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A novel disintegrin domain protein affects early cell type specification and pattern formation in *Dictyostelium*

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SUMMARY

The cellular slime mold, *Dictyostelium discoideum* is a non-metazoan organism, yet we now demonstrate that a disintegrin domain-containing protein, the product of the *ampA* gene, plays a role in cell type specification. Disintegrin domain-containing proteins are involved in Notch signaling in *Drosophila* and *C. elegans* via an ectodomain shedding mechanism that depends on a metalloprotease domain. The *Dictyostelium* protein lacks a metalloprotease domain. Nonetheless, analysis of cell type specific reporter gene expression during development of the *ampA* null strain identifies patterning defects that define two distinct roles for the AmpA protein in specifying cell fate. In the absence of a functional *ampA* gene, cells prematurely specify as prespore cells. Prestalk cell differentiation and migration are delayed. Both of these defects can be rescued by the inclusion of 10% wild-type cells in the developing null mutant aggregates, indicating that the defect is non-cell autonomous. The *ampA* gene is also demonstrated to be necessary in a cell-autonomous manner for the correct localization of anterior-like cells to the upper cup of the fruiting body. When derived from *ampA* null cells, the anterior-like cells are unable to localize to positions in the interior of the developing mounds. Wild-type cells can rescue defects in morphogenesis by substituting for null cells when they differentiate as anterior-like cells, but they cannot rescue the ability of *ampA* null cells to fill this role. Thus, in spite of its simpler structure, the *Dictyostelium ampA* protein carries out the same diversity of functions that have been observed for the ADAM and ADAMTS families in metazoans.

Key words: Differentiation, Pattern formation, Anti-adhesive, Cell type-specific gene, Expression, *Dictyostelium discoideum*, Disintegrin, ADAM

INTRODUCTION

Cell adhesions are a vital component of the tissue architecture of developing organisms. Modulation of cell adhesions to allow cell migration during embryogenesis or cell fusion during myogenesis represent extremes of a continuum of dynamic interactions between cells and their underlying matrices. The sites of cell contact are not just static structures, but are increasingly recognized as highly complex interaction sites that couple molecules that recognize the extracellular environment with molecules of the cellular signal transduction systems (Yamada and Geiger, 1997). The coupling of cell adhesions to signal transduction is central to the regulation of growth, differentiation and apoptosis.

The disintegrin domain has been increasingly identified as a motif in several molecules involved in modulating adhesive interactions between cells and extracellular matrix components, as well as in morphogenetic signaling events (Huang, 1998). Initially identified as a soluble hemorrhagic peptide in viper venom that competitively inhibits platelet integrin receptors binding to fibrin (McLane et al., 1998), disintegrin domains have now been found in transmembrane and secreted proteins from a wide spectrum of metazoan organisms (Schlondorff and Blobel, 1999). The ADAM and ADAMTS family of proteins contain a disintegrin and a metalloprotease domain coupled with a cysteine-rich region, usually containing an EGF repeat. In the ADAMTS family, the cysteine-rich region is followed by a series of thrombospondin repeats. The role played by molecules containing disintegrin-like motifs often depends upon the context in which the domain is expressed and whether or not the metalloprotease domain is active (Schlondorff and Blobel, 1999). When expressed as part of an integral membrane protein, the disintegrin domains can carry out a fusogen-like function during myogenesis and sperm-egg fusion (Töpfer-Petersen, 1999; Yagami-Hiromasa et al., 1995). ADAMs with active metalloprotease activity have been involved in ectodomain shedding, where the disintegrin domain functions to target the protease domain to its extracellular substrate (Schlondorff and Blobel, 1999). It is through this mechanism that the *Drosophila Kuzbanian* gene and the *C. elegans sup-17* gene function to cleave the Notch ligand, Delta, influencing cell-fate specification (Rooke et al., 1996; Wen et al., 1997).

A role for ADAM proteins in facilitating cell motility has also been observed. Two different members of the ADAM family appear to modulate cell motility in distinctly different ways. ADAM9, an integral membrane protein facilitates fibroblast migration by disrupting integrin binding to

extracellular matrix ligands, while the *C. elegans* ADAM, *mig-17*, is secreted and influences the route of migration of the distal tip cells (Nath et al., 2000; Nishiwaki et al., 2000). It is the metalloprotease domain that is essential for the role of *mig-17* role in cell migration, while the disintegrin domain plays a localization function.

A more widely diverged disintegrin domain protein has recently been identified in *Xenopus* where it functions in dorsal/ventral specification (Matsui et al., 2000). The *Xenopus* kielin protein is secreted and contains thrombospondin repeats followed by chordin repeats, and has a disintegrin domain at the C terminus.

We have recently described another highly diverged disintegrin domain-containing protein from a non-metazoan organism, the cellular slime mold *Dictyostelium discoidium* (Varney et al., 2002). This protein, AmpA, influences developmental progression by modulating cell-cell and cell-substrate adhesions (Varney et al., 2002). AmpA contains an N-terminal signal sequence followed by two disintegrin-like domains and four repeats, which are similar to thrombospondin repeats and share homology with the leech ornatins, a family of anti-coagulant peptides found in leech saliva (Varney et al., 2002). Unlike the ADAM and ADAMTS families AmpA does not contain a metalloprotease domain. We show that like Kuz and Sup17, AmpA also plays a role in cell fate specification, possibly through a mechanism involving lateral inhibition. However, as AmpA lacks a metalloprotease domain, it cannot do this directly through ectodomain shedding.

Dictyostelium is one of the simplest organisms to undergo true multicellular development. It is a single-celled amoeba that can be grown axenically in a shaken suspension, similar to tissue culture cells or, it can feed on bacteria plated on a solid substratum. Development is initiated by starvation. Multicellularity is achieved by chemotactic migration of single amoebae into aggregation centers, or mounds, where several different adhesion proteins mediate cell-cell contacts (reviewed by Kessin, 2001). Within the mounds cells differentiate and undergo morphogenesis to form a fruiting body consisting of spore cells supported by a stalk with a basal disc that anchors the fruiting body to the substrate.

The *ampA* gene is expressed at a low level in most growing cells but expression is rapidly restricted to anterior-like cells (ALCs) during development (Casademunt et al., 2002). These cells represent about 10% of the total cells in the fruiting body. They form the support structures of the mature fruiting body, the upper and lower cups that support the spore head and the basal disc. During the later stages of development the migrations of the anterior-like cells play a major role in morphogenesis of the fruiting body (Abe et al., 1994; Dormann et al., 1996).

The AmpA protein functions to modulate adhesions during development. Inactivation of AmpA causes increased cell-cell and cell-substrate adhesion that delays or arrests development (Varney et al., 2002). Whether development is arrested or merely delayed in the *ampA* null mutants depends upon the prior growth conditions of the cells (Varney et al., 2002). Cells that have been grown axenically in suspension culture progress normally through development to the mound stage and then are delayed by four hours in the process of forming the tip on the mound. Ultimately they complete development and form slightly aberrant fruiting bodies. Cells grown on bacteria express additional proteins on their surfaces that facilitate phagocytosis and alter their adhesive properties (Chia, 1996; Cornillon et al., 2000; Furukawa et al., 1992; Rezabek et al., 1997; Vogel et al., 1980). We postulate that this leaves them significantly more dependent upon the anti-adhesive activity of AmpA than cells grown axenically in suspension culture. As a result, bacterially grown *ampA* null cells are more adherent to the substratum which delays their migration into mounds and development completely arrests at the mound stage.

Cell migrations are important at mound stage for ‘sorting out’ of distinct cell populations that have differentiated at random locations throughout the mound (reviewed by Kessin, 2001). The differentiated cells must move to their correct locations in order to form the various structures of the fruiting body. This process is not unlike gastrulation in higher systems, where cells must reposition themselves to form the three germ layers prior to organogenesis. Cells destined to form the stalk of the fruiting body migrate from their site of initial differentiation at the periphery of the mound to the mound apex where they form the tip structure (Clow et al., 2000; Early et al., 1995). Cells that will form the basal disc and the lower cup structure assemble at the base of the mound, while cells that will constitute the spore mass sort to a position just above these cells. Cells that will form the upper cup locate to a thin layer separating the prespore cells and the apical prestalk cells (reviewed by Kessin, 2001). Once the differentiated cells have ‘sorted out’ a final series of morphogenetic movements shape the final fruiting body. The prestalk cells at the tip of the mound migrate down through the spore mass while the upper and lower cup cells move in a swirling vortex that results in the spore mass rising to the top of the extending stalk (reviewed by Kessin, 2001).

We show that in the absence of AmpA, cells are prematurely directed to assume a prespore or anterior-like cell fate and that prestalk differentiation is delayed. We show that this role of AmpA involves a non-cell autonomous mechanism and is distinct from a second cell-autonomous defect that interferes with correct localization of a subset of anterior-like cells destined to form the upper cup. We discuss models that can explain how a more primitive disintegrin domain-containing protein like AmpA, which lacks a metalloprotease activity, can still carry out the functional complexity of the more highly evolved, multi-domain ADAM and ADAMTS proteins.

MATERIALS AND METHODS

Strains, cell propagation and development

Procedures used to generate *ampA*$^{-}$ strains have been reported previously (Varney et al., 2002). Wild-type cells (Ax3) and *ampA*$^{-}$ cells were grown under axenic conditions in HL5 medium (Sussman, 1987) supplemented with 0.8 μg/ml folic acid, 20 ng/ml vitamin B12, 40 μg/ml ampicillin, 0.5 μg/ml amphotericin B and 0.3 μg/ml streptomycin (Sussman, 1987). G418 (9.6 mg/ml; Sigma) was added to the culture medium of strains carrying reporter genes, and 10 mg/ml blasticidin S HCl (ICN) was included in medium used to propagate *ampA*$^{-}$ cells. All strains were grown in the absence of antibiotics and selection drugs for 48 hours before experiments. Conditions for growth in association with *E. coli* B/r and procedures used to induce development have been described elsewhere (Varney et al., 2002).

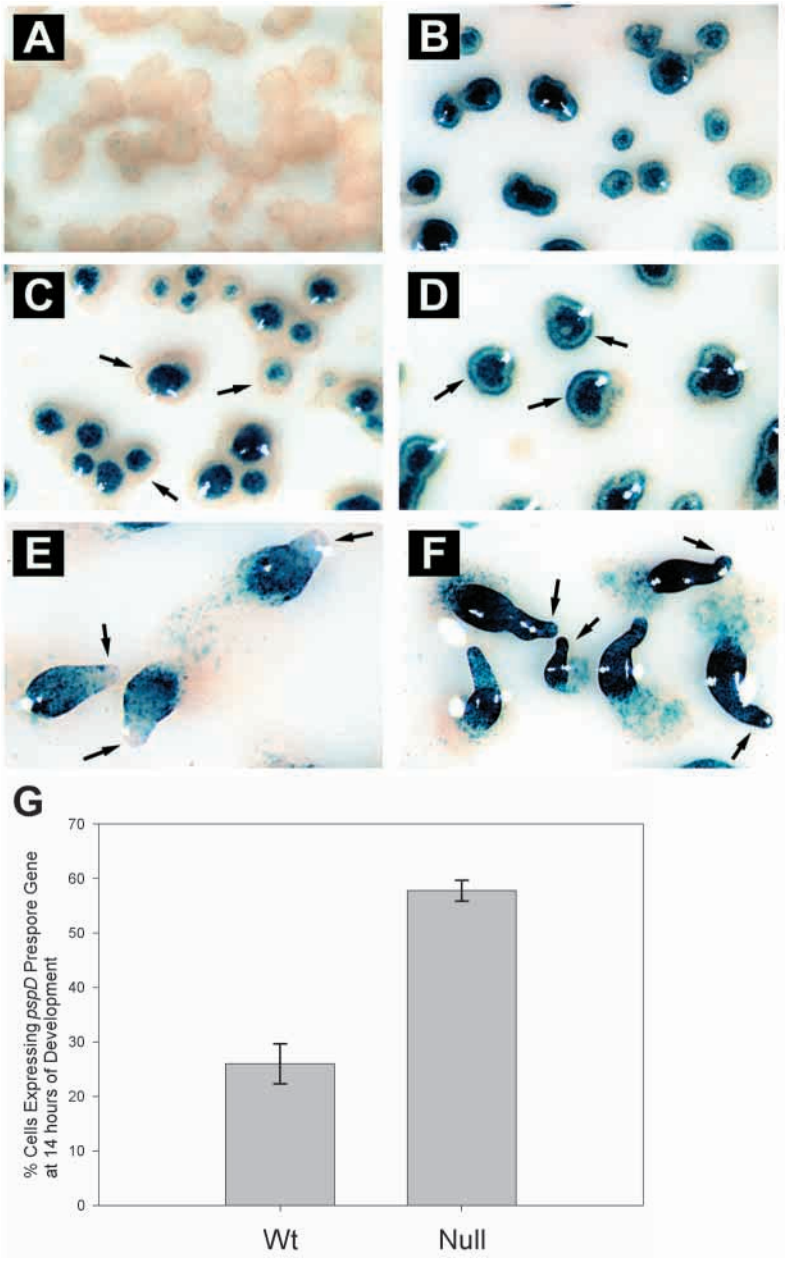

Fig. 1. During *ampA*⁻ strain development, expression of the *pspD* reporter gene is induced prematurely and prespore reporter activity is observed in cells in regions normally occupied by prestalk cells. *PspD* reporter expression patterns are shown for wild-type (left) and *ampA*⁻ structures (right) for the following developmental stages: (A,B) tight mounds (10 hours development), 4 hours staining; (C,D), tips (13.5 hours development), 2.5 hours staining; (E,F), early culminants (18 hours development), 2.5 hours staining. Arrows indicate regions of the structures where prestalk cells are usually found. (G) Quantitation of the number of cells expressing the *pspD* reporter in wild-type and *ampA* null structures harvested at 14 hours of development. Developing structures were dispersed to single cells, fixed, stained and counted as described in the Materials and Methods.

Transformation and detection of reporter activity

The construction of reporter genes marking expression of *ampA* (Casademunt et al., 2002) and *pspD* (Yoder and Blumberg, 1994) has been described. The *ecmA* reporter (Jermyn and Williams, 1991) was a kind gift from Dr Jeffrey G. Williams of University of Dundee, Scotland. This construct contains the full promoter for the *ecmA* gene and tags both prestalkA cells (pstA) and a subset of the anterior-like cells that form the upper cup. Stable transformation of *Dictyostelium* cells with reporter genes was by Ca^{2+} phosphate precipitation (Knecht et al., 1986). *ampA*⁻ and wild-type aggregates formed by cells carrying the same reporter gene were assayed in parallel. Detection of reporter gene-driven β-galactosidase activity in multicellular structures is described elsewhere (Dingermann, 1989). To quantify the percentage of cells expressing the *ampA* or *pspD* reporter gene, aggregates were dispersed to single cells by brief digestion with trypsin/EDTA (Casademunt et al., 2002) before fixing and staining. Three hundred to 2500 cells per experiment were photographed for analysis.

RESULTS

Prespore gene expression is initiated prematurely in *ampA* null cells

The *pspD* gene encodes a protein found in the coat surrounding mature spore cells (Yoder et al., 1994). Expression of this gene is initiated during post-aggregation development and is specifically restricted to prespore cells (Oyama and Blumberg, 1986; Yoder and Blumberg, 1994). When the promoter for this gene is used to drive expression of the *E. coli* β-galactosidase enzyme, reporter expression is normally first detected by in situ staining in a few widely scattered cells at about 10 hours of development, when cells have assembled into tight mounds (Yoder and Blumberg, 1994) (Fig. 1A). By 14 hours of development, many more cells have started to express the *pspD* reporter and these cells are localized to the center of the mound surrounded at the periphery of the mound by non-staining cells destined to become prestalk cells (Fig. 1C). These non-staining prestalk cells migrate to the apex of the mound where they will form the tip structure (Fig. 1E).

When the *ampA* gene is knocked out by insertional inactivation, development of axenically grown cells proceeds normally until late mound stage when there is a 4 hour delay in formation of the tip on the mound apex (Varney et al., 2002). During development of the *ampA* null cells, the *pspD* prespore gene is expressed prematurely (Fig. 1B). At 10 hours of development, when only a few scattered wild-type cells express the prespore reporter, there is already extensive expression of the *pspD* reporter in the null cells. Quantitation indicates that at 14 hours of development, only 25-30% of wild-type cells express the prespore reporter, while nearly 60% of the null cells express the reporter (Fig. 1G). This suggests that the *ampA* gene product functions early in development to suppress premature entry of cells into the prespore pathway.

Cells expressing the *pspD* prespore reporter in *ampA* null developing structures occupy prestalk cell positions

Cells staining for the *pspD* reporter are found at the periphery of mounds formed by *ampA* null cells (Fig. 1D). The arrows

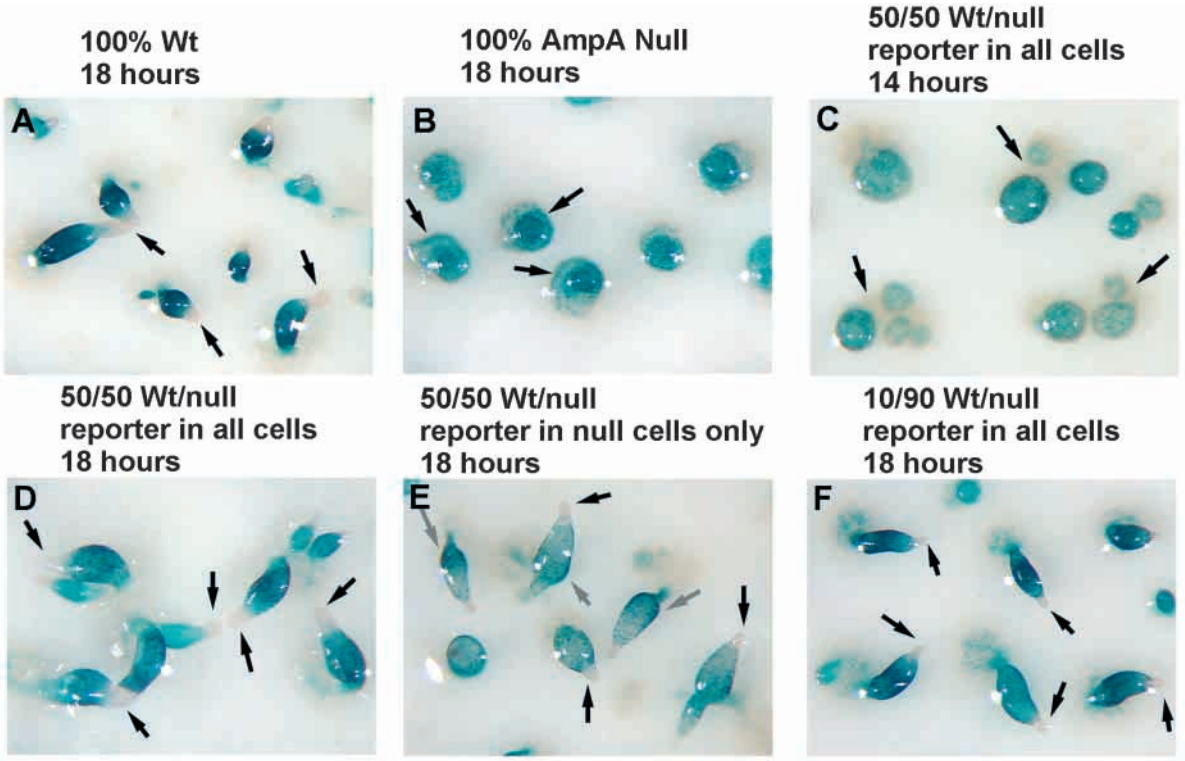

Fig. 2. Mis-expression of *pspD* reporter is rescued by the presence of wild-type cells in chimeric structures. Patterns of *pspD* reporter expression in wild-type, *ampA*⁻ and chimeric structures formed by 2 day bacteria-fed cells are shown. Samples were prepared as described in Materials and Methods. All samples shown were stained for β-galactosidase activity for 2 to 3 hours. (A) 100% wild-type cells, reporter in all cells; 18 hours of development. (B) 100% *ampA*⁻ cells, reporter in all cells; 18 hours of development (C) 50% wild-type cells/50% *ampA*⁻ cells, reporter in all cells; 14 hours of development to show mound stage. Arrows in B,C indicate the position of prestalk cells at the mound periphery at this stage. These cells are stained in B, but unstained in C. (D) 50% wild-type cells/50% *ampA*⁻ cells, reporter in all cells; 18 hours of development. (E) 50% wild-type cells/50% *ampA*⁻ cells, reporter in *ampA*⁻ cells only; 18 hours of development. (F), 10% wild-type cells/90% *ampA*⁻ cells, reporter in all cells; 18 hours of development. Black arrows in A,D,E,F indicate the tips that are composed of prestalk cells and devoid of prespore cells. Gray arrows in E indicate clustering of *ampA* null cells carrying the *pspD* reporter clustering at the base of the prespore zone.

indicate the prestalk cells at the periphery of the mounds that are unstained in wild type (Fig. 1C) and stained in *ampA*-null structures (Fig. 1D). Likewise, when the tip is formed on the mound by the migration of the prestalk cells to the mound apex, *pspD* prespore staining cells are located in the tip of the *ampA*-null mounds (Fig. 1F) but not in the tip of mounds from wild-type cells (Fig. 1E). Thus, it appears that *ampA*-null cells that initially differentiate as prespore cells respecify and assume a prestalk fate later in development.

Aberrant prespore gene expression by prestalk cells is rescued when wild-type cells are mixed with developing *ampA* null cells

We have previously demonstrated that a significant proportion of the AmpA protein is secreted and can be recovered in the wash of developing cells (Varney et al., 2002). If wild-type cells added to developing *ampA* null cells can rescue the aberrant pattern of gene expression by the *ampA* null cells, then AmpA protein would probably act extracellularly or by a 'non-cell autonomous' mechanism to suppress premature prespore gene expression.

The experiments described above were carried out using cells grown axenically in suspension culture where loss of *ampA* gene function results in only a 4 hour delay in tip formation and extension (Varney et al., 2002). When grown in contact with a solid substratum in the presence of bacteria for 2 days, *ampA*-null cells are delayed in the initiation of development by 7-8 hours, and morphogenesis is completely arrested at the tip formation stage (Varney et al., 2002). This more severe developmental defect makes it feasible to ask whether addition of wild-type cells to developing *ampA*-null cells can rescue the aberrant prespore gene expression pattern.

At 18 hours of development, bacterially grown wild-type cells are at the early culminant stage where the prestalk cells are evident in the tip region (Fig. 2A). The wild-type prestalk cells are clear because they do not express the prespore reporter gene. By contrast, bacterially grown *ampA*-null cells have arrested development at mound stage (Fig. 2B). Mis-expression of the *pspD* prespore reporter gene is observed in the prestalk cells located at the periphery of the mounds formed by the *ampA*-null cells (Fig. 2B). Inclusion of wild-type cells in a 50:50 ratio with *ampA*-null cells prior to plating for development rescues the mis-expression of the prespore marker (Fig. 2C). Because inclusion of wild-type cells also rescues the delay in developmental progression, it was necessary to look earlier (at 14 hours) to observe mound stage in the chimeric aggregates. The prestalk cells at the periphery of the chimeric wild-type/*ampA*-null mounds show little or no

expression of the prespore reporter gene (Fig. 2C). Moreover inclusion of the wild-type cells in the chimeric aggregates rescues the developmental arrest and by 18 hours early culminants are formed just as in wild type (Fig. 2D,E). The prestalk tip region in the chimeric culminants is also devoid of cells expressing the prespore reporter. In Fig. 2D, both wild-type and *ampA*-null cells carry the *pspD* reporter, while in Fig. 2E only the *ampA* null cells carry the *pspD* reporter. As in both cases the tips are devoid of prespore gene-expressing cells, the inclusion of the wild-type cells prevents the *ampA*-null cells from mis-expressing the prespore gene. A caveat to this conclusion is that wild-type cells capable of correctly suppressing prespore gene expression may exclusively differentiate as prestalk cells in the chimeras. (Later, we show that this is not the case.) The *ampA*-null cells also differentiate as prestalk cells in the presence of wild-type cells. Thus, the inclusion of wild-type cells rescues the mis-expression of the prespore gene marker by the *ampA*-null cells. The inclusion of as few as 10% wild-type cells with 90% *ampA*-null cells is sufficient to rescue the mis-expression of the prespore reporter gene in the prestalk cells (Fig. 2F).

In spite of the rescue of prespore gene mis-expression by wild-type cells, there is still a cell autonomous defect in *ampA*-null prespore cells. In culminating aggregates formed by a suspension containing an equal number of wild-type and *ampA*-null cells, the upper half of the spore mass is enriched with wild-type cells. When *pspD* reporter expression is marked only in *ampA*-null cells, the resulting structures show a cup-shaped expression pattern outlining the lower half of the spore mass at 18 hours as a result of increased numbers of *ampA*-null cells in the lower half of the prespore zone (Fig. 2E, gray arrows). However, when the cells of both strains carry the *pspD* reporter, an even distribution of staining is observed throughout the prespore zone (Fig. 2D). These results suggest that the ability of *ampA*-null cells entering the prespore pathway to migrate into the anterior regions of mixed aggregates is reduced compared with wild-type cells, presumably as a result of their increased adhesiveness.

Prestalk cell differentiation is delayed in *ampA* null structures and prestalk cells remain longer at the mound periphery

Prestalk cells express an extracellular matrix protein, EcmA (McRobbie et al., 1988a; McRobbie et al., 1988b). The *ecmA* promoter fused to the *E. coli lacZ* gene is a widely used reporter for prestalk cells and is expressed in both prestalk cells and in a subset of the anterior-like cells that form the upper cup (Jermyn et al., 1989; Jermyn and Williams, 1991). Initial expression of the *ecmA* prestalk cell reporter begins in both wild-type and *ampA*-null strains at 10 hours of development, just before the onset of tip formation. This is seen as a very faint ring of cells at the periphery of the mounds that express the *ecmA* reporter (Fig. 3A,B). In wild-type structures, the number of cells expressing the reporter gene rapidly increases as the tip emerges, and the expressing cells are progressively seen increasing in concentration towards the mound apex (Fig. 3C,E). The number of cells expressing the prestalk *ecmA* reporter in the *ampA*-null strain is reduced in the developing mounds (Fig. 3D,E). Additionally, many of the cells that express the prestalk *ecmA* reporter remain near the mound periphery longer than cells expressing the reporter in wild-type structures (Fig. 3D,E). In this experiment, cells grown axenically in suspension culture were used so that prestalk gene expression could be viewed throughout development in the *ampA*-null cells. By culmination, the pattern of *ecmA* prestalk reporter expression in *ampA*-null structures (Fig. 2H) is similar to the wild-type pattern (Fig. 2G). These results indicate that the increase in prestalk gene expression is delayed in the developing *ampA* null cells and that the cells that do differentiate as prestalk cells remain at the mound periphery longer. After the 4 hour delay in tip

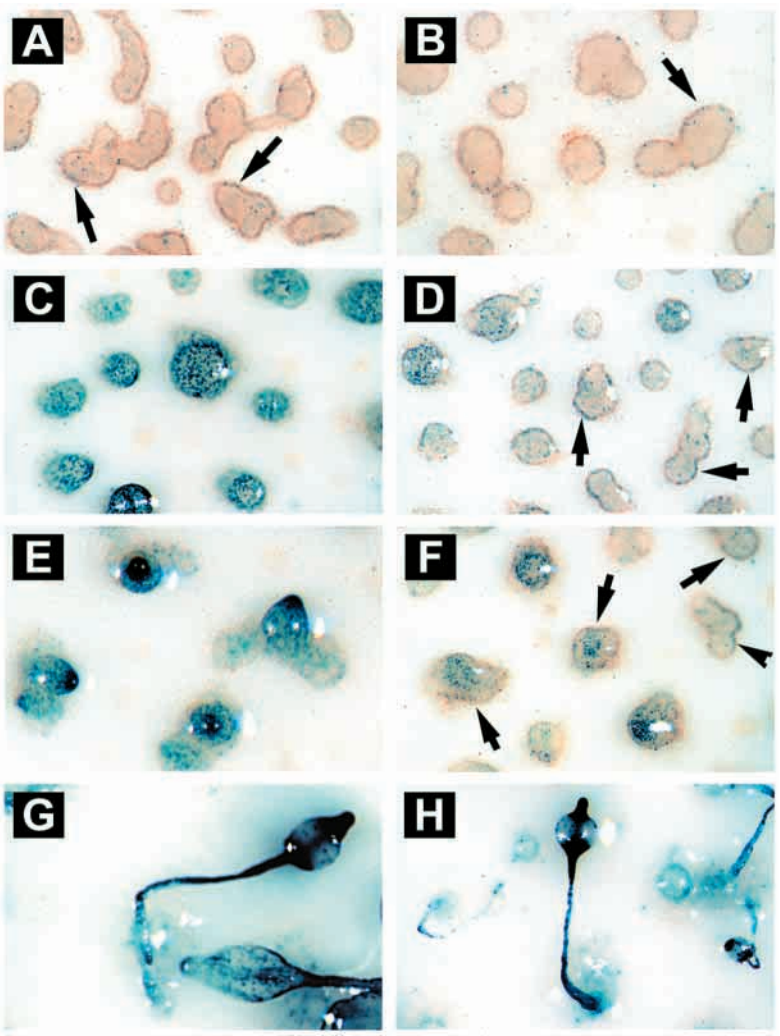

Fig. 3. Loss of AmpA function delays differentiation of prestalk cells and migration of stalk A cells from the mound periphery to the mound tip. *EcmA* reporter expression patterns are shown for wild-type structures (left) and *ampA*$^{-}$ structures (right) for the following developmental stages: (A,B), tight mounds, 10 hours of development; 2 hours staining. (C,D), late tight mounds, 12 hours of development; 1 hour staining. (E,F), tips, 13.5 hours of development; 1 hour staining. (G,H) Culminants, 22 hours of development, 1 hour staining. Arrows indicate the mound periphery where stalk A cells initially differentiate.

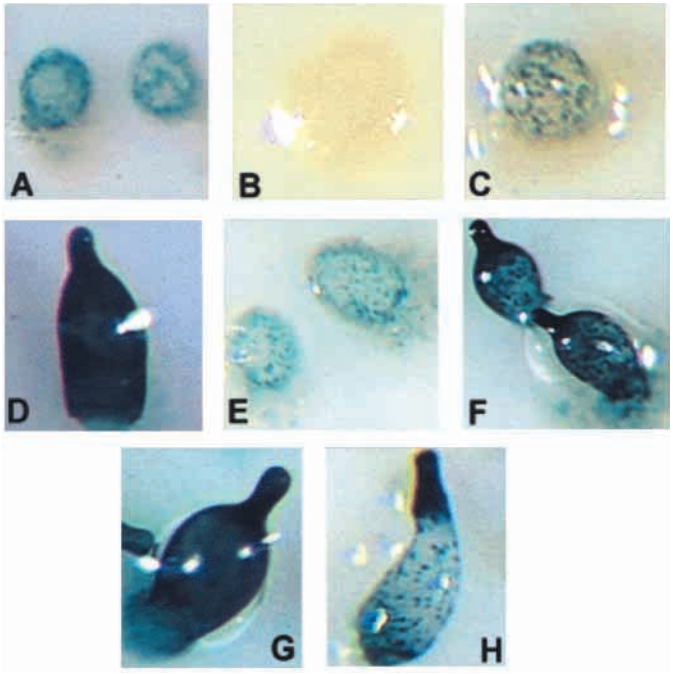

Fig. 4. The delay in prestalk gene expression and anterior prestalk cell migration in structures formed from *ampA*-null cells is rescued by the presence of wild-type cells. (A-C) Expression patterns of *ecmA* reporter after 14 hours of development by bacterially grown cells are shown. (A) Wild type; (B) *ampA*$^{-}$; (C) 50/50 wild type/null with reporter carried only in *ampA*-null cells. (D-F) 18 hours of development by bacterially grown cells is shown. (D) Wild type; (E) *ampA*-null; (F) 10/90 wild type/null chimera with reporter carried in null cells only. (G) 50:50 wild-type/*ampA*$^{-}$ cells, reporter in all cells; 18 hours development. (H) 50:50 wild-type/*ampA*$^{-}$ cells, reporter in *ampA*$^{-}$ cells only; 18 hours development. All staining was for 1 to 2 hours.

extension, however, the pattern of prestalk gene expression becomes normal.

The delay in prestalk differentiation and migration in *ampA*-null cells is rescued by inclusion of wild-type cells during development

By using bacterial growth of cells before development to produce the more severe developmental defects of the *ampA*-null mutation, it is possible to address the question of whether the inclusion of wild-type cells in the developmental plating suspension can also rescue the defects in prestalk cell differentiation. Expression of the prestalk-specific *ecmA* reporter is not observed in 14 hour aggregates formed by *ampA*-null cells that have been grown on bacterial plates prior to development (Fig. 4B). By comparison, bacterially grown wild-type cells express the prestalk *ecmA* reporter at the mound periphery and prestalk cells have started to migrate to the mound apex by this stage in development (Fig. 4A). The bacterially grown *ampA*-null cells arrest development at the mound stage, although by 18 hours they do express prestalk *ecmA* reporter but many of the prestalk cells remain at the mound periphery (Fig. 4E). By 18 hours of development, the wild-type cells have reached the early culminant stage (Fig. 4D). Inclusion of wild-type cells with *ampA*-null cells in a 50/50 mix prior to plating for development results in rescue of the ability of *ampA*-null cells to express the prestalk reporter by 14 hours (Fig. 4C). Prestalk cells appear toward the apex of the chimeric mound, suggesting that not only does the inclusion of wild-type cells rescue the delay in differentiation of prestalk cells but also the ability of the prestalk cells to migrate to the mound apex. In Fig. 4C, only *ampA*-null cells carry the prestalk reporter, indicating that the inclusion of the wild-type cells induces *ampA*-null cells, themselves, to express the prestalk cell reporter on time. Examination of the chimeric structures at 18 hours of development indicates that inclusion of only 10% wild-type cells in the plating suspension with 90% *ampA*-null cells is sufficient to rescue the developmental arrest at mound stage and to allow the *ampA*-null cells that differentiate as prestalk cells to migrate to the tip of the early culminating structure (Fig. 4F; only *ampA*-null cells carry reporter).

Fig. 4H shows 18 hour development of a 50:50 wild-type:*ampA*-null chimeric population in which the *ecmA* prestalk reporter construct is carried only in the *ampA*-null cells. The intensity of staining within the tip region of the structures suggests that *ampA*-null cells not only gain the ability to participate in tip extension in the presence of wild-type cells, but may represent the majority of the prestalk population in structures formed by an equal number of cells from each strain. In Fig. 4G, where wild-type cells also carry the *ecmA* reporter, there is more staining in the scattered areas throughout the spore mass characteristic of the staining pattern of the anterior-like cells that also express the *ecmA* reporter. This result raises the possibility that the *ampA*-null cells have a tendency to assume the prestalk fate, rather than the anterior-like cell fate in the chimeras.

Anterior-like cells that express the ampA reporter are prematurely induced and largely excluded from the upper cup region of late developing structures formed by *ampA*-null cells

While much of the AmpA protein is secreted during development and can be recovered in the wash of developing cells, some of it is found associated with the cell pellet (Varney et al., 2002). Immunofluorescence analysis indicates that AmpA protein is associated with the anterior-like cells. These are the cells that express the *ampA* gene during development (Casademunt et al., 2002). It was therefore of interest to examine the effects of the *ampA* null mutation on the differentiation and localization of these cells. We used *ampA* promoter driven expression of β-galactosidase enzyme to label the anterior-like cells. Two striking differences are observed. First, *ampA* gene expression is prematurely induced in the *ampA*-null developing cells. The expression of the *ampA* gene is initially observed in a few widely scattered cells during early development of wild type (Fig. 5A). By contrast, in the *ampA*-null strain, significantly more cells are observed to express the *ampA* gene early (Fig. 5B). Quantitation indicates that nearly four times more cells express the *ampA* gene in early development (Fig. 5C). By 12 hours of development, about 1% of the wild-type cells express the *ampA* gene, while nearly 4% of the null cells express it.

The second effect of the *ampA*-null mutation on differentiation of anterior-like cells is that they fail to localize to the upper cup region of the culminating fruiting bodies (Fig. 6A-D). Fig. 6A,C show wild-type early and mid-culminants

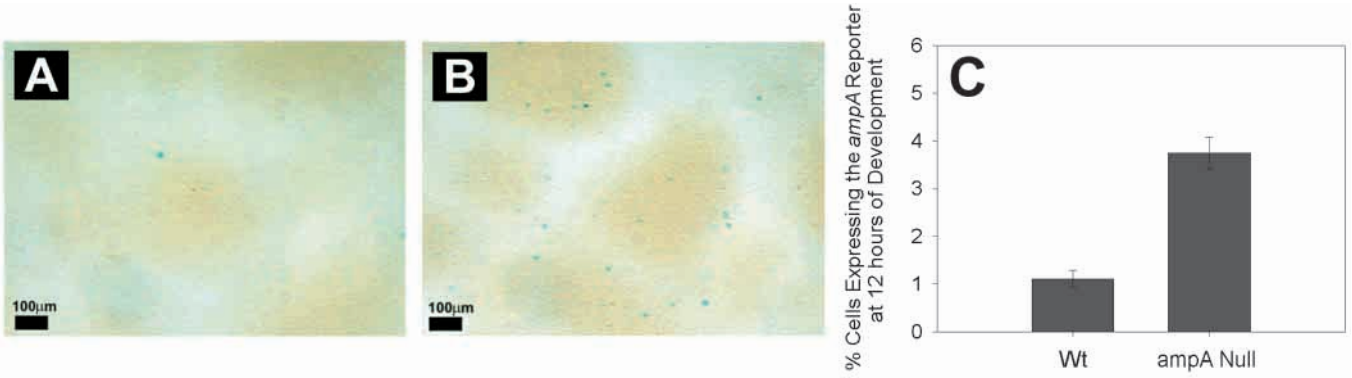

Fig. 5. Inactivation of *ampA* gene function results in a premature increase in the percentage of cells expressing the *ampA* reporter at the earliest developmental stages. Axenically growing wild-type (A) and *ampA*⁻ (B) cells carrying the *ampA* reporter gene were washed free of growth medium, plated for development on nitrocellulose filters and stained 19 hours for expression of β-galactosidase after 4 hours of development as described in the Materials and Methods. (C) Quantification of the percentage of cells expressing the *ampA* reporter in developing wild-type and *ampA*⁻ structures during development on nitrocellulose filters. Structures harvested at 12 hours of development were dispersed to single cells, and fixed, stained and counted as described in the Materials and Methods.

and the arrows indicate the *ampA*-expressing upper cup cells. By contrast, early and mid-culminants from *ampA*-null strains show a reduced number of *ampA*-expressing cells in the upper cup region (Fig. 6B,D).

This effect is even more striking when wild-type and *ampA*-null cells are grown on bacteria prior to plating for development (Fig. 6E-H). By 18 hours of development, the wild-type cells have reached the early culminant stage and *ampA*-expressing cells are clearly present in the upper and lower cup regions (Fig. 6E). The *ampA*-null cells have arrested development at mound stage, but cells expressing the *ampA* gene are almost completely excluded from the interior of the mounds and are found exclusively at the periphery (Fig. 6F). When wild-type cells are mixed with *ampA*-null cells in a 50:50 ratio prior to plating, developmental progression is rescued but it is the wild-type *ampA*-expressing cells that occupy the interior positions in the upper and lower cup regions of the early culminants. In Fig. 6G, both wild-type and null cells carry the *ampA* reporter and staining cells are clearly visible in the upper and lower cup regions. When only the *ampA* null cells carry the *ampA* reporter construct, there are no cells expressing the *ampA* gene in the upper cup regions and even the lower cup region shows few if any *ampA*-expressing cells (Fig. 6H). Thus, the *ampA* gene is required in a cell associated or cell autonomous way for the correct localization of *ampA*

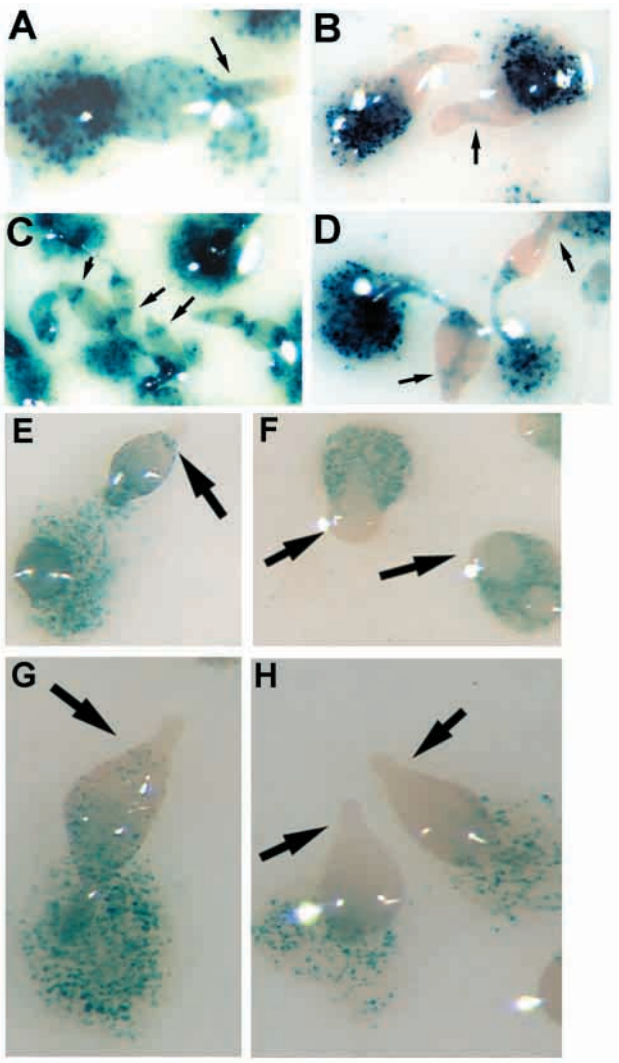

Fig. 6. Inactivation of *ampA* gene function results in a decrease in the number of cells expressing the *ampA* reporter in the region of the upper cup during late development. *ampA* reporter gene expression patterns for wild-type (A,C) and *ampA*⁻ (B,D) structures formed by axenically grown cells are shown. (A,B) Early culminants (18 hours development); 29 hours staining. (C,D) Culminants (24 hours development); 20 hours staining. Arrows indicate the regions of the upper cup where reporter expression is reduced in *ampA*⁻ aggregates relative to wild type. (E-H) Wild-type and *ampA*⁻ cells were grown in the presence of bacteria. Suspensions of wild-type cells, *ampA*⁻ cells or wild-type/*ampA*⁻ cell mixtures were plated for 18 hours of development. Samples were fixed and stained 22 to 24 hours for β-galactosidase activity. (E) 100% wild-type cells, reporter in all cells; (F) 100% *ampA*⁻ cells, reporter in all cells; (G) 50% wild-type cells/50% *ampA*⁻ cells, reporter in all cells; (H) 50% wild-type cells/50% *ampA*⁻ cells, reporter in *ampA*⁻ cells only. Arrows indicate regions of the structures where upper cup cells should be found.

expressing cells to the upper cup region and possibly to the lower cup region.

DISCUSSION

The disintegrin domain containing AmpA protein has two developmental functions (Varney et al., 2002). We have previously shown that it modulates cell-cell and cell-substrate adhesion. We now demonstrate that it also plays a role in initial cell fate specification.

A role for AmpA protein in preventing cells from prematurely assuming the prespore cell fate

Not only is the AmpA protein necessary to prevent premature prespore gene expression but, in its absence, cells that will later differentiate as prestalk cells initially express the prespore gene. This suggests that, in its absence, most cells initially enter the prespore developmental path. AmpA protein secreted by a small number of wild-type cells mixed into a population of *ampA*-null cells is sufficient to prevent mis-expression of the prespore gene.

Oohata has described low-density monolayer conditions that cause most cells to differentiate to the prespore fate (Oohata, 1992; Oohata, 1995). A prespore inducing factor has been isolated from the media of cells developed under these conditions (Oohata et al., 1997). In very preliminary experiments, we have also identified an activity in the wash from developing *ampA*-null cells that is capable of increasing the number of wild-type cells that express the prespore reporter (H. H. and D. D. B., unpublished). Under identical conditions, this activity is not present in the wash from developing wild-type cells that secrete AmpA protein. One possibility is that the AmpA protein functions to inactivate a secreted prespore inducing factor in order to allow prestalk cell differentiation.

During development, the expression of the *ampA* gene is restricted to the anterior-like cells (Casademunt et al., 2002). While some *ampA*-expressing cells are scattered in the interior of the mound, the majority of *ampA*-expressing cells are localized at the periphery (Casademunt et al., 2002). Importantly, the majority of the *ampA*-expressing cells are found in the same location, at the mound periphery, as are the cells that are the earliest to express the prestalk *ecmA* reporter (Early et al., 1995). A high concentration of AmpA-secreting cells at the mound periphery could act to prevent neighboring cells at the mound periphery from entering the prespore pathway by locally reducing the level of a prespore inducing factor, such as that described by Oohata et. al. (Oohata et al., 1997).

The prespore gene mis-expression that occurs in the absence of AmpA protein is clearly reversible, provided that the cells are grown axenically and can complete development after the 4 hour delay at the tip formation stage. Cells that have started down the prespore pathway and retain the history of prespore reporter expression end up in prestalk cell derived structures. A similar ‘transdifferentiation’ phenomenon has been observed during prolonged migration of slugs, where loss of differentiated stalk cells in the slime trail triggers a conversion of prespore cells to prestalk cells to restore the correct cell type proportions (Abe et al., 1994). It is possible that a similar proportion regulation response is invoked to correct the overexpression of prespore fates in the axenically grown *ampA*-null developing structures, allowing them to complete development after a delay.

Inactivation of the *ampA* gene results in a delay in differentiation of the maximal number of prestalk cells. A simple explanation is that cells that have erroneously entered the prespore pathway require time to respecify as prestalk cells. However, migration of the prestalk cells to the mound apex is also delayed. A three-dimensional in vivo analysis of the migration of *ecmA* prestalk cells during mound and tip formation indicates that prestalk cell differentiation actually takes place in two steps (Clow et al., 2000). Initially, prestalk *ecmA*-expressing cells are observed to form a small, randomly located cluster early in mound morphogenesis. The cluster of *ecmA*-expressing cells then migrates largely as a unit to the mound apex, presumably in response to a second directional signal. As the cluster migrates toward the apex, additional *ecmA*-expressing cells arise throughout the mound, join the cluster and move with it to the tip. It is during this period of migration that there is the significant increase in the number of cells expressing the *ecmA* prestalk marker gene. This raises the question of whether migration is necessary for differentiation of these cells, which is also a central question in the differentiation and migration of neural crest cells. A very close examination of Fig. 3 shows that the *ecmA* prestalk reporter is first expressed in a small number of cells at the very periphery of the mound at the same time in both wild-type and *ampA*-null cells. It appears that it is the second and later increase in reporter expression that is delayed in the null cells. This raises the question of whether a second function of AmpA is necessary for cluster formation and the subsequent migration and differentiation of the prestalk cells?

The ampA protein is necessary for anterior-like cells that express the *ampA* gene to localize to the upper cup region

ampA-expressing anterior-like cells are reduced in the upper cup structure when axenically grown *ampA*-null cells are plated for development. Bacterial growth leaves *ampA*-null cells adhering so strongly to the substrate during early development that mound formation is significantly delayed. Bacterial growth also results in a nearly complete exclusion of the anterior-like cells, marked by *ampA* expression, from the interior of the mounds formed by developing *ampA*-null cells. The addition of wild-type cells rescues morphogenesis, but it is the wild-type cells themselves that penetrate the interior of the mound and differentiate as the anterior-like cell-derived upper and lower cups.

The *ampA*-null cells that express the *ampA* gene cannot be induced to enter the interior of the mound by the inclusion of the wild-type cells. Thus, AmpA has two distinct functions, one a non-cell autonomous role in reducing premature prespore specification and the second, a cell-autonomous role, which allows the anterior-like cells that express the *ampA* gene to penetrate to the interior of developing mounds.

Like the multi-domain ADAM and ADAMTS proteins of higher metazoans, AmpA clearly functions at the crucial intersection of cell adhesion, cell migration and cell fate specification. However, AmpA is an evolutionarily more primitive disintegrin domain-containing protein that lacks a metalloprotease domain. It is interesting to draw an analogy

with the Discoidin domain receptor tyrosine kinases of metazoans (Vogel, 1999). These receptors take their name from the extracellular domain that is homologous to a small soluble lectin, Discoidin, initially identified in *Dictyostelium*. An interesting question is whether small, single proteins such as AmpA or Discoidin represent evolutionary precursors that recombined with other catalytic domains to form the multi-domain ADAMs or Discoidin tyrosine kinase receptor families. The AmpA protein could function as a single domain to activate or to antagonize receptor ligand interactions, or it could serve as an extracellular matrix 'adaptor' to anchor other important signaling, adhesion or catalytic molecules.

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