**, 2367-2377.

Kimmel, A. and Firtel, R. (1985). Sequence organization and developmental expression of an interspersed, repetitive element and associated single-copy DNAsequences in *Dictyostelium discoideum*. *Mol. Cell. Biol.* **5**, 2123.

Kimmel, A. R. (1987). Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. *Dev. Biol.* **122**, 163-171.

Kimmel, A. R. and Firtel, R. A. (1991). cAMP signal transduction pathways regulating development of *Dictyostelium discoideum*. *Curr. Opin. Genet. Dev.* **1**, 383-390.

Knecht, D. A., Cohen, S. M., Loomis, W. F. and Lodish, H. F. (1986). Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low-copy and high-copy transformation vectors. *Mol. Cell. Biol.* **6**, 3973-3983.

Langdon, W. Y., Hyland, C. D., Grumont, R. J. and Morse H. C. III (1989). The *c-cbl* proto-oncogene is preferentially expressed in thymus and testis tissue and encodes a nuclear protein. *J. Virol.* **63**, 5420-5424.

Langdon, W. Y., Heath, K. G. and Blake, T. J. (1992). The localization of the products of the c-cbl and v-cbl oncogenes during mitosis and transformation. *Curr Top. Microbiol. Immunol.* **182**, 467-474.

Li, M., Jan, Y. N. and Jan, L. Y. (1992). Specification of subunit assembly by the hydrophilic amino-terminal domain of the shaker potassium channel. *Science* **257**, 1225-1230.

Li, X.-J., Li, S.-H., Sharp, A. H., Nucifora, F. C., Schilling, G., Lanahan, A., Worley, P., Snyder S. H. and Ross, C. A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. *Nature* **378**, 398-402.

Loomis, W. F. (1982). The Development of *Dictyostelium discoideum*. Academic Press, New York.

Loomis, W. F. (1993) Lateral inhibition and pattern formation in *Dictyostelium*. *Curr. Top. Dev. Biol.* **28**, 1-46.

Louis, J. M., Ginsburg, G. T. and Kimmel, A. R. (1994). The cAMP receptor CAR4 regulates axial patterning and cellular differentiation during late development of *Dictyostelium*. *Genes Dev.* **8**, 2086-2096.

Maddox, J. (1994). Triplet repeat genes raise questions. *Nature* **368**, 685.

Mann, S. K. O. and Firtel, R. A. (1991). A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech. Dev.* **35**, 89-101.

Mann, S. K. O. and Firtel, R. A. (1993). cAMP-dependent protein kinase

differentially regulates prestalk and prespore differentiation during *Dictyostelium* development. *Development* **119**, 135-146.

Mayer, B. J. and Eck, M. J. (1995). Minding your p's and q's. *Current Biol.* **5**, 364-367.

Newell, P. C. and Ross, F. M. (1982). Inhibition by adenosine of aggregation centre initiation and cyclic AMP binding in *Dictyostelium*. *J. Gen. Microbiol.* **128**, 2715-2724.

Pawson, T. and Schlessinger, J. (1993). SH2 and SH3 domains. *Curr. Biol.* **3**, 434-442.

Pierce, S. B. and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development* **121**, 755-765.

Perrimon, N. (1994). The genetic basis of patterned baldness in *Drosophila*. *Cell* **76**, 781-784.

Raper, K. (1940). Pseudoplasmodium formation and organisation in *Dictyostelium discoideum*. *J. Elisha Mitchell Sci. Soc.* **59**, 241-282.

Richardson, D. L., Loomis, W. F. and Kimmel, A. R. (1994). Progression of an inductive signal activates sporulation in *Dictyostelium discoideum*. *Development* **120**, 2891-2900.

Riedel, V., Gerisch, G., Muller, E. and Beug, H. (1973). Defective cyclic adenosine-3,5′-phosphate-phosphodiesterase regulation in morphogenetic mutants of *Dictyostelium discoideum*. *J. Mol. Biol.* **74**, 573-585.

Rivero-Lezcano O., Sameshima, J. H., Marcilla, A. and Robbins, K. C. (1994). Physical association between Src homology 3 elements and the protein product of the *c-cbl* proto-oncogene. *J. Biol. Chem.* **269**, 17363-17366.

Saurin, A. J., Borden, K. L. B., Boddy, M. N. and Freemont, P. S. (1996). Does this have a familiar RING? *Trends Biochem. Sci.* **21**, 208-213.

Saxe III, C. L., Ginsburg, G. T., Louis, J. M., Johnson, R., Devreotes, P. N. and Kimmel, A. R. (1993). CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. *Genes Dev.* **7**, 262-272.

Schnitzler, G. R., Fischer, W. H. and Firtel, R. A. (1994). Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium*. *Genes Dev.* **8**, 502-514.

Sternfeld, J. (1992). A study of pstB cells during *Dictyostelium* migration and culmination reveals a unidirectional cell type conversion process. *Roux Arch. Dev. Biol.* **201**, 354-363.

Stott, K., Blackburn, J. M., Butler, P. J. G. and Perutz, M. (1995). Incorporation of glutamine repeats makes proteins oligomerize: Implications for neurodegenerarive diseases. *Proc. Nat. Acad.Sci. USA* **92**, 6509-6513.

Wharton, K. A., Yedvobnick, B., Finnerty, V. G. and Artavanis-Tsakonas, S. (1985). *opa*: A novel family of transcribed repeats shared by the Notch locus and other developmentally regulated loci in *D. melanogaster*. *Cell* **40**, 55-62.

Xi, H., Saint-Jeannet, J.-P., Woodgett, J. R., Varmus, H. E. and Dawid, I. B. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* **374**, 617-622.

Yoon, C. H., Lee, J., Jongeward, G. D. and Sternberg, P. W. (1995). Similarity of *sli-1*, a regulator of vulval development in *C. elegans*, to the mammalian proto-oncogene *c-cbl*. *Science* **269**, 1102-1105.

Zhukovskaya, N., Early, A., Kawata, T., Abe, T. and Williams, J. (1996). cAMP dependent protein kinase is required for the expression of a gene specifically expressed in *Dictyostelium* prestalk cells. *Dev. Biol.* **179**, 27-40.