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AmpA protein functions by different mechanisms to influence early cell type specification and to modulate cell adhesion and actin polymerization in *Dictyostelium discoideum*

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Abstract

The *Dictyostelium discoideum ampA* gene encodes a multifunctional regulator protein that modulates cell–cell and cell–substrate adhesions and actin polymerization during growth and is necessary for correct cell type specification and patterning during development. Insertional inactivation of the *ampA* gene results in defects that define two distinct roles for the *ampA* gene during development. *AmpA* is necessary in a non-cell autonomous manner to prevent premature expression of a prespore gene marker. It is also necessary in a cell autonomous manner for the anterior like cells, which express the *ampA* gene, to migrate to the upper cup during culmination. It is also necessary to prevent excessive cell–cell agglutination when cells are developed in a submerged suspension culture. Here, we demonstrate that a supernatant source of AmpA protein, added extracellularly, can prevent the premature mis-expression of the prespore marker. Synthetic oligopeptides are used to identify the domain of the AmpA protein that is important for preventing cells from mis-expressing the prespore gene. We further demonstrate that a factor capable of inducing additional cells to express the prespore gene marker accumulates extracellularly in the absence of AmpA protein. While the secreted AmpA acts extracellularly to suppress prespore gene expression, the effects of AmpA on cell agglutination and on actin polymerization in growing cells are not due to an extracellular role of secreted AmpA protein. Rather, these effects appear to reflect a distinct cell autonomous role of the *ampA* gene. Finally, we show that secretion of AmpA protein is brought about by elevating the levels of expression of *ampA* so that the protein accumulates to an excessive level.

Keywords

Differentiation; Pattern formation; Cell type specific gene expression; *Dictyostelium discoideum*; Cell adhesion; Actin polymerization

1. Introduction

Embryonic patterning involves a combination of mosaic and regulative mechanisms. Secreted signaling molecules which confer positional information represent one mechanism for cell type specification while differential distribution of cytoplasmic determinants to daughter cells represents a cell intrinsic or cell autonomous means of cell type specification.

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Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.diff.2013.06.003.

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Dictyostelium is one of the simplest organisms to undergo true multicellular differentiation, and, like higher eukaryotes, displays both cell autonomous and non-cell autonomous components directing its developmental program (Kessin, 2001). *Dictyostelium* combines well developed molecular genetics with a sequenced haploid genome that allows easy analysis of the effect of gene knock outs and gene replacements on the developmental program (Williams, 2010; Urushihara, 2002, 2009). These advantages have made it a powerful system for identifying and analyzing morpho-genetic factors that direct growth and differentiation.

Dictyostelium amebas grow and divide as single cells. Starvation triggers a developmental program in which cells migrate by chemotaxis into multicellular mounds (Kessin, 2001). Within the mounds cells undergo differentiation into three main cell types—prespore cells, prestalk cells and anterior like cells. These differentiated cell types sort out during a process of morphogenesis into a final fruiting body with a sorocarp of spore cells held on a stalk. The anterior like cells form the support structures, the basal disc that anchors the fruiting body to the substratum and the upper and lower cups that support the spore head on the stalk.

The initial factors that influence choice of cell fate are intrinsic or cell autonomous and are dependent on the cell's history during vegetative growth prior to development. Cells that are in the S or early G2 phase at the onset of nutrient starvation will adopt the prestalk fate while cells in the late G2 or M phase will adopt the prespore fate (Weijer et al., 1984; Maeda, 2011; Gomer and Ammann, 1996; Maeda et al., 1989, 2002). The predisposition of a cell to adopt a particular fate can also be influenced by its nutritional state. In a chimeric structure cells grown without glucose preferentially form prestalk cells while the glucose grown cells preferentially form prespore cells (Blaschke et al., 1986). These initial predispositions are further shaped by a number of secreted proteins and small molecules that function in a non-cell autonomous manner to further influence development and cell type specific gene expression. During growth, cells secrete a variety of proteins that enable the population to monitor cell density and to sense starvation, which initiates development (Gomer et al., 2011). Additional autocrine factors like PSF (prestarvaton factor) and CMF (conditioned media factor) regulate gene expression and early development (Clarke and Gomer, 1995) while a secreted complex of proteins including countin factor determines aggregate size (Brock and Gomer, 1999). Cyclic AMP, which directs cell migration into aggregation centers during early development, also functions as a morphogen to induce cell type specific gene expression (Strmecki et al., 2005; Kimmel and Firtel, 2004). Cyclic AMP additionally induces competence for cells to respond to DIF, a chlorinated hexaphenone which induces the expression of a subset of prestalk genes and suppresses prespore genes (Kay et al., 1999).

We have previously described another small protein, AmpA, which appears to be a multifunctional regulator of a number of aspects of growth and differentiation in *Dictyostelium*. Secretion of the AmpA protein is developmentally regulated. During development AmpA protein is largely secreted, although some of it is retained within cell pellets. Insertional inactivation of the *ampA* gene results in several distinct developmental phenotypes. *ampA* null cells form excessively large agglutinates relative to wild type cells when developed in suspension culture (Varney et al., 2002a). Conversely, over expression of the *ampA* gene reduces the size of the agglutinates and significantly increases the number of single cells. When plated for development, *ampA* null cells also show increased adhesion to the substratum that interferes with cell migration, delaying the migration of prestalk cells to the mound apex and preventing the migration of the anterior like cells to the upper cup. Finally, in the absence of the AmpA protein, cells destined to assume non-prespore fates initially express prespore genes (Varney et al., 2002b). The addition of 10% wild type cells

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to a population of developing *ampA* null cells rescues the defect in cell fate determination, suggesting that the AmpA protein effects initial cell fate determination in a non-cell autonomous manner. The wild type cells in chimeras, however, do not rescue the migration of the anterior like cells to the upper cup region, suggesting that AmpA also plays a cell autonomous role in development (Varney et al., 2002b).

In contrast to development, where most of the AmpA protein is secreted, in growing cells the AmpA protein is not found extracellularly (Varney et al., 2002a). The *ampA* gene is expressed in all growing cells, especially as they reach high density, and it influences the level of actin polymerization and cell–substrate adhesion (Noratel et al., 2012). Loss of AmpA function results in decreased actin polymerization and increased cell–substrate adhesion while over expression of AmpA increases actin polymerization and decreases cell–substrate adhesion (Noratel et al., 2012).

Here, we demonstrate that it is indeed the AmpA protein, itself, that functions extracellularly (non-cell autonomously) to prevent the premature differentiation of cells as prespore cells rather than a secondary effect of the null mutation. We identify a nine amino acid sequence of the AmpA protein that is capable of inhibiting the excessive prespore cell differentiation of the *ampA* null cells. We propose that secreted AmpA protein acts by a localized lateral inhibition mechanism to prevent mis-specification non-prespore cells during early development. We also demonstrate that when AmpA is over expressed in growing cells, some of the excess AmpA is secreted to the extracellular media. We propose that secretion of AmpA protein both in growing and developing cells is entirely determined by the protein accumulating above a critical level.

2. Methods

2.1. Dictyostelium strains, cell propagation and induction of development

Procedures used to generate *ampA*$^{-}$ strains were reported (Varney et al., 2002a). 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. G418 (9.6 μ/mL) was added to the culture medium of strains carrying reporter genes, and 10 μg/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 h before experiments. Cells were passed over bacteria and not kept in axenic culture for more than 3–4 weeks. Cells were starved and plated for development on nitrocellulose filters according to standard procedures (Varney et al., 2002a; Sussman 1987).

The AmpA–Tap tag–Wt , AmpA–Tap tag–OE and mRFP–AmpA strains carrying AmpA–Tap tag or mRFP–AmpA fusion protein constructs are described in Noratel et al. (2012) (plasmid maps in Additional Files 11 and 12 of Noratel et al. (2012).

Western analysis was described (Varney et al., 2002a). When AmpA–Tap tag or mRFP–AmpA fusion proteins were analyzed, anti-TAP tag antibody (1:1000, Open Biosystems) or anti–RFP antibody (1:1000, Chromotek) was the primary antibody. The secondary antibody was goat anti-mouse conjugated to alkaline phosphatase (Jackson Immunoresearch). For Western blots measuring secreted AmpA protein from growing cells, the medium in which the cells were growing was concentrated in Centricon/Centriprep concentrators (10 k MWCO) (Fisher Scientific) such that supernatant from 1×10^6 cells was applied to individual lanes of the gel.

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2.2. Preparation of the extracellular supernatant fractions from Wt and ampA null strains

Growing cells were harvested, washed free of nutrients, resuspended in $KMgPO_4$ buffer (20 mM KCl, 40 mM NaK_2PO_4, 2.5 mM $MgSO_4$, pH 6.0) at 1.4×10^8 cells/mL, and 0.5 mL of the cell suspension was plated for development on 47 mm Millipore filters sitting on five Whatman #3 pads moist with buffer. Cells were developed for 10 h in a moist chamber at 22 °C, harvested by successively vortexing cells from filters into a tube containing 20 mM Na_2KHPO_4, pH 6.0 (made from 1 M stock solution of 17.5 g Na_2HPO_4 dibasic anhydrous, 119.4 g KH_2PO_4 L^{-}) so that the final concentration of harvested cells was 2×10^8 cell/mL. Cells were harvested by centrifugation at 1.5 K (DPR6000 or Beckman J6B centrifuge) for 3 min at 4 °C. The supernatant was saved and the cells resuspended and washed in an equal volume of 20 mM Na_2KHPO_4. After centrifugation to remove the cells, the second supernatant was combined with the first so that the concentration of "cell equivalents" from which the supernatant was harvested was 1×10^8 cells/mL. The supernatant was recentrifuged to remove any remaining cells, saved in a clean conical tube, and kept on ice. No freezing occurred, and the supernatant was immediately used for assays.

2.3. Assay of the effect of extracellular supernatant fractions or oligopeptides on prespore cell differentiation

Cells washed free of nutrient broth were resuspended at 1.33×10^8 cells/mL in either $KMgPO_4$ buffer or in supernatant from 10 h developing wild type or *ampA* null cells or in phosphate buffer with oligopeptides at the indicated concentrations. An aliquot of cell suspension (75 μL) was plated on each of four 10 mm nitrocellulose filters on top of 5 Whatman (no. 2) filters (70 mm diameter) in a Petri dish. The filters were presoaked with 4 mL of either supernatant, $KMgPO_4$ buffer or $KMgPO_4$ buffer with peptides at the indicated concentrations. Cells were developed in a moist chamber for 14 h. The cells were harvested by transferring the nitrocellulose filters to a Petri dish where they were covered with a 5× trypsin (1.25% trypsin)/10 mM EDTA solution from Sigma completely covering the developing structures and soaked at room temperature for 5 min prior to harvesting the cells from the filters by vortexing.

Synthetic AmpA oligopeptides were either from the Biopolymer Laboratory (University of Maryland at Baltimore, Department of Microbiology and Immunology) or United Biochemical Research, Inc. (Seattle, WA 98125). The peptides were purified by HPLC to at least 80% purity and resuspended in 20 mM Na_2HPO_4 at 500 μg/mL, aliquoted in 1 mL volumes into cryogenic tubes (Sardstet), and stored at –80 °C. Peptide sequences are indicated in Figs. 3B and 5A.

2.4. Quantification of the percentage of cells differentiating as prespore cells

Reporter constructs in which expression of the *Escherichia coli* β-galactosidase gene is driven by the full length prespore cell specific *pspD* promoter (Yoder et al., 1994) were transformed into wild type Ax3 and *ampA* null cells by $CaPO_4$ precipitation (Knecht et al., 1986) as modified by Joly et al. (1993) and used to assay for prespore differentiation. Cells developed on nitrocellulose filters, as described above, were harvested at the late mound to tipped mound stage (10–16 h of development), dispersed to single cells and stained for β-galactosidase activity as described (Casademunt et al., 2002). Per experiment, 300–2500 cells were imaged and photographed for analysis as described below. Alternatively, developmental structures were stained for β-galactosidase activity as described (Casademunt et al., 2002).

Cell agglutination assays were carried out as described (Varney et al., 2002a).

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Phalloidin binding assays to determine the amount of polymerized F-actin were carried out as described (Noratel et al., 2012).

2.5. Microscopy and image analysis

Starved cell agglutinates were imaged using a Leica DM IRB microscope with either a 10× or 20× objective. *Dictyostelium* cells staining for β-galactosidase activity were imaged using a 40× objective. Images were obtained using a DC330 video camera (DAGE-MTI, Inc., Michigan City, IN, USA) and frame grabber. Images were digitized, processed, and analyzed using the Metamorph image processing system, (Universal Imaging, West Chester, PA, USA).

3. Results

3.1. Supernatant source of AmpA protein can reduce the number of ampA null cells that assume a prespore fate

In the absence of an active *ampA* gene, cells differentiate prematurely and assume a prespore fate. Previous work has shown that the inclusion of 10% wild type cells in a chimera with 90% *ampA* null cells is sufficient to rescue the premature entry of cells into the prespore pathway. This raises the question of whether it could be extracellularly secreted AmpA protein that is capable of suppressing premature entry of cells into the prespore pathway (Blumberg et al., 2002; Varney et al., 2002b). To test this possibility, Wt or *ampA* null cells carrying a prespore-specific *pspD–β-galactosidase* reporter were induced to enter development by starvation in the presence or absence of wild type or *ampA* null cell supernatant. At various times after starvation, the developing structures were fixed and stained with X-gal to detect prespore cells (Fig. 1A and B). Cells that had adopted the prespore fate expressed the prespore-specific *β-galactosidase* marker and appeared blue (indicated by the blue arrows). Cells that had not entered the prespore pathway did not have any *β-galactosidase* activity and remained clear (indicated by the white arrows). Under control conditions (starvation in phosphate buffer) wild type cells exhibit normal pattern formation. At the mound stage, the periphery of the structure was occupied by the prestalk and anterior like cells (clear) while the center of the structure was occupied by the prespore cells (blue) (Fig. 1A, WT in PO_4 buffer). Similarly, the anterior region of the wild type finger remained unstained while the posterior prespore region was stained blue (Fig. 1B, WT in PO_4 buffer). In the *ampA*-null structures, by contrast, the cells at the periphery of the mounds and the anterior region of the finger mis-expressed the prespore marker and stained blue (Fig. 1A and B, KO in PO_4 buffer).

The AmpA containing supernatant collected from wild type cells prevented *ampA null* cells from mis-expressing the prespore marker at the periphery of the mounds or the anterior tips of the fingers/slugs (Fig. 1A and B, $ampA^-$ in WT sup). In wild type cells, this supernatant caused no change in the normal pattern of prespore gene expression (Fig. 1A and B, WT in WT sup). By contrast, the supernatant collected from *ampA null* cells lacked the ability to suppress the misexpression of the prespore marker in the *ampA* null prestalk cells (Fig. 1A and B, $ampA^-$ in KO sup). Interestingly, when wild type cells were developed in the *ampA null* supernatant, regions occupied by prestalk and anterior like cells showed the activity of the prespore marker, indicated by the blue regions of β-galactosidase activity at the mound periphery and anterior tip of the finger (Fig. 1A and B, WT in KO sup).

These results suggest that there is a factor that induces expression of a prespore marker in the absence of AmpA. Such a factor becomes inactivated or eliminated by the presence of AmpA protein since supernatant from wild type cells expressing the *ampA* gene do not have this activity. This factor is likely unstable because batches of supernatant harvested from

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ampA null cells varied significantly in their ability to induce the prespore activity and the activity was not stable to freezing. By contrast, the wild type *ampA* containing supernatant was very consistent and reproducible in its ability to inhibit the mis-expression of the prespore activity. Western analysis confirmed that the concentrated supernatant harvested from wild type cells contained AmpA protein while supernatant from *ampA* null cells lacked detectable levels of *ampA* protein (Fig. 2D).

To quantify the suppression of prespore differentiation, the experiment was repeated with the following changes. Fourteen hours after the initiation of starvation, the developing structures, instead of being stained as whole units, were trypsinized and disaggregated to single cells. The individual cells were stained for β-galactosidase activity and the percentage of cells expressing the prespore marker was determined. Fig. 2A and B shows an example of the individual cell staining. The quantification is shown in Fig. 2C. In phosphate buffer, about 30% of the wild type cells expressed the prespore marker while nearly 60% of the *ampA null* cells showed activity for the marker. When developed in supernatant collected from wild type cells, the percentage of *ampA* null cells expressing the marker is reduced to about the wild type level of 30%. There was no further reduction of prespore expressing cells when wild type cells were developed in wild type supernatant. The supernatant collected from *null* cells failed to reduce prespore expression in *null* cells. It actually increased the percentage of wild type cells expressing the prespore marker to the level of *null* cells expressing the marker in phosphate buffer. Changes in the number of cells expressing the *pspD* prespore specific reporter could not reflect contamination by wild type or *ampA* null cells in the supernatant fraction because the cells used to prepare the supernatant fractions did not carry the *pspD* reporter. These numbers corroborate the results of whole mound staining shown in Fig. 1A and B and clearly indicate that a supernatant source of AmpA can suppress the mis-expression of the prespore marker by *ampA* null cells. They also indicate that supernatant from *ampA* null cells can induce wild type cells to mis-express the prespore gene. Since we have not found a way to stabilize this activity we have not pursued it further. Instead we have focused on further characterizing the extracellular role of AmpA in suppressing prespore gene mis-expression.

3.2. Synthetic oligopeptides from specific domains of the AmpA protein are capable of efficiently inhibiting excess prespore cell induction by ampA null cells

In order to confirm that it is the AmpA protein, itself, and not something else in the supernatant that acts to prevent excessive or premature entry of cells into the prespore pathway, we analyzed the ability of synthetic oligopeptides to replicate the behavior of the supernatant from the *ampA* wild type cells. The full length AmpA protein is 24 kD and contains a hydrophobic leader sequence followed by a series of repeated peptide sequences depicted by green, yellow and blue bars in Fig. 3A. Current BLAST searches show nothing that shares significant homology with the AmpA protein; however, BLAST searches on data bases at the time that the AmpA protein was first described indicated that the two repeated domains (shown in Fig. 3A, in green/yellow) possess a cysteine repeat with similarities to those found in some snake venom disintegrins, sperm fertilins and the von Willebrand factor disintegrin domain. The 4 carboxy terminal repeats (Fig. 3A, shown in blue/yellow) share limited homology with the anticoagulant ornatin peptides from leech saliva (Blumberg et al., 2002; Varney et al., 2002b). A Clustal W alignment of the four AmpA carboxy terminal repeats with four of the ornatin peptides is shown in Supplemental Fig. 1. We refer to these domains as the disintegrin and ornatin domains of AmpA (Blumberg et al., 2002). Because of size constraints for synthesis, we could not examine the potential activity of the full disintegrin domain, but we were able to examine the potential activity of an oligopeptide corresponding to a portion of the first of the two disintegrin repeats of the AmpA protein. This peptide (#1 in Fig. 3B) comprised the sequences that showed the highest homology to

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the von Willebrand factor disintegrin domain. A second peptide comprising the entire sequence of the first of the four ornatin repeats (peptide #2 in Fig. 3B) was also synthesized. We found that the ornatin peptide (peptide #2) is very effective at reducing the number of *ampA* null cells that express the *pspD* prespore reporter, and, like the wild type supernatant, it also has no effect on the number of wild type cells expressing the prespore reporter (Fig. 4A). By contrast, the specific disintegrin domain peptide we examined is less effective (Fig. 4A).

Fig. 4B shows the percentage inhibition of prespore reporter expression, relative to that seen with wild type supernatant, as a function of peptide concentration. Peptide #2 at the highest concentrations is maximally efficient at inhibiting the number of *ampA* null cells expressing the prespore reporter. Half maximal inhibition is achieved at 33 μM. Peptide #1, gave 80% inhibition with half maximal inhibition requiring at least 120 μM. In the assay, cells are resuspended in the supernatant or in the peptides and then plated for development on filters that have been soaked in the peptide or supernatant solution. The cells are then allowed to develop for 10–12 h. Limiting factors in the assay may be the stability of the peptides or of the AmpA protein in the supernatant as well as physical access of all cells to the peptide or supernatant source of AmpA. Nonetheless, the assay clearly demonstrates that either intact AmpA protein in supernatant from wild type cells or the synthetic peptide from the ornatin domain of the AmpA protein is capable of inhibiting excessive numbers of *ampA* null cells from assuming an initial prespore fate. The ornatin domain peptide does this in a concentration dependent manner that exhibits saturation behavior, raising the possibility the AmpA acts to inhibit initial prespore differentiation by binding to a receptor or other factor.

In order to determine if a smaller portion of the AmpA gene could function to inhibit prespore gene misexpression, two nine amino acid peptides from the disintegrin and ornatin domains were tested. Since the B domain (yellow bar in Fig. 3A) is largely conserved in both disintegrin and ornatin domains of AmpA and repeated six times in the 24 kD AmpA protein, we chose a nine amino acid sequence from this part of the molecule (Fig. 3B). The decision to choose this peptide was guided in part by the observation that when the ornatin domains of the AmpA protein are aligned with the ornatin peptides from leech saliva, the EGE tripeptides (blue in Fig. 3B) are in the same position as the “active site” RGD tripeptide of the ornatins (Varney et al., 2002b see Fig. 3). A variant of this tripeptide motif in which charged amino acids flank a glycine, DGE is also present in the B repeat of the two AmpA disintegrin domains (Fig. 3B blue). The two nine amino acid peptides, peptide #4 from the first disintegrin repeat and peptide #3 from the first ornatin repeat are indicated in Fig. 3B. At the highest concentrations tested, peptide #3 inhibited prespore cell differentiation by *ampA* null cells only a little less efficiently than did the full length ornatin peptide #2. Half maximal inhibition by peptide #3 occurred at about 60 μM, about half as efficiently as the full ornatin peptide and twice as efficiently as the disintegrin domain peptide. When a scrambled version of peptide #3 was used there was almost no inhibition (peptide #7 sequence in Fig. 3B), consistent with the inhibition depending on the correct AmpA sequence (Fig. 4B). Interestingly, peptide #4 from the disintegrin domain is about as efficient as the larger disintegrin domain peptide #1 and was at least two-fold less efficient than the nine amino acid ornatin peptide (peptide #3). Comparison of the two nine amino acid peptide sequences indicates that only three conserved amino acid differences exist between the two sequences (compare peptides 3 and 4 in Fig. 3B). This suggests that subtle variations in these sequences can alter the efficiency of interaction of the AmpA protein with its target/s.

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3.3. Mutational analysis of the nine amino acid ornatin domain peptide indicates that the three charged amino acids and the conserved glycine are essential for activity

In order to identify key amino acids that were essential for the activity of the ornatin domain peptide, a series of alanine substitution peptides were prepared. Fig. 5A shows the sequences of these variant peptides. Changing a conserved cysteine residue at the fourth position of the nine amino acid peptide reduced the prespore inhibitory activity more than two-fold (peptide #6, Fig. 5B triangles) suggesting the possibility that a disulfide bond may stabilize the interaction of the AmpA protein with its target or be necessary for optimal activity. The nine amino acid sequence contains three charged amino acids (K, D, and E). It is possible that the charges on these residues are involved in alignment of the AmpA protein with its interacting partner. When these three residues were changed to alanines, there was a complete loss of prespore inhibitory activity (Fig. 5B squares, peptide #5). Changing the conserved tyrosine in position 3 of the nine amino acid peptide to alanine (peptide #8, Fig. 5A), resulted in a reduction of at least three-fold in the peptide's activity (Fig. 5C, squares). When the glycine in position 7 of the peptide was changed to alanine (peptide #9, Fig. 5A), virtually all activity was lost (Fig. 5C, triangles). Interestingly, the glycine in this position is absolutely conserved in all of the ornatin and disintegrin repeats (Fig. 3B). This suggests that this glycine plays an essential role in the peptide's prespore inhibitory activity.

3.4. Extracellular AmpA cannot act to reduce cell–cell adhesion in early development

Previous work has demonstrated that *ampA* null cells developed by starving in suspension culture for 6 h form much larger agglomerates than wild type cells (Varney et al., 2002a). Conversely, overexpression of AmpA protein results in agglomerates that are half the size of those formed by wild type cells and many more cells remain single. In order to determine whether the effects of the *ampA* gene upon cell–cell adhesion were due to secreted, extracellular AmpA, we developed *ampA* null cells by starvation in suspension. We supplemented them with the same supernatant fractions from 10 h developing wild type cells that were effective in preventing the *ampA* null cells from mis-expressing the prespore reporter. The supernatant failed to cause any reduction in the size of the agglomerates formed. Fig. 6A shows agglomerates formed by wild type and *ampA* null cells developed in suspension in phosphate buffer and in supernatant fractions from developing wild type cells (AmpA containing) and *ampA* null cells (lacking AmpA protein); Fig. 6B shows the quantification of agglomerate size.

We have previously shown that synergistic mixing of 10% wild type cells with a population of *ampA* null cells was sufficient to prevent the excessive premature entry of *ampA* null cells into the prespore pathway. Synergistic mixing of 10% wild type cells and even 20% wild type cells in the population of *ampA* null cells did not reduce the size of the *ampA* null agglomerates formed by starvation in suspension culture (Fig. 6C shows the agglomerates; Fig. 6D shows the quantitation of agglomerate size).

As a further approach, *ampA* null cells were developed by suspension starvation in the presence of the highest concentrations of peptide #2, the most efficient inhibitor of prespore differentiation by *ampA* null cells. Again, there was no reduction in the size of the agglomerates formed by the *ampA* null cells. In case it was the disintegrin like domain and not the ornatin domain responsible for this function, the highest concentration of peptide #1 was also tested. It too did not prevent agglomerate formation. Fig. 7A shows the agglomerates; Fig. 7B shows the quantification of agglomerate size. It thus appears that AmpA functions intracellularly to modulate cell adhesion, in contrast to its effect on pre-spore gene expression where it clearly functions extracellularly.

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3.5. Over expressed AmpA protein is secreted by growing cells but this extracellular AmpA does not increase F-actin levels in growing cells

The AmpA protein is only found secreted in developing cells. In wild type growing cells it is not secreted although its hydrophobic leader appears to be cleaved (Varney et al., 2002a). In growing cells, AmpA is associated with Golgi derived vesicles; it is transiently associated with the cell surface and then is endocytosed and found in a perinuclear, slow recycling compartment (Noratel et al., 2012). AmpA influences a number of parameters in growing cells. Among these, loss of AmpA results in a significant (approximately 2.5-fold) loss of F-actin while overexpression of AmpA in growing cells increases the amount of F-actin to almost twice the level found in wild type cells (Fig. 8C; Noratel et al., 2012). Since AmpA is not secreted in growing cells it was assumed that its effects on these cells were entirely cell autonomous. However, when AmpA is overexpressed, the excess AmpA protein is indeed secreted (Fig. 8A and B).

Because of the difficulty of producing AmpA antibodies (only one in six rabbits produced an anti-AmpA antibody and no successful antibodies were produced using multiple different peptides from the AmpA protein), we generated an AmpA–Tap tag fusion protein construct to use for studies localizing AmpA in cells (Noratel et al., 2012). Two different cell lines were generated with this AmpA–Tap tag fusion protein construct. One cell line, AmpA–Tap–Wt was generated by introducing a linearized restriction fragment of the plasmid DNA that contained only the AmpA gene fused to the Tap tag protein and a blastocidin resistance cassette. This linear restriction fragment of DNA integrated into the genome. In all AmpA functions this strain was entirely wild type (Noratel et al., 2012). The second cell line, AmpA–Tap–OE was one in which the AmpA–Tap tag vector DNA was introduced as a circular plasmid. This cell line behaved phenotypically as an AmpA over expressing cell line and produced three times more AmpA protein than the AmpA–Tap–Wt strain (Noratel et al., 2012). Surprisingly, when AmpA is over expressed in growing cells, significant amounts of AmpA protein are found secreted into the media as detected on western blots (Fig. 8A). This is not seen with the AmpA–Tap–Wt construct (Fig. 8A). With this wild type like strain no AmpA protein is detected in the extracellular media (Fig. 8A). We also constructed a vector containing an AmpA fusion protein that had the mRFP protein fused to the N terminus of the AmpA protein immediately after the hydrophobic leader sequence (Noratel et al., 2012). When this plasmid was introduced into wild type cells as a circular plasmid the cells also displayed an AmpA overexpresser phenotype (Noratel et al., 2012). With this construct as well, the mRFP–AmpA fusion protein was secreted during vegetative growth (Fig. 8B). This suggests that in AmpA over-expressing cells the excess AmpA protein is secreted. However, when AmpA is expressed at or near wild type levels the AmpA protein is not secreted. Since AmpA appears transiently on the extracellular surface of wild type cells (Noratel et al., 2012); a possible explanation is that AmpA interacts with a membrane protein that is limiting and, when AmpA is overexpressed, the excess AmpA is then found in the extracellular media.

The presence of extracellular secreted AmpA in the overexpres-sing strains in growing cells raises the question of whether the extracellular AmpA is responsible for the increased F-actin polymerization in these cells (Noratel et al., 2012). We grew wild type and *ampA* null cells overnight in the presence of supernatant from AmpA overexpressing cells; the AmpA containing supernatant did not increase the level of F-actin polymerization as measured by phalloidin binding to cell extracts (Fig. 8C). High levels of peptides 1 and 2 were also added to wild type or *ampA* null cells and these also could not increase the amount of F-actin polymerized (data not shown). In addition to overnight incubation, a variety of other ways of adding the AmpA supernatant were tried and none resulted in an increase in F-actin polymerization in either Wt or *ampA* null cells (not shown). This suggests that while AmpA may function extracellularly to effect prespore gene expression, it has a second, cell

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autonomous function. This latter function is responsible for the level of polymerized F-actin in growing cells and for cell–cell agglutination in suspension starved developing cells.

4. Discussion

4.1. AmpA prevents prespore gene mis-expression in cells not destine to become prespore cells

A direct role of AmpA in the regulation of cell fate determination is supported by pattern formation in chimeric structures. Addition of 10% wild type cells to *ampA* null cells prevents cells at the mound periphery from differentiating as prespore cells, allowing these cells to respond to other morphogens and to differentiate as prestalk cells (Varney et al., 2002b). Since AmpA is expressed in the anterior like cells that are also found at the mound periphery, this finding suggested the possibility that secreted extracellular AmpA inhibits prespore fate in neighboring cells in a non cell-autonomous manner.

Here we have confirmed this possibility by demonstrating that an exogenous source of AmpA, either the supernatant collected from developing wild type cells or AmpA specific oligopeptides, can prevent the excessive expression of a prespore-specific *pspD* marker in the *ampA* null cells. The number of prespore-expressing cells could never be reduced past the wild type value, regardless of the concentration of the exogenous AmpA in the form of super-natant or of synthetic AmpA oligopeptides. This suggests that there is a population of cells that are predetermined to adopt the spore fate and a second smaller population of cells that arrive late to the mounds and would normally adopt an alternative fate. This second population seems to depend on the activity of AmpA to suppress expression of prespore genes.

Two other proteins, like AmpA, have been identified that not only modulate adhesions but also regulate cell fate. The *Dictyostelium* adhesion molecule DdCAD1 (gp24) mediates early side-to-side EDTA-sensitive cohesion during the early stages of development (Wong et al., 1996; Siu, 1990; Siu et al., 1997; Siu and Lam, 1988). Disruption of the gene was shown not only to reduce cell–cell adhesion but also to reduce the number of cells that enter the prespore pathway (Wong et al., 2002). This is exactly the opposite of the *ampA* null phenotype, which increases adhesion and increases the number of cells that adopt the prespore fate. Another protein, Countin factor, is a secreted complex that is involved in quorum sensing to determine aggregate size (Brock et al., 2002). CF50 is a component of countin factor and has been demonstrated to be necessary to reduce premature entry of cells into the prespore pathway (Brock et al., 2002). CF50, like AmpA, can only reduce the number of cells entering the prespore pathway to wild type level, suggesting again that there is a population of cells that are predetermined to assume the prespore fate and are not responsive to these developmental morphogens.

4.2. Synthetic oligopeptides identify domains of the AmpA protein responsible for activity

A synthetic peptide containing the full AmpA ornatin repeat could effectively inhibit *ampA* null cells from mis-expressing the prespore marker. The disintegrin domain peptide was less active. One possible explanation for this is that we have not used the entire disintegrin domain since the full disintegrin domain was too long to be efficiently synthesized. A nine amino acid peptide was identified within the ornatin domain that retained most of the activity of the full ornatin peptide. Interestingly, the variant of this peptide found in the disintegrin domains differed from the ornatin peptide by only three conservative amino acid substitutions and showed the same lower activity as the larger disintegrin domain peptide. A scrambled version of the nine-amino acid active site peptide showed much reduced activity, indicating specificity for the AmpA sequence. Since a nine-amino acid sequence with no

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known catalytic activity can block initial prespore differentiation by a subset of *ampA* null cells, it is likely that it does so by binding to a factor or receptor that plays a role in inducing prespore differentiation.

Alanine substitution mutagenesis identified key amino acids in the 9-amino acid sequence. The conserved cysteine residue probably plays an important role in the interaction of AmpA protein since its mutation to alanine resulted in significant loss of activity. When the charged amino acids (K, D, and E) were alanine-substituted, there was a complete loss of activity suggesting that interaction of AmpA with a receptor or partner requires an initial step of alignment of charged amino acids on the proteins, followed by formation of a disulfide bond that stabilizes AmpA in interacting with its receptor or binding partner. Additionally, the conserved glycine is absolutely essential

4.2.1. Is there a factor, inactivated by AmpA, which induces prespore gene expression?—While supernatant from *ampA* null cells failed to reduce the excessive prespore expression of the *ampA* null cells, this super-natant could increase prespore gene expression in wild type cells. It is possible that there is a factor that accumulates in the extracellular supernatant of developing *ampA* null cells that upregulates expression of prespore genes. This prespore-inducing factor (PIF) could be inactivated or eliminated when AmpA protein is present in wild type cells. In our hands this PIF activity is unstable. Oohata has described low-density monolayer conditions that cause most cells to differentiate into prespore cells (Oohata, 1995; Oohata et al., 1997). A prespore inducing factor has been isolated from the media of cells developed under these conditions (Yamada et al., 2010). It is possible that PIF is this factor. It is also possible that Oohata had to use these low density conditions to identify PIF because PIF does not accumulate to high levels extracellularly in the presence of AmpA in wild type cells.

4.2.2. Does AmpA function as a lateral inhibitor of prespore differentiation at the mound periphery?—At mound stage the anterior like cells are concentrated at the mound periphery (Casademunt et al., 2002). Cells that differentiate as prestalk cells are also initially located at the mound periphery and it is here that they first activate the expression of the *stkA* reporter gene *ecmA* (Early et al., 1995). It is possible that AmpA protein secreted from anterior like cells at the mound periphery acts to either prevent release of PIF or binds to and inactivates PIF in this region at the mound periphery. A reduced amount of PIF in the mound periphery may prevent the late arriving cells from assuming a prespore fate. In the absence of AmpA, PIF could accumulate at the mound periphery and the late arriving cells could respond to it and assume a prespore fate. In the absence of AmpA, these cells later transdetermine to the prestalk fate and can complete development after a delay (Varney et al., 2002a). They continue to stain for the stable β-galactosidase reporter but form normal stalks. This is likely a result of a later proportion regulation response know to be invoked to correct the over-expression of prespore fates in migrating slugs that have lost prestalk cells in the slime trail (Detterbeck et al., 1994; Jermyn and Williams, 1991; Williams and Jermyn, 1991; Williams and Morrison, 1994).

4.3. Cell autonomous role for AmpA in cell adhesion and growth phase actin polymerization suggests a model for AmpA's dual function

When starved cells are deposited on a solid substratum they progress through development to form fruiting bodies. Development can also be induced by starving cells in a suspension culture without any nutrient. In this case, the lack of air/water interface does not allow the starved cells to undergo correct morphogenesis for fruiting body formation. The cells adhere to each other to form agglutinates, making tight cell–cell contacts and start to differentiate. Deletion of the *ampA* gene resulted in excessively large agglutinates while over expression

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of the protein resulted into dramatically smaller agglutinates. Although reintroduction of the *ampA* gene could prevent the formation of excessively large agglutinates formed by the *ampA*$^{-}$ cells, extracellular sources of AmpA protein failed to have any effect on these agglutinates. These findings strongly suggest that the down-modulation of an adhesion system to prevent excessive cell–cell agglutination during axenic growth and early development reflects a cell-associated role for AmpA protein. We have previously shown that AmpA is transiently present on the cell surface and is then endocytosed and traffics to a slow endocytic compartment (Noratel et al., 2012). Since AmpA does not have any transmembrane domains its association with the cell surface must be by interaction with a cell membrane protein. It is likely that as AmpA traffics to the cell surface it binds to a cell membrane protein which presents AmpA on the cell surface. The fact that when AmpA is overexpressed, the excess is secreted and found free in the extracellular media suggests that the amount of the membrane protein partner is limiting. We have demonstrated here that the secreted AmpA is not responsible for the excessive actin polymerization seen in the AmpA overexpressing strains. We suggest that this membrane partner may play a role in cell adhesion and actin polymerization and that interaction with AmpA may control its rate of membrane recycling and thus account for the cell autonomous effect of AmpA on cell–cell adhesion and actin polymerization as well as for the effects of AmpA on cell–substrate adhesion and cell migration described by Noratel et al. (2012).

During development AmpA mRNA levels increase 15-fold, but AmpA expression is confined to only the anterior like cells (Casademunt et al., 2002). As a result, the amount of AmpA per cell is significantly higher in the anterior like cells during development than it is in growing cells. During development AmpA protein is largely secreted (Varney et al., 2002a). This suggest that what determines whether AmpA is secreted or retained on the cell surface (or within the cellular vesicle compartments) is the amount of AmpA that is made in the cells relative to the amount of its interacting partner. This suggests that the developmental specific secretion of AmpA it is not due to a developmentally regulated secretion mechanism but rather is due to the level of AmpA protein being made relative to the level of a potential interacting partner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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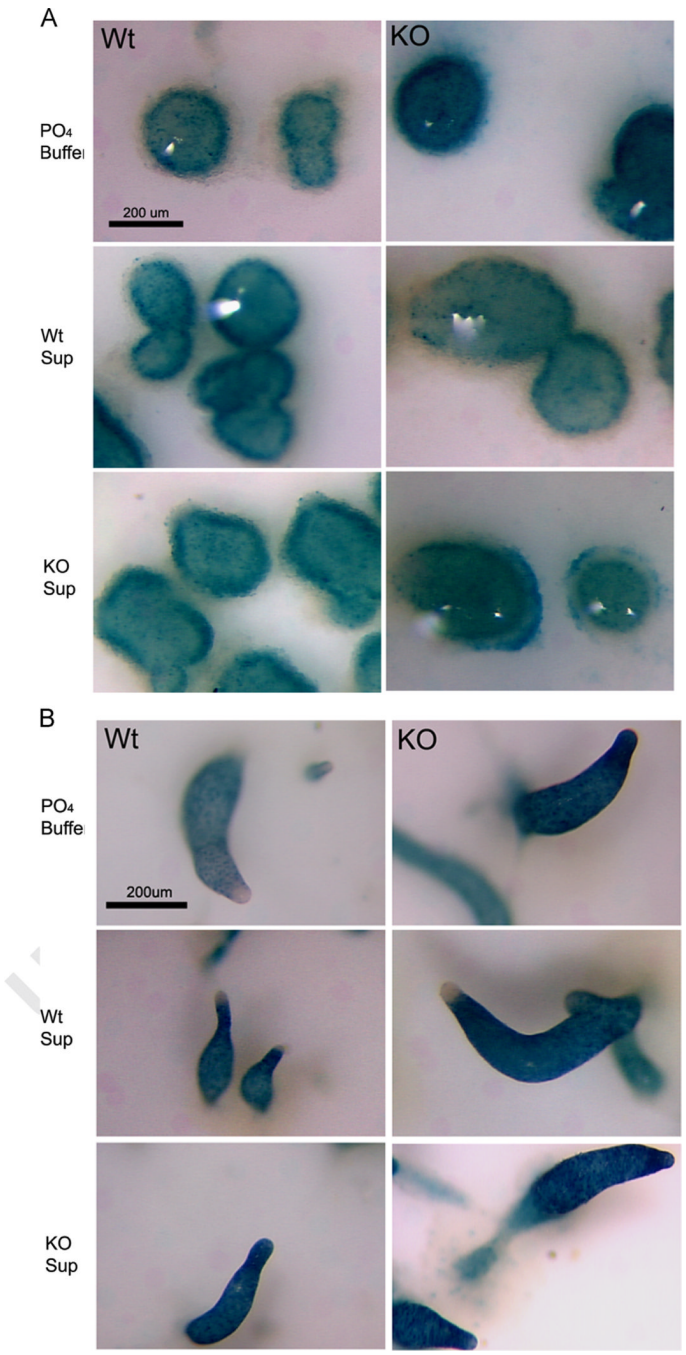

Fig. 1.

ampA null cells show prespore gene expression in cells not destined to become prespore cells. This aberrant expression can be suppressed by *ampA* containing supernatant from wild type cells. Supernatant from *ampA* null cells can induce prespore gene expression in prestalk cells of wild type mounds and fingers. Wild type or *ampA* null cells containing a prespore-specific β-galactosidase reporter were plated for development in buffer or in buffer containing supernatants collected from 10-h developing wild type or *ampA* null cells. 12 h (A) or 20 h (B) after the initiation of starvation, filters containing developing structures were fixed and stained for β-galactosidase activity. Cells that had adopted the prespore fate stained blue, while cells that had not entered the prespore pathway were unstained and

remained clear. White arrows indicate regions of clear unstained cells; blue arrows indicate regions of blue stained cells. Scale bars are 200 μm. Images shown are representative of at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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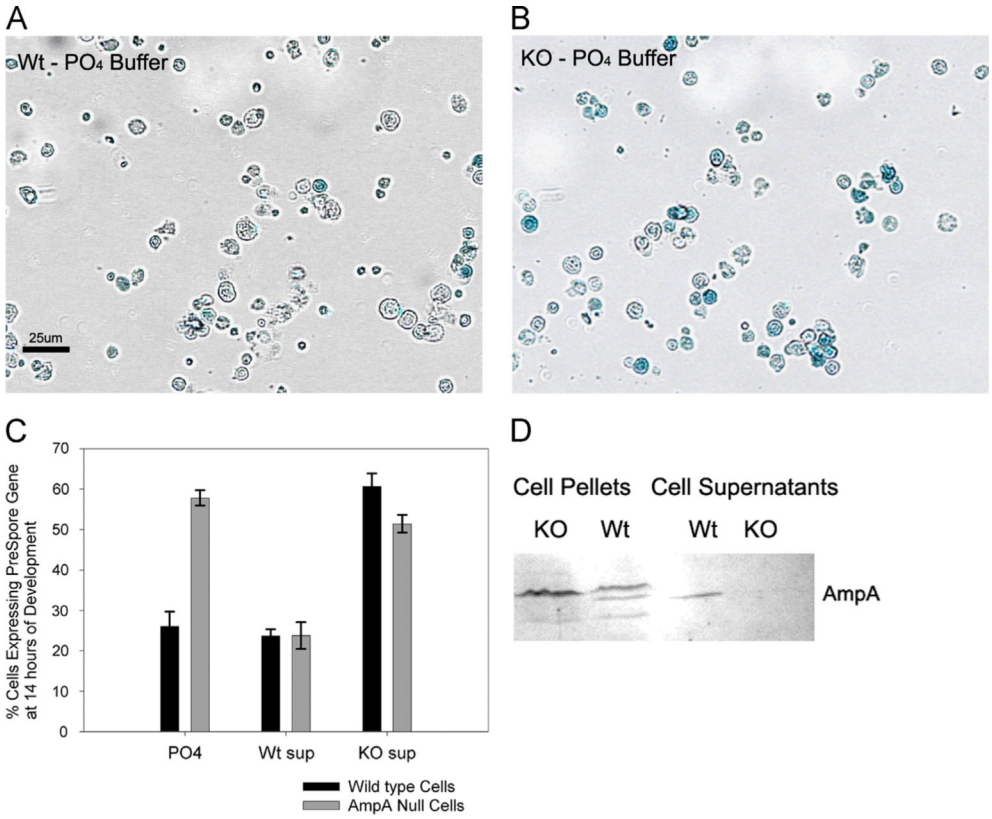

Fig. 2.

Effect of extracellular supernatant on the number of cells assuming a prespore fate. Wild type and *ampA*⁻ cells carrying a prespore-specific marker were plated for development in starvation buffer or in buffer containing either wild type or *ampA*⁻ (KO) supernatants from 10 h developing cells. At 14 h, the structures were harvested, disaggregated into single cells, and stained for the prespore-specific marker activity. Stained cells were imaged and the percentages of prespore cells were calculated. (A,B) an example of single cells dispersed from Wt (A) and *ampA* null (B) 14 h late mound stage developing structures and stained for β-galactosidase expression. Scale bar is 25 μm. Images were background subtracted in order to enable clear identification of blue and white cells. Blue arrows point out some of the blue cells. (C) The effect of extracellular supernatant on the number of cells assuming a prespore fate. Wild type (black) or *ampA* null cells (gray); error bars are standard error of the mean. All determinations were repeated on at least three different batches of cells grown and developed on different days. Per condition 300–3500 cells were counted. (D) Western analysis demonstrating that supernatant collected from 10-h developing wild type cells contains AmpA, while no AmpA can be detected in the supernatant collected from *ampA* null cells. Extracellular supernatants were harvested and concentrated from wild type and *ampA* null cells at mound stage of development (10 h). Protein or supernatant from equal numbers of cells were loaded on gels. AmpA protein was detected using a 1:300 dilution of anti-AmpA antibody and a 1:8000 dilution of goat-anti rabbit second antibody. Approximately equal loading was verified by Coomassie staining of a parallel gel (not shown). Arrow indicates the position of AmpA protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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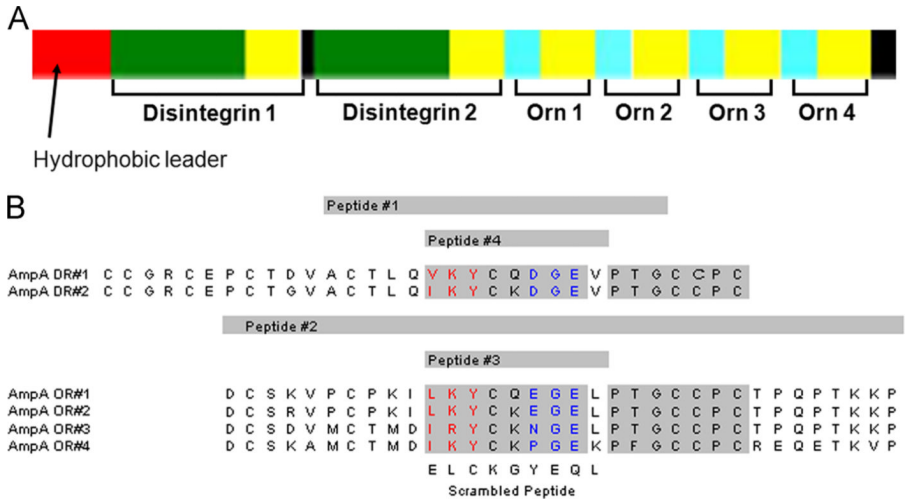

Fig. 3.

(A) Diagram of the repeat structure of the *ampA* gene. The *ampA* gene encodes a 24 kD protein that contains a hydrophobic leader sequence (red) followed by two disintegrin like repeats (green and yellow) and four ornatin like repeats (blue and yellow). Imbedded in both the disintegrin and the ornatin like domains is a highly conserved sequence (yellow). There are two short amino acid sequences that are not repeats indicated in black separating the disintegrin and ornatin like repeats and at the carboxy-terminus of the protein. (B) Sequence alignment of the two disintegrin and four ornatin repeats that comprise the AmpA protein. The disintegrin repeats are labeled DR#1 and #2, with the #1 repeat being the most N terminal of the two repeats. The four ornatin repeats are labeled N-terminus to C-terminus OR#1-4. The tripeptides VKY and IKY occupy the same position in the disintegrin repeat of AmpA as the “active site” RGD tripeptides in the fertilins and disintegrins of metazoans relative to the conserved cysteine organization and are indicated in red. These tripeptides are also present in the ornatin repeats (in red). In the ornatin domain of AmpA, the EGE, NGE and PGE tripeptides occupy the same site as the RGD active site tripeptide in the leech saliva ornatins and are indicated in blue (see Clustal W alignment in Supplemental Fig. 1). A variant of this sequence, DGE is present in the disintegrin domains of AmpA (indicated in blue). Areas of conserved sequence between the disintegrin and ornatin domains of AmpA are shaded in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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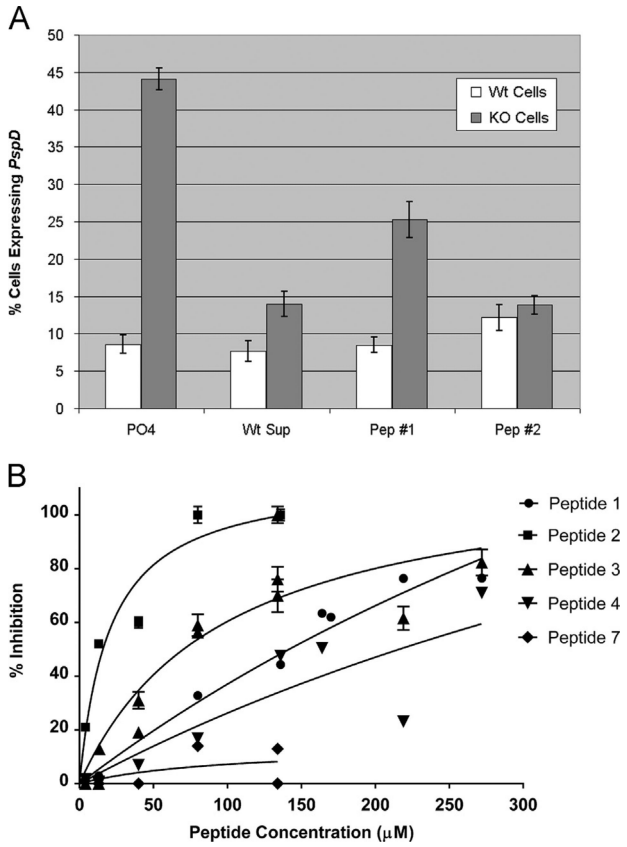

Fig. 4.

Oligopeptides from domains of the AmpA protein can reduce the number of *ampA*$^{-}$ cells that express a prespore-specific marker. (A) Wild-type cells or *ampA*$^{-}$ cells containing a prespore-specific marker were plated for development in 20 mM Na_2KPO_4 starvation buffer, or supernatant harvested from 10-h developing wild-type cells, or in starvation buffer containing 500 μg/mL of either disintegrin peptide (peptide #1) or ornatin peptide (peptide #2). At 14 h, cells were harvested, disaggregated to single cells, fixed, and stained for β-galactosidase activity. Stained cells were imaged and the percentages of cells staining for the prespore-specific marker were calculated. Error bars represent standard error of the mean. The results represent the averages of at least three independent determinations. (B) Synthetic AmpA oligopeptides can reduce the number of *ampA* null cells assuming a prespore fate in a concentration dependent manner. *ampA* null cells were resuspended and plated for development for 14 h in either phosphate buffer (PO4), or supernatant harvested from 10 h developing wild type cells (Wt sup) as controls or they were incubated in the indicated concentrations of the disintegrin domain peptides (peptides #1 and #4), the ornatin domain peptides (peptides #2 and #3) or a scrambled peptide (peptide #7) as detailed in Fig. 3B. The % inhibition of prespore fate was calculated by setting the percentage of *ampA* null cells expressing the pspD prespore reporter in phosphate buffer equal to 0% inhibition and the percentage of *ampA* null cells expressing the reporter in the presence of supernatant from 10 h developing wild type cells equal to 100% inhibition. The reduction in the number of *ampA* null cells expressing the pspD prespore reporter in the presence of each of the oligopeptides at each concentration was normalized to these controls. Error bars represent standard error of the mean. For some data points the error bars are obscured by the data symbol. Between 300 and 3500 cells were counted for each data point. Multiple repeats were done on

different batches of cells prepared on different days. For peptide #3 the determinations include two different batches of peptide prepared by two different sources.

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A

#	Sequence	Description
3	LKYCQEGEL	Ornatin active site
5	LAYCQAGAL	Mutated charged amino acids
6	LKYAGEGEL	Mutated cysteine
8	LKACQEGEL	Mutated Tyr to Ala
9	LKYCQEAEL	Mutated Gly to Ala

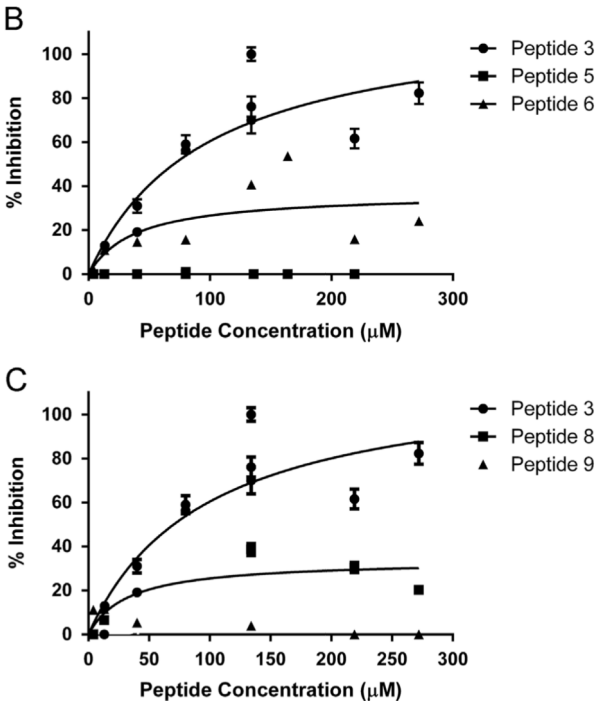

Fig. 5.

A nine amino acid peptide of AmpA inhibits initial prespore cell differentiation. Mutations in key amino acids reduce or abolish the activity of the peptide. (A) Amino acid sequence of the wild type ornatin domain peptide and the sequences of the peptides containing alanine substitution mutations. (B,C) *ampA* null cells were plated for development in the presence of the indicated concentrations of the wild type and mutant oligopeptides and the percentage inhibition of prespore fate was calculated as described in the legend to Fig. 4. (B) Compares the wild type peptide #3 to mutant peptides #5 and #6. (C) Compares the wild type peptide #3 to mutant peptides #8 and #9. Error bars represent standard error of the mean. For some data points the error bars are obscured by the data symbol. Between 300 and 3500 cells were counted for each data point. Multiple repeats were done on different batches of cells prepared on different days.

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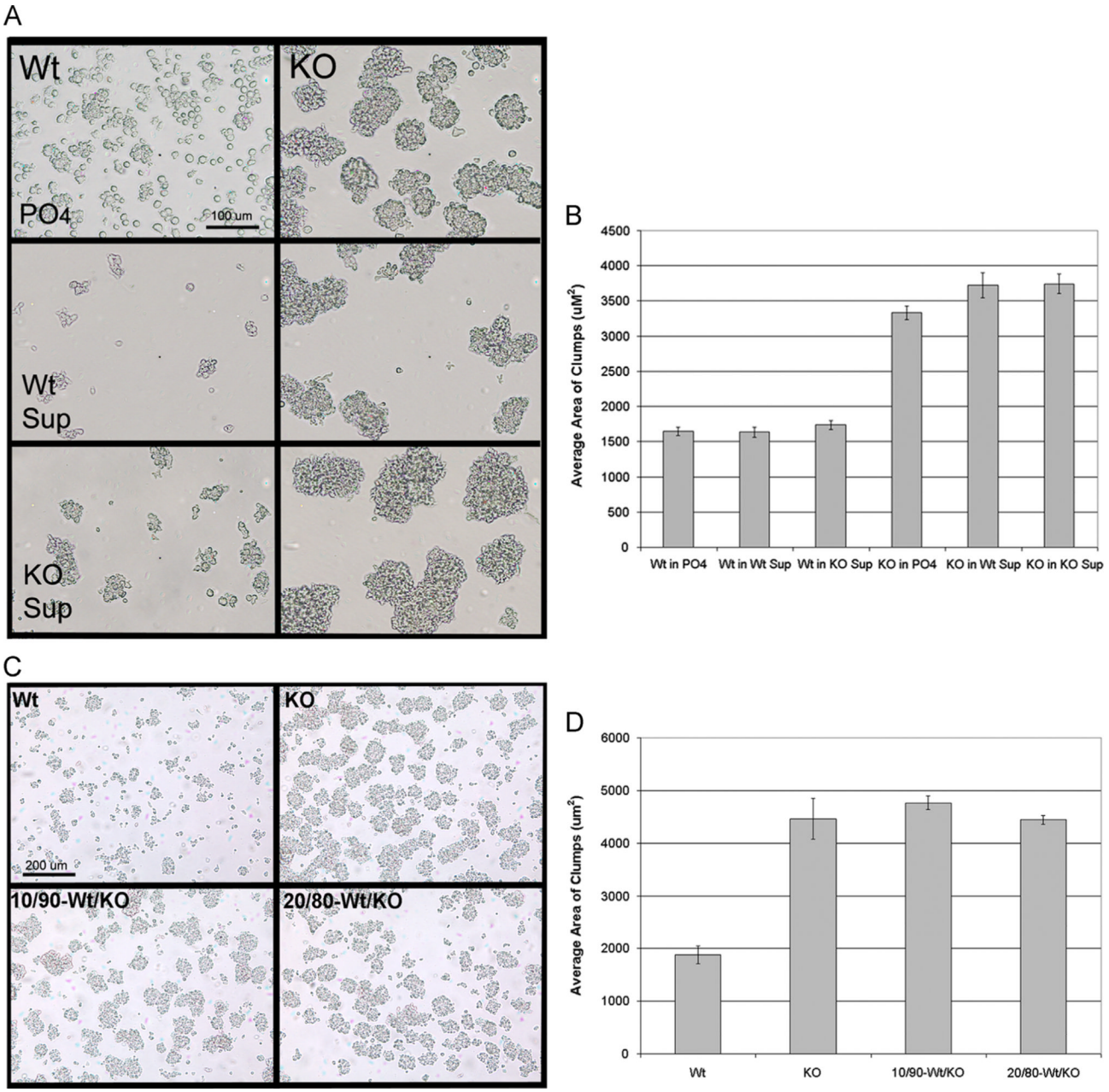

Fig. 6.

Externally added supernatant fractions that block the prespore gene mis-expression fail to block the over-adhesive *ampA* null phenotype. Wild type and *ampA* null cells were developed by starvation in slowly shaken suspension culture in either phosphate buffer (PO_4), supernatant harvested from 10 h developing wild type cells (Wt sup) or 15 h developing *ampA* null cells (KO sup). (A) Representative micrographs of the agglutinates formed imaged with a 20× objective. Scale bars are 100 μm. (B) Quantification of agglutinate cross-sectional area. The cross-sectional area of cell agglutinates depicted in A was determined. Over 60 cell agglutinates were measured for each strain and condition in several independent experiments. Error bars are the standard error of the mean. (C) The anti-adhesive effect of AmpA is cell autonomous and cannot be rescued by the addition of wild type cells. Wild type and *ampA* null cells were developed by starvation in slowly shaken

suspension culture. Agglutinates formed by wild type cells (Wt), *ampA* null cells (KO), 90% *ampA* null cells mixed with 10% wild type cells (10/90—Wt/KO) or 80% *ampA* null cells mixed with 20% wild type cells (20/80—Wt/KO). Agglutinates were imaged with a 10× objective. Scale bars are 200 μm. (D) Quantification of agglutinate cross-sectional area as described in B.

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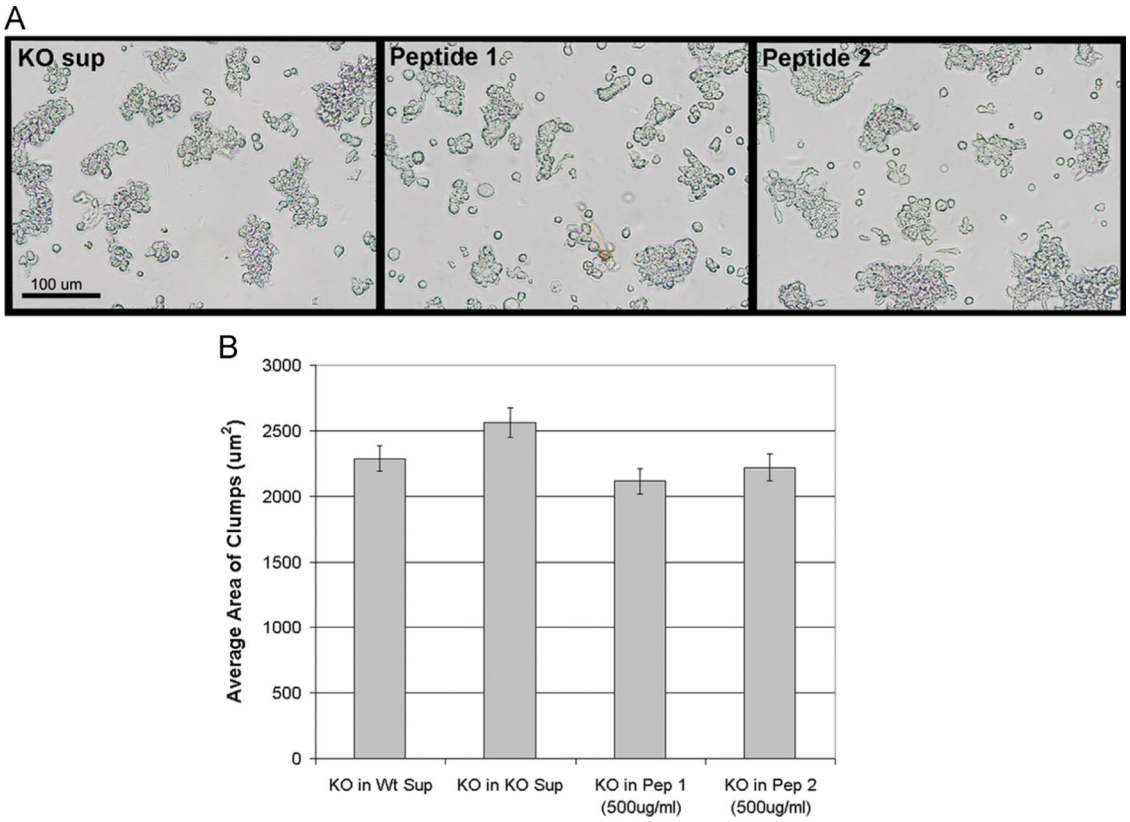

Fig. 7.

Externally added oligopeptides that block the prespore gene mis-expression phenotype of *ampA* null cells fail to block the over-adhesive *ampA* null phenotype. Wild type and *ampA* null cells were developed by starvation in slowly shaken suspension culture in either supernatant harvested from 10 h *ampA* null cells (KO sup) or 134 μM oligopeptide #1 or oligopeptide #2 described in Fig. 3B. (A) Representative micrographs of the agglutinates imaged with a 20× objective. Scale bars are 100 μm. (B) Quantification of agglutinate cross-sectional area. The cross-sectional area of cell agglutinates depicted in A was determined as in Fig. 6.

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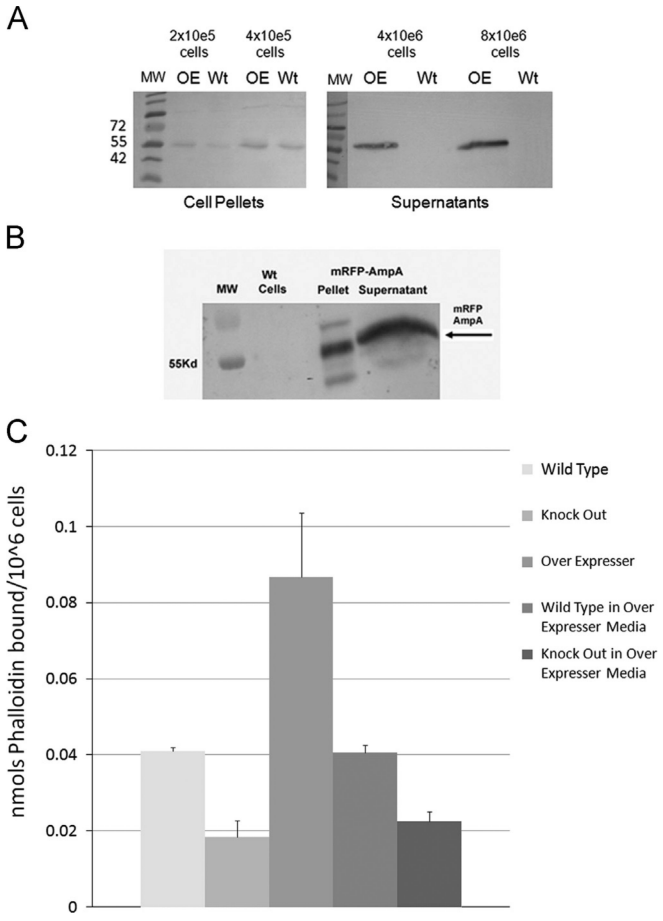

Fig. 8.

AmpA is secreted during growth only in overexpressing cells. Extracellularly it cannot induce actin polymerization. (A) Western blot of the AmpA–Tap tag fusion protein from AmpA–Tap fusion protein (Wt) and AmpA–Tap tag overexpressing cells (OE). The cells were harvested at a density of 4×10^6 cells/mL and the supernatants were concentrated on Centricon concentrators. Cell pellets and supernatants from the indicated number of cells were loaded on polyacrylamide gels. For western blots the primary antibody was rabbit anti-Tap and secondary antibody was goat anti rabbit conjugated to alkaline phosphatase. The amount of cells loaded in the cell pellet lanes were in a range where the amount of signal was linearly dependent on the amount of protein loaded on the gel. The supernatant lanes were overloaded to try to detect any secreted AmpA–Tap tag protein from the AmpA–Tap tag-Wt cells. (B) A mRFP-AmpA fusion protein that is overexpressed in growing cells also is secreted. Westerns were preformed as in A but probed with anti-RFP primary antibody. (C) The amount of F-actin polymerized per 10^6 cells was measured using phalloidin binding assays. Wt, *ampA* null and AmpA overexpres-sing cells were grown and incubated in their own media prior to harvesting and assay. Additionally, Wt and *ampA* null cells were grown overnight or incubated for 2 h in supernatant from AmpA overexpressing cells that contained secreted AmpA protein prior to harvesting and assay. Error bars indicate standard error of the mean. Assays were repeated three times on different days with different batches of cells. Wt and *ampA* null cells incubated in supernatant from AmpA overexpressing cells showed levels of F-actin that were no different than the same cells kept in their own media (*p*-values¼0.5–0.9 with a significant difference being <0.05 using a paired two-tailed Student's *t*-test).