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The novel ankyrin-repeat containing kinase ARCK-1 acts as a suppressor of the Spalten signaling pathway during *Dictyostelium* development

Laurence Aubry,[a,b] Susan Lee,[b] Kissia Ravanel,[a] and Richard A. Firtel[b,*]

[a] *Laboratoire de Biochimie et Biophysique des Systèmes Intégrés (UMR 5092 CNRS-CEA-UJF), DRDC/BBSI, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France*

[b] *Section of Cell and Developmental Biology, Division of Biological Sciences, Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0634, USA*

Abstract

Spalten (Spn), a member of the PP2C family of Ser/Thr protein phosphatases, is required for *Dictyostelium* cell-type differentiation and morphogenesis. We have identified a new protein kinase, ARCK-1, through a second site suppressor screen for mutants that allow *spn* null cells to proceed further through development. ARCK-1 has a C-terminal kinase domain most closely related to Ser/Thr protein kinases and an N-terminal putative regulatory domain with ankyrin repeats, a 14-3-3 binding domain, and a C1 domain, which is required for binding to RasB$^{\text{GTP}}$ in a two-hybrid assay. Disruption of the gene encoding ARCK-1 results in weak, late developmental defects. However, overexpression of ARCK-1 phenocopies the *spn* null phenotype, consistent with Spn and ARCK-1 being on the same developmental pathway. Our previous analyses of Spn and the present analysis of ARCK-1 suggest a model in which Spn and ARCK-1 differentially control the phosphorylation state of a protein that regulates cell-type differentiation. Dephosphorylation of the substrate by Spn is required for cell-type differentiation. Control of ARCK-1 and Spn activities by upstream signals is proposed to be part of the developmental regulatory program mediating cell-fate decisions in *Dictyostelium*.

Keywords: Dictyostelium; Protein kinase; Ankyrin repeats; Cell-type differentiation

Introduction

Reversible protein phosphorylation catalyzed by protein kinases and phosphatases is a major regulatory event that controls many aspects of cell function. The resulting effects are multiple, as phosphorylation can activate or inactivate the biological activity of the target protein, modify its subcellular localization, induce or disrupt interactions with partners, and trigger its destabilization and destruction.

Dictyostelium discoideum provides examples of signaling cascades in which protein kinases play key roles in controlling developmental processes. Upon starvation, *Dictyostelium* initiates a multicellular developmental program leading to the formation of a differentiated fruiting body composed of a

vacuolated stalk supporting a mass of viable spores (Aubry and Firtel, 1999; Firtel, 1995; Loomis, 1996). Multicellularity is achieved by aggregation of individual amoebae, a process that is dependent on the cAMP-dependent protein kinase PKA (Aubry and Firtel, 1999; Mann and Firtel, 1993; Williams et al., 1993) and the MAP kinase ERK2 (Aubry et al., 1997; Gaskins et al., 1996; Segall et al., 1995) as well as the signaling pathways that regulate chemotaxis. Later in development, cell-type specification requires the additional participation of the glycogen synthase kinase-3 (GSK3) (Harwood et al., 1995; Kim et al., 2002) and the tyrosine kinase ZAK-1 (Kim et al., 1999). Spatial patterning of the different cell subtypes also involves signaling components such as the MEK kinase MEKKα (Chung et al., 1998). Whereas molecular and biochemical data are available regarding the role of Ser/Thr kinases in *Dictyostelium,* much less is known about the Ser/Thr protein phosphatases that control the reciprocal processes. In
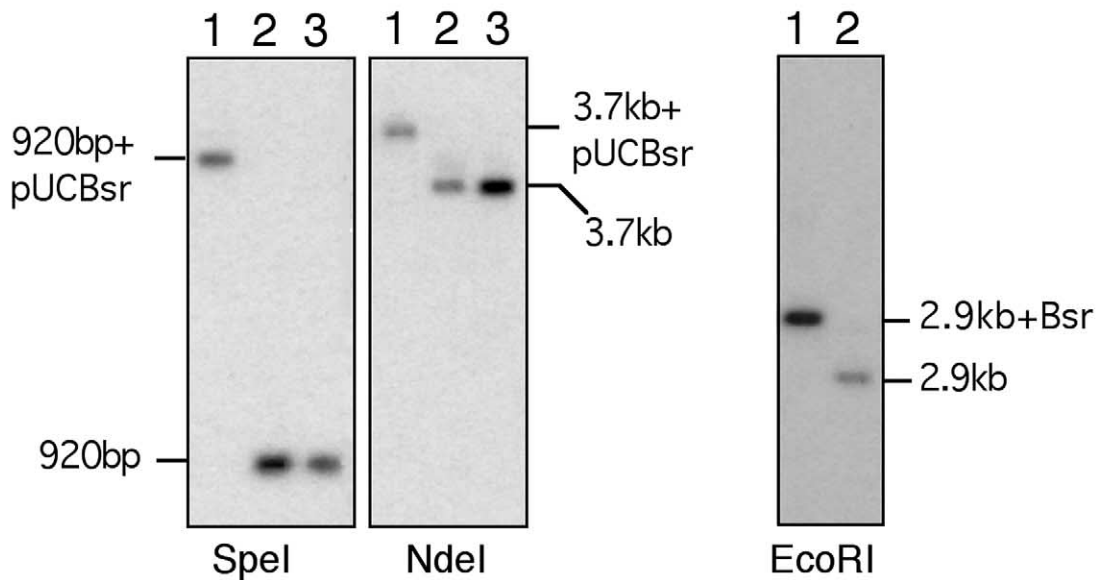
A
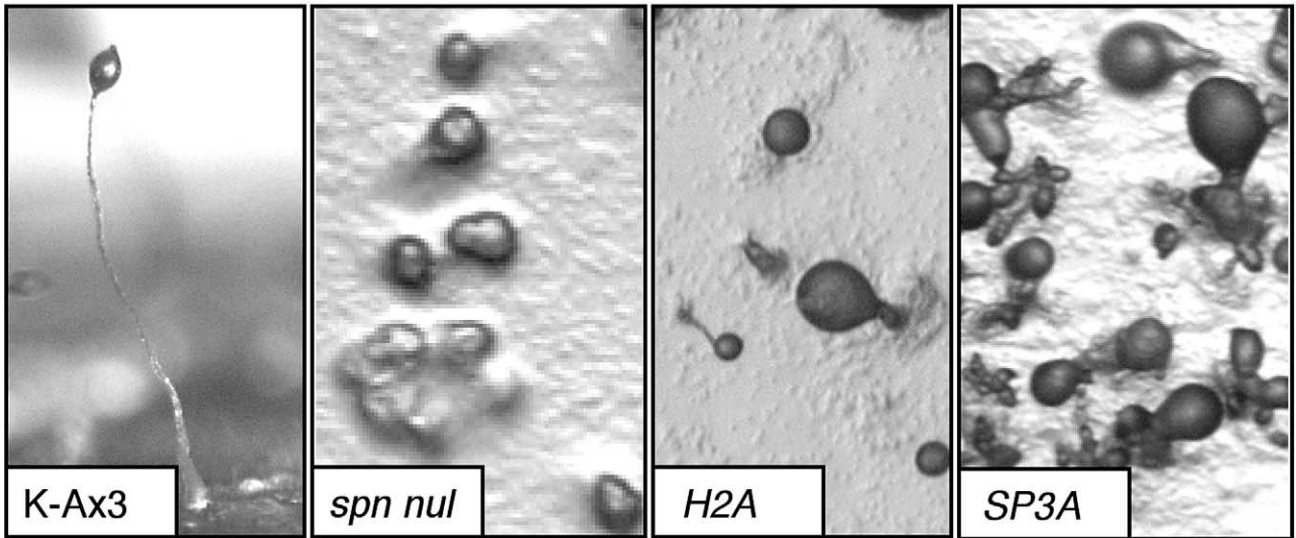
B

Fig. 1. Analysis of *spn* null cell suppressors. (A) Suppressors of the *spn* null mutation. A mutagenesis screen was performed on the *spn* null mutant to isolate double knockouts able to differentiate fruiting bodies. Two distinct clones were obtained, H2A and SP3A, that were mutated in the same ORF. (B) Southern blot analysis. The SP3A mutation was created in the *spn* null mutant by homologous recombination of the rescued plasmid (left panel) in the *ArkA* locus. The genomic DNA was digested with either *Spe*I or *Nde*I and probed with a ^{32}P-labeled *Spe*I–*Spe*I fragment of the *ArkA* gene (see map in Fig. 2A, *Spe*I sites are boxed). Disruption of *ArkA* in the parental strain was made by insertion of the blasticidin resistance cassette in the ORF (*Bam*HI site; panel on the far right). The genomic DNA was digested with *Eco*RI and probed with the same *Spe*I–*Spe*I fragment. Lane 1, *arkA/spn* double knockout strain; lane 2, KAx-3; lane 3, *spn* null strain.

A

B

C

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MSIKLPLSNINSGGNSNNSSSSNSTSNNNININIGNGIPTNIEKMTFEDSETQKLNIKQQ        60
SNNNTSTSVCVSSIHSSSPISSPTSHQVNKSSGSLPPVIKRSPTTTHHHNSSGSNSSSSS       120
SGSNNNNNQIKTSNGMNKPNPTGFLFGSKPRENSQNKIDDNKGVNLAVSTSNSSNNFHKS       180
HSESNIININAPPVETVNMEEIYNNIPSSISMENIRENENNNNSSNNNSNNNNNNNNNNN       240
NSNNINNVNGNKSSLASSTSSISSLSSVSTLSTSNAATNTNTNANTTSTTKTTSTVRSTS       300
                             AKT
PTQFKPRVEFDENNPNAIILS[RQRCKSI]SSPSDFRLNPPSNVHMPTSSLSTSTSTTNVNG      360
LLLPNGVGGVSMSFTPSGLPMTPPTNSSQVDTLQMSTESITIQPSSLDSSSESVSDGLQS       420
VSGSPVTASPSPTISNNTNATTTNNNNNTNTNNNNNNNNNQHNHHHHQHSHSQQHDVYQI       480
        MAPK
KKENF[PSSPTSP]TLLPSETHDFSSEYSSNPGGKCAICRKPLWSFPISDKSRRCRDCSLVV      540
                                                         14-3-3
HRACVPLATECPSAKKSPSKLSVPNGNQSNSSSSSSSSSSSSSSSSSSNSSSSNTKGHS[RTPS]   600
[SPS]VSSIPGFQLTSNASQNLHVNSHTLSLLVNGATIDSNHYKRNKKSLEAGARDFHFIFR      660
GCGIPLDEFPLDSYVCGLYSYFAHGRLYLTESYVCFYDGFVFDRTKERTKIIKVSNIASI       720
EKRSSGLNPSAIKIKTFDDQSFIFTHFMHREVAFDDLEGLLIHQESLHFAHSIVANNFPG       780
MNEAIRGQTKRLLSRARLDGHYQIHSKIRCPMPSKEILLMSAIKNNNLDMVVTLLNYYCQ       840
                                            ①
VNSDEINSVDSKGYTPLHNAVFSECSDQIFMHLLNQKEVRVRERNMDGNTPLHYFCQKFK       900
                          ②
SPECQRIVQAMIEKGANINEQNYNGETPLHKAIFNHSVRLLMVYILLKNNANVNIVNNAG       960
      ③                                      ④
ESPLHYAVRLGRLDVAKMLLAAGADPTIISLRDRKTALALAVDYDVCPEISDLLRRLDTI      1020
              ⑤
TTSLEMYELEKFQASLVVEELQKENVVARLDEKLLDKIGCTDKEERRKFLSLKSNRLLIH      1080
HPARTQGAKIILKEMETMDIKNGKLIISETELEYTEKI**GSGASGKVFKGIYRGRVVAIKV**     1140
**LKSADDEMTREDFLKEFGVLASLESHTIVGLYGVVLEPKICLVMEYCSNGSIYHSIRKNP**    1200
**PSWERFFSFVQQMLAGINALHQSTPQVLHRDIKTLNFLVNHNNKVKVADFGLSRFNTESN**    1260
**QETLNKTRGTSVYCAPEVFEGKEYNERSDMYSMGIVMWEIVYCVVYGCYMIPYQEYNKMF**    1320
**NAFQVALLVNSSKRVLRPTIPIGVPQVLKDLIYCLWDHDVSSRPTATEAMTALAVCEKEY**    1380
**KQNKAQWELVITKKEPTPLPRVAPLPAFPGYRQFYEQNLDIKPIPEEEQIYQEAMEKQRR**    1440
**NQEASANRNQKNKELLNNNN**                                          1460
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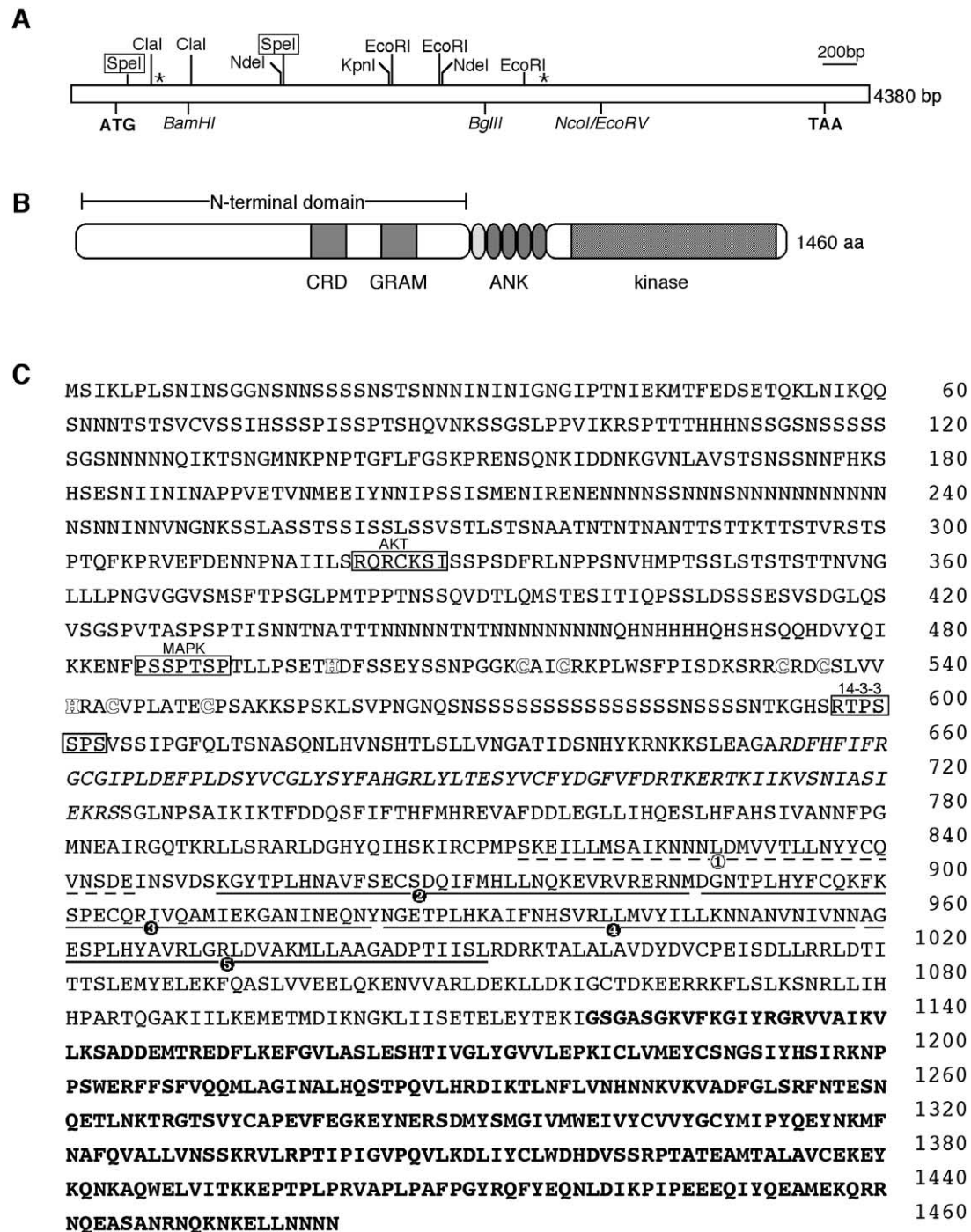
Fig. 2. Map and amino acid sequence. (A) Map of the *ArkA* ORF. The restriction sites present in the *ArkA* gene are represented in regular letters; those in italics have been created as silent mutations for subcloning convenience. The asterisks represent the insertion sites of pUCBsr in the original REMI mutants SP3A (5′ side) and H2A (3′ side). (B) Primary subdomain organization of ARCK-1. The ARCK-1 protein contains several domains of interest: a long N-terminal extension with a cysteine-rich motif and a GRAM domain, 5 ankyrin repeats, and a putative protein kinase activity in the C-terminal part of the protein. (C) Amino acid sequence derived from the *ArkA* gene. The putative kinase domain is shown in bold letters. The ankyrin repeats are underlined, the GRAM is represented with italic letters, and the cysteine and histidine residues that belong to the cysteine-rich domain appear in outlined letters. Potential phosphorylation sites by MAP kinases and Akt as well as the putative 14-3-3 interaction site are boxed.

Dictyostelium, the protein phosphatase Spalten (Spn), an atypical member of the PP2C family, is required for proper development (Aubry and Firtel, 1998). Spn contains a C-terminal PP2C phosphatase domain and an N-terminal regulatory domain that is homologous to heterotrimeric Gα protein subunits. Abrogation of Spn function leads to the inability of the mutant to differentiate prespore and prestalk cells and to achieve terminal differentiation and morphogenesis. Cells arrest their
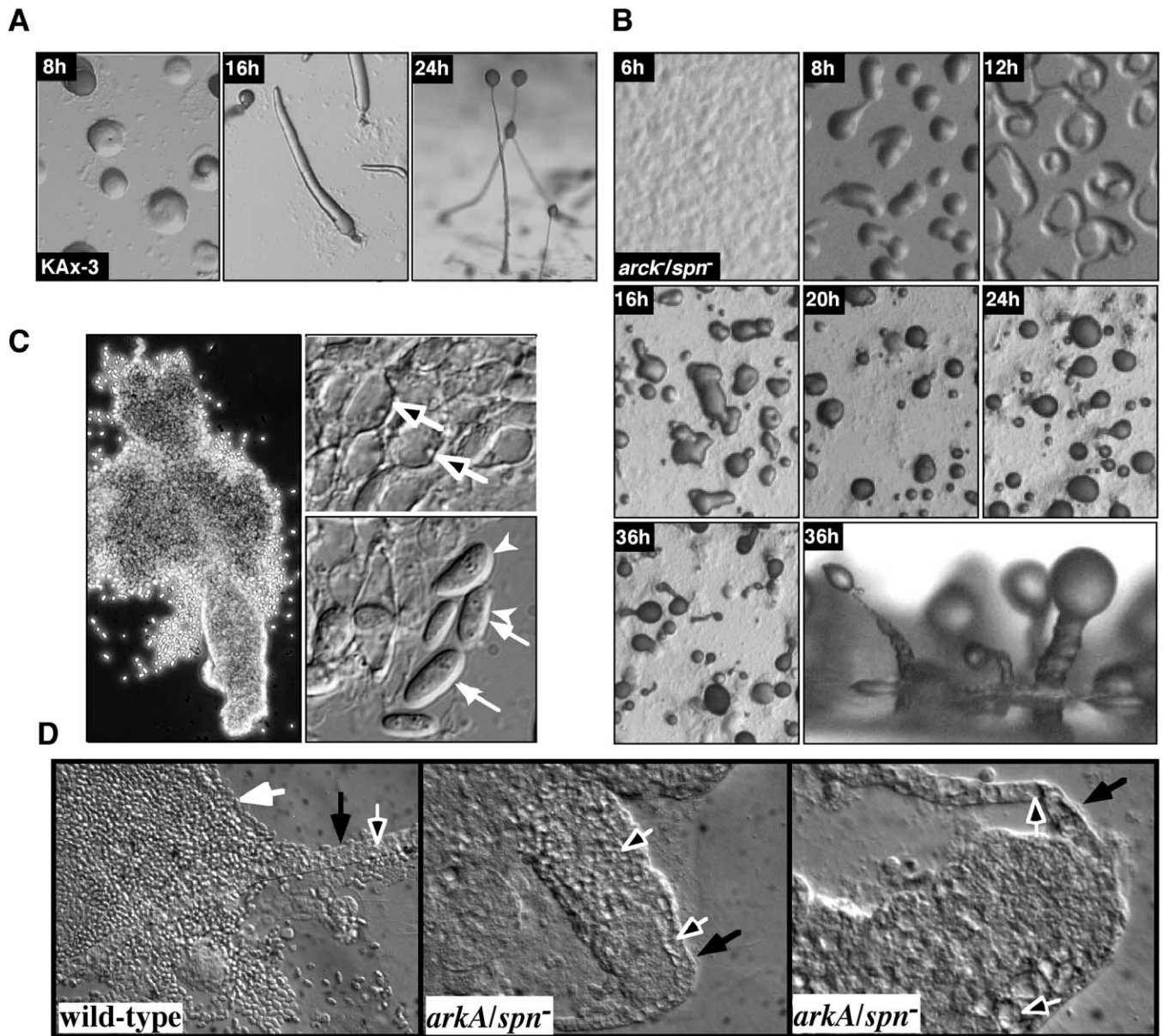
Fig. 3. Sequence comparisons of ARCK-1. (A) Amino acid sequence comparison of ARCK-1 with the catalytic domains of Ser/Thr and Tyr kinases. Alignment was performed by using the SMART consensus sequences SMART 00220 for Ser/Thr kinases and SMART 00219 for Tyr-kinases. The major conserved subdomains present in protein kinases are boxed and labeled I to X. (B) Alignment of the 5 ankyrin repeats of ARCK-1 with a consensus sequence of the ankyrin domains. (C) Alignment of the ARCK-1 CRD with the C1 domain of human Raf (P04049), murine Ksr (NP_038599), and bovine PKCγ (C1b) (P05128). The top arrows depict the position of the secondary structure (β-strands β-1 to 4 and α-helix α-1) of the Raf CRD obtained from NMR analysis (Mott et al., 1996). (D) Alignment of the ARCK-1 GRAM domain with the GRAM domain of the ABA-responsive-element-binding protein T31B5 from *A. thaliana* (CAB86627), the glucosyl-transferase UGT51B1b from *P. pastoris* (Q9Y751b), the protein YHO0 from *S. cerevisiae* (P38800), and the Rab-like GTPase activator Y45F10A.6a from *C. elegans* (O62462). Identical amino acids are boxed in black; homologous amino acids are boxed in gray.

A

B

C

D

Fig. 4. Developmental phenotype of the *arck/spn* null mutant. Vegetative cells were washed several times in 12 mM NaKPO$_4$ buffer, pH 6.1, to remove nutrients and were plated on nonnutrient agar plates to induce development. Pictures were taken at various times of development. (A) Parental strain KAx-3 and (B) *arkA/spn* double knockout. (C, D) Microscopic observation of the *arkA/spn* double null and wild-type fruiting bodies. Spores (solid white arrows) and vacuolated stalk cells (open arrows) are visible. A solid black arrow marks the stalk tube. Images in part C are phase contrast and part D are DIC.

multicellular development at the mound stage. Chimeric analysis of *spn* null cells suggested that the defect was in the ability to initiate the differentiation of prestalk cells.

To further characterize the phosphorylation-dependent pathway uncovered by the *spn* null mutation, we chose a genetic approach that relied on a second-site suppressor screen (Shaulsky et al., 1996). The objective was to generate second null mutations in the *spn* null strain and identify mutants able to develop further than the original strain. Such a strategy could reveal activators or negative regulators of the down-stream pathways. In this paper, we describe the isolation and characterization of a suppressor of Spalten signaling. The gene

(ARCK-1, for Ankyrin-Repeat Containing Kinase) encodes a novel protein kinase that shares homology with Raf kinases and carries multiple ankyrin repeats.

Materials and methods

Dictyostelium cell culture

Dictyostelium KAx-3 and the various null cells were grown at 21°C in axenic HL5 medium in shaking culture (150 rpm) or on tissue culture plates. When necessary,

A

arkA⁻ pArkA::ArkA **arkA⁻/spn⁻ pArkA::ArkA** **arkA⁻/spn⁻ ΔpSpn::Spn**

B

arkA⁻ cells
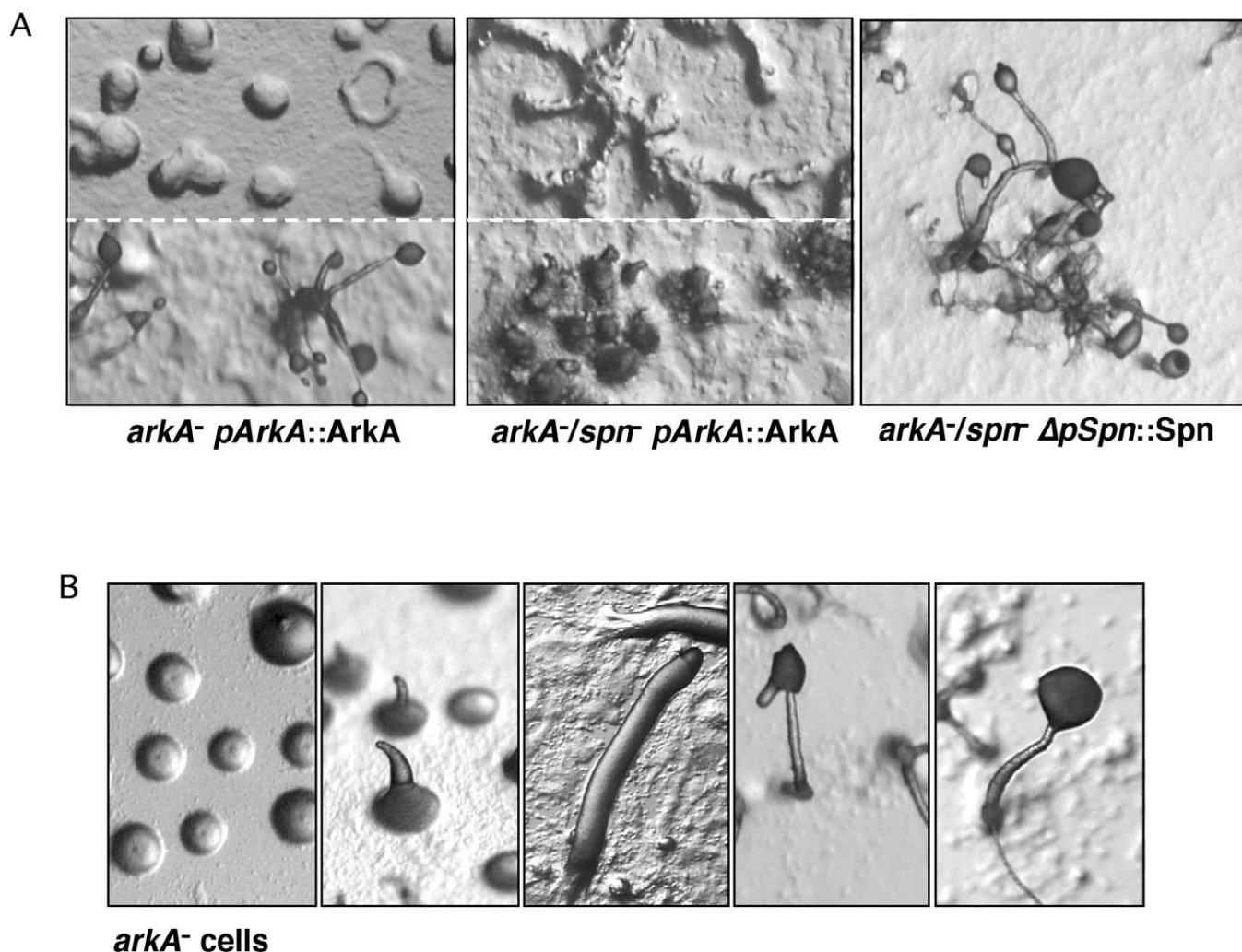
Fig. 5. Overexpression analysis. (A) *arkA/spn* and *arkA* null cells overexpressing either Spn or ARCK-1 under their specific promoter were plated for development on nonnutrient NaKPO₄-buffered agar plates. Pictures represent an intermediate (top panel) and the final (lower panel) stage of development for the different strains. (B) Developmental phenotype of the *arkA* null strain. Cells were plated on nonnutrient agar plates and pictures were taken at various times of multicellular development (aggregation, early first finger, slug, and fruiting body stages). The terminal structure displayed a distorted thick stalk and an abnormal spore mass.

blasticidin or geneticin was added to the medium at the final concentration of 7.5 μg/ml or 20 μg/ml, respectively. For clonal isolation, cells were grown in association with *Klebsiella aerogenes* on rich nutrient agar plates. Isolated colonies were transferred in liquid culture for amplification.

To induce differentiation, cells were washed several times in 12 mM Na/KPO₄ buffer, pH 6.2, to remove all traces of nutrients. Cells were plated (at a density of 2×10^6 cells/cm²) on Na/KPO₄-buffered agar plates and developed over a period of 24 h. For experiments using vegetative cells, cells were harvested from shaking culture at a density of $0.5–1 \times 10^7$ cells/ml.

Insertional mutagenesis

Mutagenesis was produced by using the restriction enzyme mediated insertion (REMI) technique in order to generate suppressors of the *spn* null mutation (Kuspa and Loomis, 1992).

For that purpose, an *spn* null mutant was created by using the JH10 thymidine auxotrophic strain as the parental strain and the *thy1* cassette for selection (Dynes and Firtel, 1989). Mutagenesis was performed as described previously (Aubry and Firtel, 1998) by using the *Bam*HI-linearized pUCBsr plasmid and the restriction enzyme *Dpn*II. Transformants were selected for their resistance to blasticidin conferred by the insertion of pUCBsr in the genome. Cells were cloned on a *Klebsiella aerogenes* lawn and potential suppressors were screened visually for rescue of the *spn* null phenotype and transferred into liquid culture. Our report focuses on two mutants, H2A and SP3A. Plasmid rescue was performed as described by Kuspa and Loomis (1992) using the *Spe*I enzyme for recircularization of SP3A genomic DNA and *Spe*I/*Xba*I for H2A. This allowed the isolation of part of the *ArkA* genomic DNA. The *Nde*I genomic fragment was used as a probe to screen a 12- to 16-h developmental λ-ZAP cDNA library (Schnitzler et al., 1994).
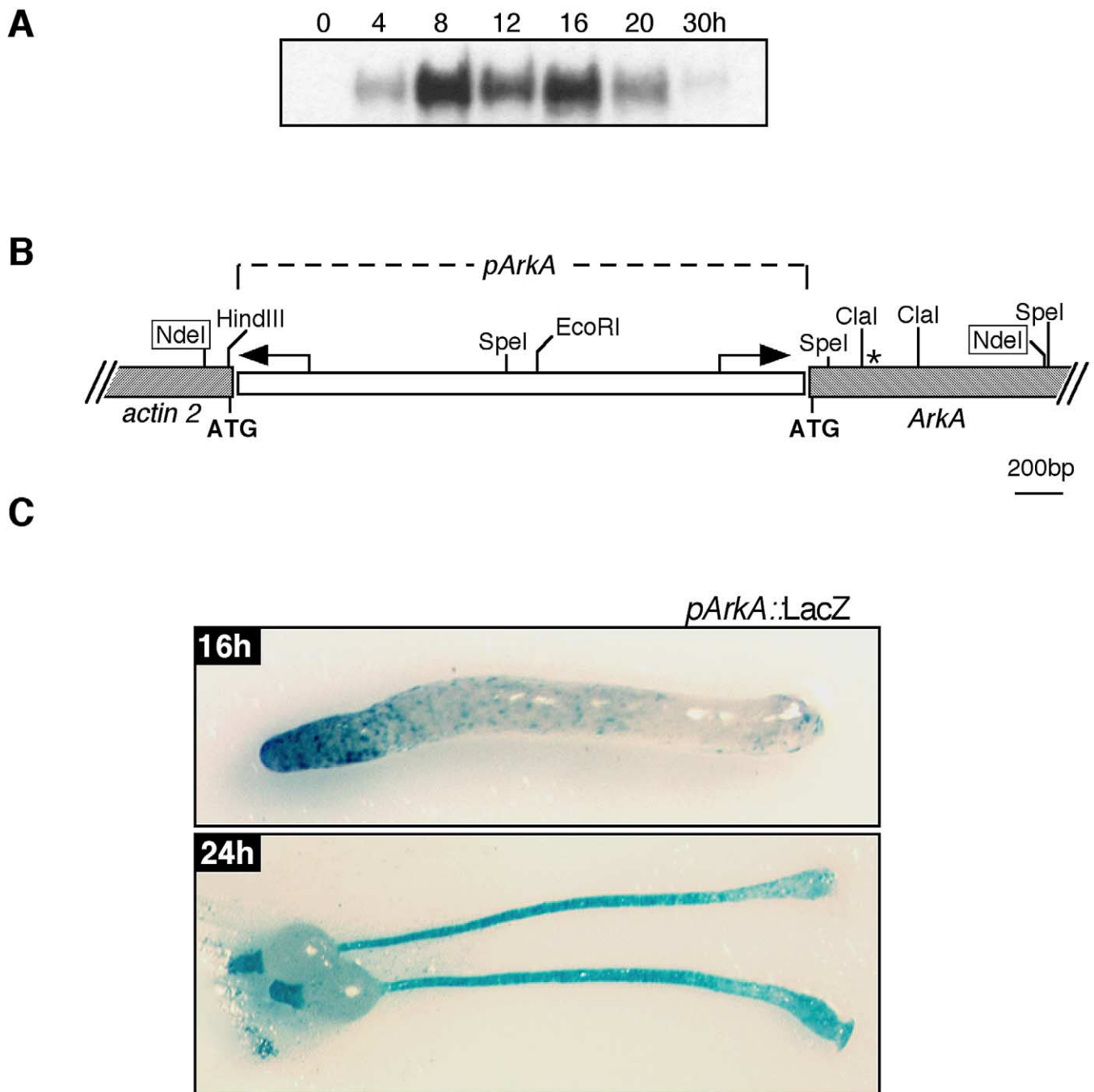
A

B

C

Fig. 6. Temporal and spatial expression analysis. (A) Northern blot analysis. Total RNAs were purified from cells developed for the indicated periods of time on nitrocellulose filters. After separation on a formaldehyde-containing gel, the RNA blot was probed with an *ArkA SpeI-SpeI* fragment labeled with ^{32}P. (B) Cloning of the *ArkA* promoter. The ArkA promoter was cloned by plasmid rescue using the *NdeI* restriction enzyme to digest the genomic DNA. The intergenic region that separated *ArkA* from the upstream *Actin 2* gene was used to control the expression of the *lacZ* reporter gene. *lacZ* was subcloned into the first *ClaI* site in-frame with ARCK-1 ATG. (C) Spatial expression of ARCK-1. Wild-type cells carrying *pArkA::lacZ* were allowed to develop on nitrocellulose filters and were histochemically stained at slug and fruiting body stages for their β-galactosidase activity.

Plasmid constructs

In order to facilitate the subcloning steps, four silent mutations were created to generate the restriction sites that are mentioned in Fig. 1A: *Bam*HI at position 439 (GG-GAGT into GGATCC), *Bgl*II at position 2167 (CGTTCA in

AGATCT), *Nco*I at position 3290 (CTATGG in CCATGG), and *Eco*RV at position 3295 (GATATT in GATATC). The *ArkA* gene disruption construct was made by insertion of the blasticidin-resistance cassette Bsr in the *Bam*HI site. The promoter region of *ArkA (pArkA)* was cloned from the original REMI mutant SP3A by recircularization of the
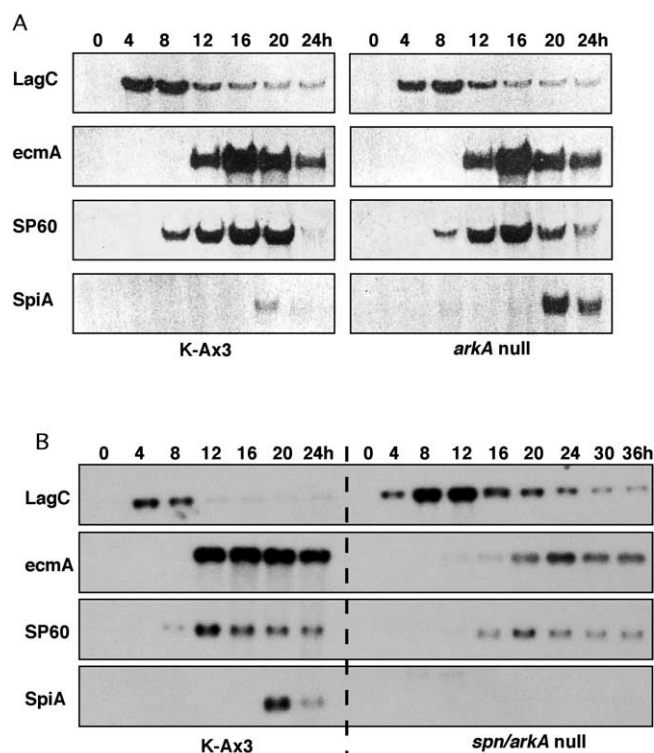
Fig. 7. Northern blot analysis of developmental marker expression. Total RNAs were purified from the wild-type and the *arkA* null (part A) and *arkA/spn* (part B) double null strains harvested at specific times of development. Northern blots were probed with ^{32}P-labeled DNA fragments specific for the postaggregative marker *LagC*, the prestalk gene *ecmA*, the prespore gene *SP60*, and the spore-specific marker *SpiA*.

flanking genomic DNA with the *Nde*I restriction enzyme. This upstream region that included part of the Actin 2 open reading frame (ORF) was shortened to the noncoding region using the upstream endogenous *Hin*dIII site. It was used for β-galactosidase staining experiments, to drive the expression of the *lacZ* reporter gene and for overexpression studies. For the *pArkA:lacZ* construct, the *lacZ* gene was subcloned into the *ArkA ClaI* site (216) in-frame with ARCK-1 ATG. For overexpression studies, various deletion constructs were subcloned into EXP4+ under the control of the ARCK-1 (*pArkA*) or Actin 15 (*pAct15*) promoter. The *pAct15::ARCK-1* and *pArkA::ARCK-1* constructs encompass the full-length cDNA sequence of ArkA, *pAct15::Nt* encompasses residues 1 to 822, *pAct15::kinase* encompasses residues 1097 to 4380, and *pAct15::ank2–5* includes residues 838 to 1103. All of the constructs were tagged with a C-terminal myc2 epitope. Constructs that required PCR amplification were verified by sequencing.

Nucleic acid analysis

The knockout strain genotype was confirmed by Southern blot analysis as described previously (Mann et al., 1998). Genomic DNA from vegetative cells was digested with appropriate restriction enzymes and electrophoresed on a 1% agarose gel. DNA fragments were transferred onto a nitrocellulose membrane and hybridized with a ^{32}P-labeled *ArkA* probe (*Spe*I-*Spe*I fragment). For Northern blot analysis, cells were plated onto nitrocellulose filters and developed for various periods of time. Total RNA was purified and separated on a formaldehyde-containing gel (Mann et al., 1998). The same fragment used for the Southern blot was also used to probe the Northern blot to follow Arck RNA expression during multicellular development.

Western blot analysis

Whole cell lysates were loaded on a 12% SDS-polyacrylamide gel. Proteins were transferred onto an Immobilon-P membrane (Millipore) and incubated sequentially with an mouse anti-myc monoclonal antibody (9E10 Boehringer 1:1000) for 1 h and a goat anti-mouse HRP-conjugated IgG (Biorad 1:3000). Proteins were detected by enhanced chemiluminescence (ECL from Amersham Biosciences).

Immunofluorescence

Cells carrying the various myc-tagged constructs were allowed to attach to a multichamber slide (Labteck) for 10 min in Na/KPO$_4$ buffer. Cells were fixed in 4% paraformaldehyde/40 mM Mes-Na, pH 6.5, for 10 min and permeabilized in 0.2% Triton X-100/40 mM Mes-Na, pH 6.5, for 2 min. After several washes in PBS-0.5% BSA, cells were incubated for 1 h at room temperature in the presence of a mouse anti-myc antibody (9E10 Boehringer 1:500 dilution), washed in PBS–0.1% Tween 20, and incubated for 1 h with an anti-mouse FITC-coupled goat antibody (Jackson Immunoresearch, 1:200 dilution). Cells were washed several times, mounted using the Cityfluor mounting solution, and observed under a Zeiss Axiovert 200M microscope (100×). For F-actin staining, fixed cells were incubated in PBS containing 1 μM phalloidin-TRITC for 1 h at room temperature. For nuclear staining, fixed cells were incubated with 4′-6-diamidine-2-phenylindole (DAPI) stain (1 μM) for 10 min at room temperature just prior to the last washes.

Subcellular fractionation

Twelve-hour developed cells expressing *pArkA::ARCK-1* were resuspended in 20 mM triethanolamine, pH 7.5, containing protease inhibitors and lysed by vortexing in the presence of 0.18-mm glass beads. The lysate was centrifuged at 800*g* for 10 min to remove unbroken cells, nuclei, and glass beads. A centrifugation at 100,000*g* for 1 h at 4°C allowed the soluble fraction (C) to separate from the particulate fraction (P) that contained both membranes and cytoskeletal matrix components. The resulting pellet (P) was resuspended in a volume of lysis buffer equivalent to that of the supernatant fraction. The same volume of each fraction was loaded on a 12% SDS-PAGE gel and analyzed by Western blot.

β-Galactosidase staining

Cells carrying the *pArkA::lacZ* construct were allowed to develop on nitrocellulose filters laid on Na/KPO$_4$ buffered-agar plates. *β*-Galactosidase staining of the developed structures was performed as described previously (Mann et al., 1998).

Results

Isolation of REMI suppressors of the spn null mutation and cloning of the gene encoding ARCK-1

In *Dictyostelium,* disruption of the gene encoding Spalten (Spn), a PP2C serine-threonine protein phosphatase, leads to a marked developmental defect, as cells arrest at mound stage and fail to differentiate into either prestalk or prespore cells (Aubry and Firtel, 1998). To determine the components of the Spn signaling pathway, we carried out a suppressor screen (Shaulsky et al., 1996) using the *spn* null mutant as a template strain for a second round of mutagenesis. Mutagenesis was achieved by using REMI insertional mutagenesis (Kuspa and Loomis, 1992) to generate knockout suppressors of the *spn* null phenotype. The transformants were cloned on a lawn of *Klebsiella aerogenes* spread onto agar plates and screened visually as they proceeded through multicellular development. Out of ~4000 transformants, 5 clones were found to develop beyond the mound stage. Two of these, SP3A and H2A, were able to form small-sized fruiting bodies and were further analyzed (Fig. 1A).

Regions of the gene flanking the insertion site were cloned by plasmid rescue using the enzymes *Spe*I and *Spe*I/*Xba*I for SP3A and H2A mutant strains, respectively, and the resultant genomic DNA was used to screen a 12- to 16-h developmental λZAP cDNA library (Schnitzler et al., 1994). Sequence analysis of the cDNA clones revealed that SP3A and H2A were both mutated in the same ORF, but insertion had occurred in distinct sites within the gene and thus represented independent mutagenesis events (Fig. 2A). This gene was designated *ArkA* and the protein is ARCK-1. To confirm that suppression of the *spn* null phenotype was due to an insertion into the *ArkA* ORF, the plasmid rescued from SP3A was linearized with *Spe*I and used for homologous recombination in the original *spn* null strain (Fig. 1B). Disruption of *ArkA* led to the same phenotype as that of the REMI mutant. This recapitulated *arkA* null strain was used for subsequent analysis.

Primary structure of ARCK-1

ArkA encodes a novel protein of 1460 amino acids (Fig. 2B and C). It contains short homopolymer runs of serines and asparagines, a common feature of many *Dictyostelium* proteins. The overall richness in serine and threonine residues is around 20%. Comparison of the derived amino acid sequence of ARCK-1 to GenBank databases using BLAST and SMART revealed four major previously described domains (Fig. 2B). The C-terminal region of ARCK-1 is highly homologous to the catalytic domain of protein kinases within regions common to Tyr and Ser/Thr protein kinases (Fig. 3A). Fig. 3A represents an alignment of the ARCK-1 C-terminal portion with a consensus sequence of Ser/Thr kinases (SMART 00220) and Tyr kinases (SMART 00219). Kinase subdomains VI and VIII, which contain residues specific for either Tyr or Ser/Thr kinases, have been more closely analyzed to classify ARCK-1 into a protein kinase subfamily (Fig. 3A). The ARCK-1 sequence is identical to the consensus for Ser/Thr protein kinases, suggesting that it may be specific for this type of hydroxy-amino acid. The central region of ARCK-1 contains five ankyrin repeats, the first one being more degenerated than the others (Fig. 3B). The N-terminal part of the protein contains a number of identifiable motifs, including putative phosphorylation sites for MAP kinases and Akt/PKB (Fig. 2C) and a cysteine-rich region (CRD) homologous to the C1 domain of PKC and Raf (Fig. 3C). The hallmark of C1 domains is an ~50-amino-acid sequence (organized as $HX_{10-13}CX_2CX_{11-19}CX_2CX_4H_{2-4}CX_{5-9}$) in which cysteine and histidine residues allow coordination of two zinc atoms (Hurley et al., 1997). C1 domains fall into two categories. The typical ones that are found in most PKC isozymes interact with diacylglycerol and phorbol esters (typical PKCs), whereas the atypical ones, present in Raf, Vav, and atypical PKCs, do not. Diacylglycerol/phorbol ester binding requires a flexibility of the β1-β2 and β3-β4 loops of the C1 domain (Fig. 3C; Mott et al., 1996). In the Raf and Ksr C1 domains, the β3-β4 loop is immobilized due to a deletion in the protein sequence (Mott et al., 1996; Zhou et al., 2002). In the CRD of ARCK-1, the putative β3-β4 loop is closer to the length of the PKC C1.

Between the CRD and the ankyrin repeats, ARCK-1 also has a GRAM domain (Doerks et al., 2000), named after the glucosyltransferases, Rab-like GTPase activators, and myotubularins that are proposed to mediate protein or lipid binding in membrane-associated signaling processes. In *Dictyostelium,* three other proteins have been identified that contain GRAM domains: the sterol glucosyltransferase UGT52 (Doerks et al., 2000) and two cGMP-binding proteins, GBPA and GBPB (Goldberg et al., 2002). To our knowledge, no protein with an organization similar to that of ARCK-1 was previously identified in the databases of *Dictyostelium* and other organisms.

Disruption of ArkA partially rescues the spn null phenotype

Fig. 4A shows the developmental phenotype of the double *arkA/spn* null strain. The parental cells aggregate and form mounds in 8 h, followed by morphogenesis steps that eventually lead to a mature fruiting body (Fig. 4A). The

arkA/spn cells aggregate normally, as assayed by the formation of mounds on nonnutrient agar plates, but the mounds disaggregate slightly to form smaller structures, a phenotype reminiscent of that of the *spn* null phenotype (Aubry and Firtel, 1998). Whereas the *spn* null cells arrest at the mound stage (Fig. 1A), the double knockout strain was able to generate a fruiting-body-like structure that formed directly from the mound stage, bypassing the slug stage (Fig. 4B). When compared with the parental strain, the fruiting body was abnormal, with a very short, thick stalk and a large mass of cells (Fig. 4C). To determine the cellular structure of these fruiting bodies, culminates were picked and gently squashed in phosphate buffer and examined under phase and DIC microscopy (Fig. 4C and D, respectively). Wild-type fruiting bodies had a stalk tube with vacuolated stalk cells that extends through the spore mass (Fig. 4D). The *arkA/spn* null structures had a short stalk tube with vacuolated cells. This structure was very short but appeared to enter the larger mass of cells, which may be prespore cells that did not differentiate into spores. A few mature spores were seen as ellipsoid-shaped cells (Fig. 4C). Further, there appeared to be other vacuolated cells that were not aligned within a stalk tube, suggesting that terminal morphogenesis was very abnormal, consistent with the abnormal morphology of the terminal structures (Fig. 4C and D).

Overexpression of Spn in *arkA/spn* null cells under its own promoter, Δ*pSpn* (Aubry and Firtel, 1998), partially complemented the *arkA/spn* null strain developmental phenotypes; the fruiting bodies that formed, though small, were better-proportioned (Fig. 5A). In contrast, overexpression of ARCK-1 in *arkA/spn* null cells under its own promoter, *pArkA* (see Materials and methods), caused abnormal development (Fig. 5A). The aggregation step was impaired, with aggregation streams failing to form nicely shaped mounds. Rudimentary morphogenesis occurred within the aggregation streams to generate short fingers that represent the final phenotype of development for that strain. Many of the cells did not undergo further development (see below).

ArkA gene disruption and ARCK-1 overexpression lead to developmental defects

To evaluate the role of the ARCK-1 protein in the KAx-3 parental strain, an *ArkA* knockout strain was generated by homologous recombination, which was confirmed by Southern blot analysis (data not shown). Loss of *ArkA* function did not visibly affect development until culmination, when the fruiting bodies displayed an abnormal structure with a fatter and distorted stalk as well as a non-round-shaped spore mass (Fig. 5B). After an extended period of time on agar plates, some of the multicellular structures progressively lost their integrity and the spore mass collapsed along the stalk (data not shown).

Northern blot analysis of the expression of stage- and cell-type-specific developmental markers did not reveal any

strong defects. The postaggregative gene LagC was expressed with a time course similar to that observed in the parental strain, as were the prestalk-specific marker *ecmA* and the prespore-specific marker *SP60/CotC*. The level of expression of the spore-specific marker *SpiA* was higher in the mutant than in the parental strain, an observation that may reflect the late developmental phenotype observed for the *arkA* null mutant (Fig. 6A).

Overexpression of wild-type or myc-tagged ARCK-1 under its own promoter ($pArkA$::ARCK-1$_{myc}$) in the *arkA* null or wild-type strains resulted in more severe developmental defects. The aggregates displayed a rough mound phenotype as observed with the *spn* null strain, and most of the cells did not develop further. Any fruiting bodies that formed were of a much smaller size than the parental ones, with several emerging from a single mound (Fig. 5B; data not shown). As overexpression of ARCK-1 in wild-type cells produced phenotypes that were similar to those of ARCK-1 overexpression in *arkA* null cells, we expect that the inability to complement the *arkA* null phenotypes with $pArkA$::ARCK-1 is the result of ARCK-1 overexpression.

The morphological analysis indicates that disruption of *ArkA* in the *spn* null background results in a partial suppression of the *spn* null morphogenesis defects. To determine whether the *arkA/spn* null strain subsequently expresses cell-type-specific genes, we undertook a Northern blot analysis of the selected mound stage, prestalk, prespore, and spore cell markers as shown in Fig. 6A. In contrast to *spn* null cells, which exhibited no detectable prestalk (*ecmA*) or prespore (*SP60/CotC*) gene expression (Aubry and Firtel, 1998), both cell-type-specific markers were expressed in the *arkA/spn* null strain. The expression of both genes was delayed compared with wild-type cells, and the expression level was reduced, as might be expected from the observation that the morphological phenotype of the *arkA/spn* null strain was very abnormal, some cells did not enter the aggregate, and morphogenesis was delayed. The mound-stage marker *LagC* was induced normally but the transcript did not decrease as rapidly in the *arkA/spn* null strain as it did in wild-type cells. The expression level was higher, possibly because some of the mounds did not proceed through development. The spore-specific marker *SpiA* was not detectably expressed in the *arkA/spn* null strain, consistent with the absence of mature spores.

### ArkA expression is spatially and temporally regulated

As shown in Fig. 7A, the *ArkA* transcript was not detected during axenic growth but was rapidly induced during aggregation with transcript levels exhibiting two peaks, one at 8 h at the end of aggregation and one at 15 h during later morphogenesis. Transcript levels then decreased (Fig. 7A). To determine the spatial pattern of *ArkA* expression during development, we cloned the ~2.5-kb region upstream of the ARCK-1 ORF by plasmid rescue of the SP3A mutant using the *Nde*I restriction enzyme for recircularization (Fig. 7B).
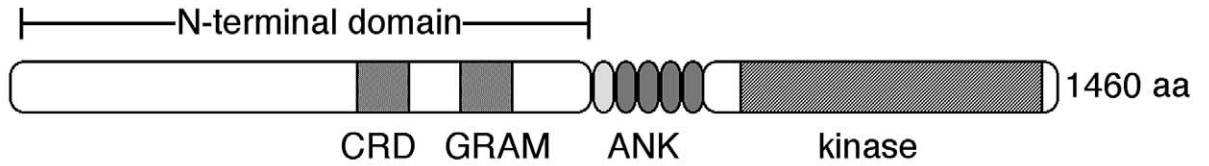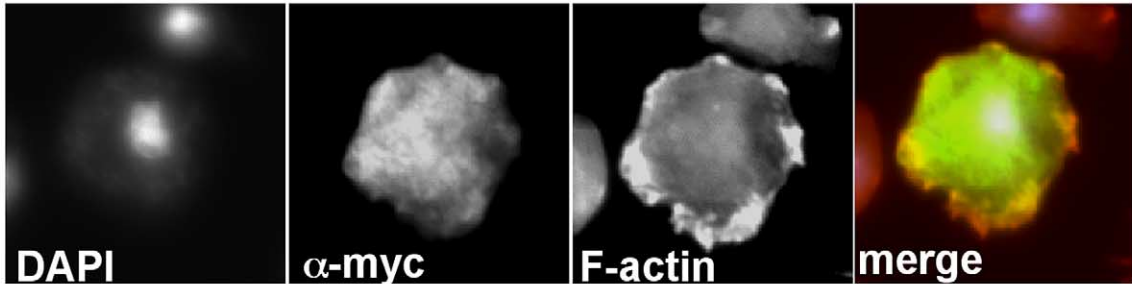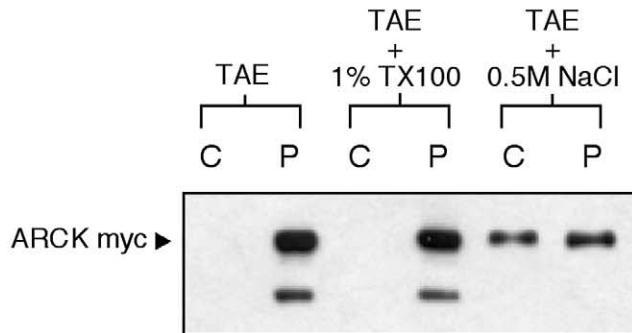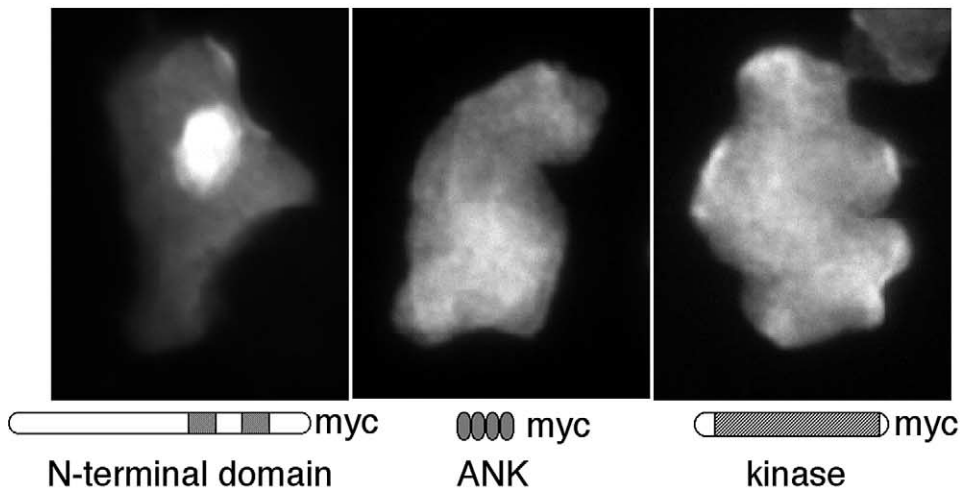
Fig. 8. Subcellular localization of ARCK-1. (A) Immunofluorescence analysis of KAx-3 cells overexpressing *pAct15::Arck$_{myc}$*. Cells were allowed to adhere and were fixed in paraformaldehyde. After permeabilization with Triton X-100, cells were successively incubated with an anti-myc antibody and a FITC-conjugated secondary antibody. A costaining was performed with phalloidin-TRITC and DAPI. Staining was visualized on a Zeiss Aviovert 200M microscope. (B) Subcellular fractionation. Fractionation experiments were performed on KAx-3 cells overexpressing ARCK-1$_{myc}$ after 12 h of development on nonnutritive agar plates. Cells were harvested in 20 mM triethanolamine at pH 7.5 and lysed by vortexing cells with glass beads. Nuclei and unbroken cells were removed by an 800g centrifugation. The remaining supernatant was treated with 1% Triton X-100 or 0.5 M NaCl and centrifuged at 100,000g for 1 h to separate the cytosol (C) from the particulate fraction (P). The pellet was resuspended in the same volume of buffer as the cytosolic fraction. Aliquots of each fraction were separated on an SDS gel and analyzed by Western blot using an anti-myc antibody. (C) Immunolocalization of ArckNt$_{myc}$, Ank2-5$_{myc}$, and kinase$_{myc}$. Staining was performed on vegetative cells as described in (A).

The fragment was sequenced and contained the starts of the *ArkA* and *Actin 2* genes in a head-to-head orientation.

The intergenic sequence was used to drive the expression of the *lacZ* reporter gene in the parental strain. No β-galactosidase activity was detected in vegetative cells (data not shown). During development, β-galactosidase-positive cells were found scattered throughout the slug with a higher density in the prestalk region (Fig. 6C). As development proceeded, the more deeply stained cells were excluded from the spore mass and mainly localized in the stalk and the tip of the fruiting body. The overall bluish cast of the sorocarps suggests that there may be some *ArkA*-expression in spores. No staining was observable in the upper and lower cups of the fruiting body, suggesting that *ArkA* is preferentially expressed in a subclass of prestalk cells.

Subcellular localization of ARCK-1

Attempts to generate specific ARCK-1 antibodies for immunofluorescence experiments were unsuccessful. Localization studies were therefore performed on cells carrying the *pAct15::ARCK-1$_{myc}$* construct. Indirect immunofluorescence using an anti-myc antibody indicated that ARCK-1 is localized in the subcortical region of the cell with some enrichment in cell protrusions (Fig. 8A). Subcellular fractionation experiments on the same cells developed for 12 h on agar plates showed that ARCK-1$_{myc}$ is predominantly, if not totally, present in the 100,000g pellet fraction that contains membranes and components of the cytoskeleton (Fig. 8B). Treatment of the extract with 0.5 M NaCl released part of the protein into the supernatant fraction. To test the possibility of an association of ARCK-1$_{myc}$ with the cytoskeleton, the cellular lysate was solubilized in 1% Triton X-100 prior to ultracentrifugation, a treatment known to maintain the integrity of the cytoskeleton. Such treatment failed to separate ARCK-1$_{myc}$ from the pellet fraction, suggesting that ARCK-1$_{myc}$ may be associated with the cytoskeletal matrix. However, ARCK-1 and F-actin did not completely colocalize as determined by costaining of ARCK-1$_{myc}$ and F-actin using phalloidin-TRITC (Fig. 8A).

To identify the domain(s) of ARCK-1 responsible for that localization, deletion mutants were generated and the deleted proteins were expressed in KAx-3 cells from the Actin 15 promoter (Fig. 8C). Observation of immunostained KAx-3 overexpressing the kinase domain revealed a distribution similar to that of ARCK-1$_{myc}$ with a stronger staining in the cytosol (Fig. 8C), a result consistent with the findings of the subcellular fractionation experiments in which kinase$_{myc}$ equally partitioned between the cytosolic and the pellet fractions (data not shown). Surprisingly, the N-terminal domain (Fig. 8C), which contains the cysteine-rich motif, predominantly distributed in the nucleus, as confirmed by the colocalization with 4′-6-diamidine-2-phenylindole (DAPI) staining (data not shown). Such localization may illustrate the ability of the full-length protein to translocate

Table 1
Binding of Ras to the putative Ras binding domain (RBD) of ARCK-1

Ras isoform	RBD ARCK-1	RBD PI3K1
RasB^{Q61L}	$++++$	$-$
RasB^{N17S}	$-$	$-$
RasD^{Q61L}	$-$	$++$
RasG^{Q61L}	$-$	$++++$
RasG^{N17S}	$-$	$-$
RasS^{Q61L}	$-$	$-$

Note. The table summarizes the results of two-hybrid analyses of the interaction of several constitutively active Ras (Q61L) and dominant negative Ras (N17S) isoforms with the Ras binding domain (RBD) of ARCK-1. The RBD of PI3K1 was used as a control in these assays. The results of the binding of the PI3K1 RBD have been published previously (Lee et al., 1999).

in the nucleus in response to a regulatory signal. A construct containing ankyrin motifs ank2 to ank5 was cytosolic.

ARCK-1 interacts with RasB and 14-3-3

The N-terminal regulatory domain of Raf kinase interacts with the small G protein Ras through two distinct domains. The first Ras-binding domain encompasses residues 55–131 and the second binding site is included in the cysteine-rich C1 domain of human Raf (residues 139–184) (Brtva et al., 1995). In addition, this same region interacts with the scaffolding protein 14-3-3 (Clark et al., 1997). Analysis of the ARCK-1 sequence in the region surrounding the C1 domain allowed us to identify a potential 14-3-3 interaction site within the sequence ^{597}RTPSSP. The *Dictyostelium* genome contains several *Ras* genes (*RasD, G, S,C,* and *B,* and *Rap*) (Chubb and Insall, 2001) and a single isoform of *14-3-3* (Knetsch et al., 1997) that were used in a yeast two-hybrid assay to test the interaction of the corresponding proteins with an internal region of ARCK-1 (amino acids 467–667) encompassing the CRD and the putative 14-3-3 binding site.

The fragment 467–667 including both motifs (CRD + 14-3-3 site) as well as the larger N-terminal region (Fig. 2B) were able to interact with *Dictyostelium* 14-3-3 as indicated by yeast growth and β-galactosidase activity on protein-interaction selective media (GAL-UHTLXgal; data not shown). However, deletion of either motif (fragments 467–571 and 562–667) completely abolished interaction. The putative Ras binding domain (RBD, residues 467–667) was tested for its ability to bind the constitutively active (GTP-bound) and dominant negative (GDP-bound) forms of several *Dictyostelium* Ras proteins. As shown in Table 1, the RBD binds to constitutively active RasB (RasB^{Q61L}) but not dominant negative RasB (RasB^{N17S}), nor does it bind the constitutively active forms of *Dictyostelium* RasD, RasG, and RasS.
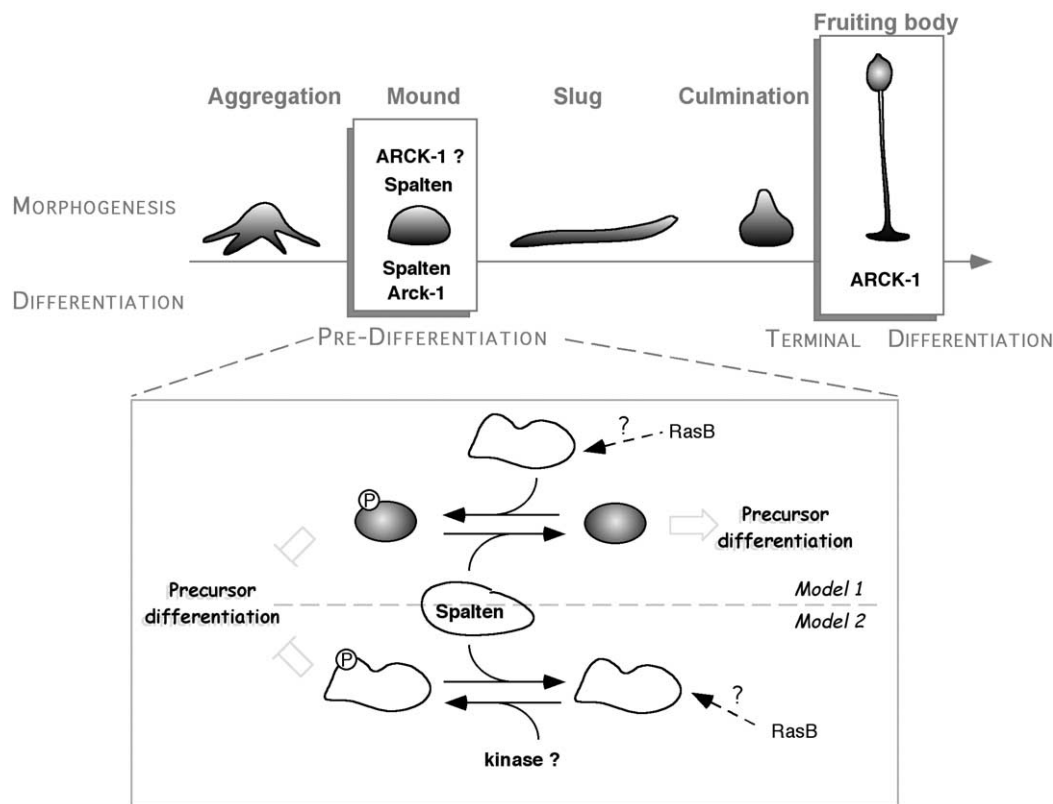
Fig. 9. Model for ARCK-1 function. *ArkA* disruption is able to rescue cell-type differentiation in the *spn* null mutant but is insufficient to restore normal morphogenesis. *ArkA* disruption in the parental strain is hardly affected except in the latest steps of development. These data suggest several periods of activity for ARCK-1 at the mound stage when induction of the precursor cell differentiation takes place and in the final stages of development. At the mound stage, a residual kinase activity prevents a complete rescue, suggesting the involvement of an additional and still-unknown protein kinase that inhibits morphogenesis. In the predifferentiation process, ARCK-1 and Spn could regulate the level of phosphorylation of a common substrate, the nonphosphorylated form being indispensable for differentiation to occur. ARCK-1 could also be the substrate of Spn, this hypothesis implying that ARCK-1 inhibits differentiation when phosphorylated.

Discussion

In *Dictyostelium,* multicellular development is dependent on the PP2C protein phosphatase Spalten (Spn) (Aubry and Firtel, 1998). Disruption of the Spn gene leads to a complete arrest of development prior to cell-type differentiation with *spn* null cell aggregates lacking the ability to produce prespore and prestalk cells. The preferential expression of Spn in prestalk cells, combined with chimeric studies, suggested that the lack of prespore cells in the *spn* null strain is due to the strain's inability to produce prestalk cells, supporting models in which prespore cell differentiation requires a cell nonautonomous signal from developing prestalk cells. These data suggested a molecular model in which the dephosphorylation of a presently unknown substrate by Spn is essential for cell-type differentiation. To dissect this phosphorylation-dependent pathway, we performed a second-site suppressor screen and selected mutants that were able to proceed further into development. This strategy allowed the identification of ARCK-1, a novel ankyrin repeat-containing, putative Ser/Thr kinase.

Disruption of *ArkA* in the *spn* null mutant background restores its ability to differentiate prestalk and prespore cells. Vacuolated stalk cells are formed but no spores. Abrogation of ARCK-1 kinase activity in the *spn* null strain is thus sufficient to allow the differentiation of the precursor cells, in agreement with a model in which ARCK-1 activity prevents induction of the predifferentiation pathway. Disruption of *ArkA* in the parental wild-type strain results in a weak and late developmental phenotype and no detectable effect on the expression of preculmination cell-type-specific markers. These observations suggest that ARCK-1 kinase activity is not essential for the early steps of development. The simplest hypothesis to explain the Spn-ARCK-1 relationship, considering that both proteins are preferentially expressed in ALC-prestalk cells, is that ARCK-1 antagonizes the activity of Spn by regulating the level of phosphorylation of a common substrate (Fig. 9). This substrate would act either as an activator of differentiation in its nonphosphorylated form or as an inhibitor of differentiation in its phosphorylated form. In one model, ARCK-1 would phosphorylate the substrate, and the function of the phosphorylated substrate would be to regulate the progression of development. Spn would relieve this block by dephosphorylating the substrate. In the absence of Spn, development would be unable to proceed. In support of this model,

overexpression of ARCK-1 results in early developmental defects. It is also possible that ARCK-1 is the substrate of Spn, although such a hypothesis would require ARCK-1 acting as a negative regulator of development when phosphorylated. We cannot exclude the possibility that these two proteins belong to distinct signaling pathways and ARCK-1 mutation could bypass the developmental block due to the loss of Spn function, allowing cell-type differentiation.

In the *arkA/spn* double null mutant, the developmental phenotype is only partially rescued, with multicellular structures developing directly from aggregates to form a short and massive fruiting body with a spherical shape. Morphogenesis remains strongly impaired. These data, together with the absence of major morphogenesis defects in the *arkA* null strain, suggest that Spn is necessary for the morphogenesis process and it is likely to antagonize the activity of another protein kinase in addition to ARCK-1 in that particular aspect of development. Analysis of *Dictyostelium* genomic DNA databases indicates the existence of a homolog of ARCK-1 (which we designate ARCK-2) that carries at least five ankyrin repeats and a putative C-terminal Ser/Thr kinase domain with 36% identity to that of ARCK-1. It is therefore possible that ARCK-1 and this homolog share redundant functions in the early steps of development. A knockout of the ARCK-1 homolog or possibly of both genes might be necessary to fully restore *spn* null morphogenetic defects. From the analysis of the *arkA* null mutant, ARCK-1 seems to function in the later stages of development. Alternatively, ARCK-1 may function in the Spalten pathway and other pathways to control development. It is also possible that Spalten may function in multiple pathways, only one of which is suppressed in the *spn/arkA* null strain. Further study is required to determine whether Spn is involved as well.

In addition to its kinase domain, ARCK-1 harbors five ankyrin repeats (Sedgwick and Smerdon, 1999) and a long N-terminal extension carrying a cysteine-rich domain (CRD) and a GRAM domain (Doerks et al., 2000). The cysteine-rich sequence is most closely related to the tandemly repeated CRD of the PKC regulatory domains (Hurley et al., 1997) and thus may bind diacylglycerol. The cysteine-rich motif of ARCK-1 is flanked by a consensus binding site for the adaptor protein 14-3-3. Yeast two-hybrid experiments indicate that a domain including both motifs can bind *Dictyostelium* 14-3-3 and the GTP-bound form of *Dictyostelium* RasB, one of several *Dictyostelium* Ras proteins. We expect the binding of RasB^{GTP} may take place through the CRD, but this has not been demonstrated by further deletion analysis or the use of point mutants. This domain does not bind GTP-bound RasD, RasG, RasS, and RasC, which function in signaling pathways associated with cell movement, phototaxis, and phagocytosis (Chubb et al., 2000; Khosla et al., 2000; Tuxworth et al., 1997; Wilkins et al., 2000). Similar data have been obtained on the Raf kinase whose activity is regulated by interactions with Ras and 14-3-3 through its N-terminal domain (Brtva et al.,

1995; Clark et al., 1997). RasB has been described as translocating to the nucleus (Sutherland et al., 2001). The interaction of ARCK-1$_{467-667}$ with RasB may explain the nuclear enrichment of the ARCK-1 N-terminal domain when deleted from its whole complex C-terminal domain. For the full-length protein, this localization within the nucleus may be transitory and under the tight control of a domain included in the C-terminal extremity. Approaches with subcellular fractionation performed on developed cells revealed that ARCK-1 is mostly associated with a Triton X-100-insoluble fraction in which it colocalizes with the protein Spn (data not shown).

At this step of the work, the identification of the partners that interact with ARCK-1 through domains such as the ankyrin repeats and the GRAM domain will provide necessary clues as to what role is played by the putative kinase ARCK-1 and how the coordinated action of the coupled Spn-ARCK-1 regulates differentiation during multicellular development.

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