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***Dictyostelium discoideum* Paxillin Regulates Actin-Based Processes**

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Abstract

Paxillin is a key player in integrating the actin cytoskeleton with adhesion, and thus is essential to numerous cellular processes including proliferation, differentiation and migration in animal cells. PaxB, the *Dictyostelium discoideum* orthologue of paxillin, has been shown to be important for adhesion and development, much like its mammalian counterpart. Here, we use the overproduction of PaxB to gain better insight into its role in regulating the actin cytoskeleton and adhesion. We find that PaxB overexpressing (PaxBOE) cells can aggregate and form mounds normally, but are blocked in subsequent development. This arrest can be rescued by addition of wild-type cells, indicating a non-cell autonomous role for PaxB. PaxBOE cells also have alterations in several actin-based processes, including adhesion, endocytosis, motility and chemotaxis. PaxBOE cells exhibit an EDTA-sensitive increase in cell-cell cohesion, suggesting that PaxB-mediated adhesion is Ca²⁺ or Mg²⁺ dependent. Interestingly, cells overexpressing *paxB* are less adhesive to the substratum. In addition, PaxBOE cells display decreased motility under starved conditions, decreased endocytosis, and are unable to efficiently chemotax up a folate gradient. Taken together, the data suggest that proper expression of PaxB is vital for the regulation of development and actin-dependent processes.

Keywords

actin; adhesion; chemotaxis; *Dictyostelium*; PaxB; paxillin

Introduction

In eukaryotic organisms, developmental processes are often characterized by the regulation of the actin cytoskeleton and associated cell adhesion molecules (CAMs). Actin and CAMs are essential in the physiological processes of development and differentiation, as well as in wound healing, lymphocyte migration, metastasis, and phagocytosis (Dinc et al. 2006; Gomer et al. 2002; Katsumi et al. 2004; Lin et al. 2006; Schwartz 2001; Shtutman et al. 2006). These complex events involve cell surface receptors, signaling molecules at focal adhesion sites, the actin cytoskeleton, and many associated proteins (Brown et al. 2002; Gebbie et al. 2004; Ginger et al. 1998; Liu et al. 2005). Briefly, cell surface proteins bind to the extracellular matrix and connect it to the actin cytoskeleton via a complex network of cytosolic proteins (Schwartz 2001).

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The mammalian protein paxillin, is a focal adhesion molecule which serves as an adapter and anchor protein at the plasma membrane for an array of signaling and structural proteins. It is important for the integration and processing of adhesion and signal transduction (Breen 1998; Brown et al. 1998; Salgia et al. 1995; Turner 2000; West et al. 2001). The interaction of paxillin with numerous proteins leads to changes in the organization of the actin cytoskeleton that are necessary for cell motility associated with embryonic development, wound repair, and tumor metastasis (Brown et al. 2002; Turner 2000). It is speculated that these proteins probably use paxillin both as a substrate and as a scaffold protein to disrupt and even bypass the normal adhesion and growth factor signaling cascades necessary for controlled cell proliferation.

Paxillin is an essential protein for normal development in mice (Hagel 2002) and for the metastasis of numerous types of cancer (Jackson and Young 2003; Salgia et al. 1999; Scibelli et al. 2003; Yano et al. 2000). However, its function and regulation have not been completely characterized. Being that paxillin is highly conserved between species, we studied its ortholog, PaxB, in the model organism *Dictyostelium discoideum* in order to further elucidate its role and regulation. PaxB shares 33% amino acid identity and 46% homology with mammalian paxillin (Bukharova 2005; Tumbarello et al. 2002). Many of the processes that paxillin is involved in, such as cell motility and morphogenesis, are important in the life cycle of *Dictyostelium discoideum*.

D. discoideum is a simple eukaryote with a well defined life cycle that displays many of the features of animal embryogenesis (Bowers-Morrow et al. 2002; Siu et al. 2004; Wong et al. 2002). It feeds on bacteria and grows as single amoeboid cells, but multicellular development is triggered subsequently by starvation. Through multiple stages of development, cells aggregate to form a multicellular fruiting body composed of differentiated spore and stalk cells. These stages involve regulated cell-cell and cell-substrate adhesions, motility, differentiation, and morphogenesis (Bracco 2000; Ginger et al. 1998). Similar to paxillin, PaxB has been shown to play a role in cell-substrate adhesion, cell sorting and migration during *D. discoideum* development (Bukharova 2005). These characteristics and similarities to paxillin make *D. discoideum* a perfect candidate for the study of adhesion.

Dictyostelium growth phase cells are barely cohesive, forming transient contacts or loose aggregates, which are easily dissociated with 10 mM EDTA or EGTA (Secko et al. 2006; Yuan et al. 2001). Aggregating cells differ from growth-phase cells by their stronger cohesion, which leads to formation of large EDTA-resistant aggregates in shaken suspension (Kamboj et al. 1989; Siu et al. 2004). The molecular basis of the post-aggregative adhesion system is less well understood. The tipped mound or slug shows a much more compact, tissue-like structure than aggregates from the aggregation stage. In addition, prestalk and prespore cell populations within the slug display different degrees of adhesiveness (Bowers-Morrow et al. 2002). It is not known whether the differences between prestalk and prespore cells result from the expression of new cell type-specific adhesion molecules or from quantitative differences in adhesion molecules common to both cell types.

As a model organism *D. discoideum* is ideal for the study of adhesion and its role in differentiation and morphogenesis. Here, we show that overexpression of *paxB* blocks development past the mound stage and prevents differentiation of cells into spores. In addition, we present evidence that overexpression of *paxB* induces the misregulation of several actin-based processes. Specifically, it increases cell-cell cohesion, but decreases cell-substrate adhesion. Also, it decreases endocytosis, motility in starved cells and efficient chemotaxis towards folate.

Results

Overexpression of *paxB* Using the Tet-off System

To better understand the function of *paxB* we created an overexpressor system. Given that paxillin has been shown to be important to numerous cellular functions, we decided to use the Tet-off, inducible system (Blaauw et al. 2000) in case constitutive *paxB* expression was lethal. To confirm that the Tet-off system works for the overexpression of *paxB*, we measured its transcript levels in wild-type cells and *paxB* overexpressing cells (PaxBOE). There is no significant difference in *paxB* transcript levels in wild-type cells in the presence or absence of Tet (Fig. 1), demonstrating that addition of Tet does not affect transcription of the endogenous *paxB* gene. In the presence of Tet, PaxBOE cells show slightly higher *paxB* transcript levels than the wild-type cells. However, when induced by the removal of Tet, this expression increases approximately 5 fold. This increase is not due to an increase in overall transcription as the transcription of the control gene, IG7, is unaffected by Tet (Fig. 1). To equate the increased transcript level with increased production of the protein, we performed western blots using an α -PaxB peptide purified antibody. In the wild-type cells, similar production of PaxB is observed in the absence and presence of Tet (Fig. 1). Consistent with the northern blots, PaxBOE cells in the presence of Tet show slightly higher protein production than wild-type cells. However, in the absence of Tet this expression is 6 fold higher than in the wild-type cells (Fig. 1). Thus the increased transcript level of PaxB correlates with an increased production of the protein. This confirms that we successfully created a tetracycline-regulated system for the overproduction of PaxB protein.

Overexpression of the *paxB* Gene Arrests Development at the Mound Stage

The integration of single-celled amoebae into a multicellular organism is a major part of the developmental program of *D. discoideum*. Thus, a protein capable of modulating cell-cell adhesion is a candidate for influencing developmental progression and morphogenesis. Therefore, we examined the effect of overexpressing *paxB* on development. Vegetative wild-type cells and PaxBOE cells were plated on filter pads and agar, and developmental progression was observed. The initial stages of development were normal in the PaxBOE cells in that, like wild-type cells, they reach the mound stage by 12 hours (data not shown). However, there is a drastic difference after this point. Wild-type cells form fruiting bodies by 24 hours (Fig. 2A). In contrast, PaxBOE cells remained arrested at the mound stage (Fig. 2B). This arrested phenotype is able to be rescued by addition of 20% wild-type cells (Fig. 2C), suggesting that the defect may be non-cell autonomous. Chimeras consisting of only 5% wild-type cells remained arrested (data not shown), indicating that the simple secretion of some factor is not sufficient to allow development to proceed. This suggests normal production of PaxB is required for development past the mound stage and that wild-type cells can provide something which will allow PaxBOE cells to progress to full development.

paxB Overexpressing Cells Preferentially Differentiate into Stalk Cells in Chimeric Structures

Given that wild-type cells were able to rescue the developmental defect in PaxBOE cells, we wanted to know whether the wild-type cells were relegated to a specific cell fate. Therefore, we investigated the localization of the wild-type, β -galactosidase expressing cells in chimeric fruiting bodies. Chimeras composed completely of wild-type cells showed random distribution of the wild-type, β -galactosidase expressing cells in fruiting body (Fig. 3A), demonstrating that expression of β -galactosidase had no effect on cell fate. In chimeric fruiting bodies containing the PaxBOE cells, the wild-type, β -galactosidase expressing cells were predominantly localized to the upper and middle section of the spore mass (Fig. 3B). Therefore, the overexpression of the *paxB* gene appears to interfere with cell sorting or differentiation during the development of *D. discoideum*. Specifically, PaxBOE cells preferentially sort away from the spore mass.

After observing that PaxBOE cells predominantly sorted out from the spore mass of the fruiting body, we investigated whether PaxBOE cells were deficient in spore formation. To test this, we created chimeras of wild-type β -galactosidase expressing cells with wild-type cells or PaxBOE cells. We then monitored the number of blue and unstained spores. In wild-type chimeras, approximately 40 percent of the spores came from non- β -galactosidase expressing wild-type cells (Fig. 4). However, in PaxBOE chimeras, the percentage of spores coming from PaxBOE cells was decreased to approximately 23 percent. This is in accordance with their exclusion from the spore mass of the chimeric fruiting body. This suggests that *paxB* plays a role in cell-type differentiation. Specifically, overexpression of *paxB* interferes with the ability of cells to differentiate into spores.

Given that PaxB appeared to be involved in spore differentiation, we wanted to test whether PaxBOE cells could produce viable spores. Wild-type and PaxBOE cells were developed for 36 hours. The cells were then heated in a detergent solution to kill non-spores, and the surviving spores were plated on bacteria and allowed to form plaques. Table 1 illustrates that while wild-type and PaxBOE cells produce equivalent number of viable cells, more than 10^6 viable spores germinated from the wild-type cells, versus only 10^2 viable spores from PaxBOE cells. This suggests that the *paxB* gene plays a role in the development of viable spores.

***paxB* Overexpression Increases Cell-Cell Cohesion but Decrease Cell-Substrate Adhesion**

Since paxillin is involved in adhesion, we examined whether overexpression of *paxB* affects cell-cell cohesion. It has been shown that cell agglomerates form when cells are washed free of growth medium, resuspended in non-nutrient buffer, and allowed to develop in suspension (Secko et al. 2006). We found that wild-type cells formed a few small agglomerates but, for the most part, remained as single cells after three hours of starvation (Fig. 5A). In contrast, PaxBOE cells formed huge agglomerates (approximately 0.4mm diameter), with very few cells remaining as individuals (Fig. 5B). This suggests that overexpression of *paxB* increases the cohesion of cells.

To quantify the increased cell-cell cohesion, cells were allowed to adhere to each other under gentle agitation. The percentage of cells found in adhering complexes was determined at specific time points. We found that approximately 5 percent of wild-type cells adhered to each other within five minutes (Fig. 6). This increased to 45 percent by 60 minutes. However, PaxBOE cells agglomerated faster and to a greater extent than wild-type with approximately 55 percent adhered to each other by 5 minutes, and close to 85 percent by 60 minutes (Fig. 6). This suggests that *paxB* plays a positive role in cell-cell cohesion.

Knowing that *D. discoideum* cells display both Ca^{2+} dependent and independent adhesion, we wanted to determine whether PaxB-mediated cell-cell cohesion is Ca^{2+} or Mg^{2+} dependent. Therefore, we examined cohesion as described above in the presence of 10 mM EDTA. As expected, in wild-type cells there was decreased cell-cell cohesion in the presence of 10 mM EDTA (Fig. 6). Interestingly, the PaxBOE cells also exhibited decreased cell-cell cohesion in the presence of EDTA. It is important to note that the increase in cell-cell cohesion caused by overexpressing *paxB* is completely negated by addition of EDTA. This suggests that the cohesion of cells mediated by PaxB is Ca^{2+} or Mg^{2+} dependent.

PaxB⁻ cells have been shown to be less adhesive to various substrates, when exposed to moderate conditions of shear stress (Bukharova 2005). We therefore examined whether overexpressing *paxB* had an opposite effect. To test the adhesion of cells to the substratum, we allowed wild-type and PaxBOE cells to adhere to a glass surface and then monitored the percentage of adhering cells after exposure to shear stress. The wild-type cells had approximately 40 percent of the cells remaining adhered to the underlying surface (Fig. 7). Surprisingly, when *paxB* was overexpressed, adhesion to the underlying substrate was reduced

to approximately 20 percent (Fig. 7). This suggests that overexpression of *paxB* disrupts cell-substrate adhesion.

Overexpression of *paxB* Decreases Random Motility in Starved Cells and Efficient Chemotaxis to Folate in Vegetative Cells

Regulated assembly and disassembly of actin-based adhesion complexes is required for cell motility. Since overexpression of *paxB* affects adhesion, we wanted to investigate the effects of overexpressing *paxB* on motility. Vegetative and starved wild-type and PaxBOE cells were seeded at low cell density on plastic dishes. The cells were allowed to adhere for one hour, and then the average speed of the cells was measured using time lapse microscopy. We found that both vegetative wild-type and PaxBOE cells have an average speed of approximately 5 μm per minute (Fig. 8), which is consistent with previous studies (Song 2006). However, when the cells were starved, there is a statistically significant 35% decrease in the motility of PaxBOE cells compared to wild-type cells (Fig. 8). This suggests that the overexpression of *paxB* does not affect the motility rate of the cell under vegetative conditions, but has an effect on cells under starved conditions.

Since overexpressing *paxB* decreased random motility in starved cells, we decided to study its effect on directed motility. It has been well established that vegetative cells chemotax towards pteridines such as folate (Pan et al. 1975). We therefore examined how overexpression of *paxB* affected chemotaxis towards folate using an under agarose assay. We found that overexpression of *paxB* had little to no effect on the speed at which cells moved when placed in a folate gradient (Fig. 9A). However, the directionality of cells overexpressing *paxB* was reduced by half, meaning they wandered more while moving up the folate gradient (Fig. 9B). This defect is evident when traces of chemotaxing cells are examined (Fig. 9C). Thus, while cells overexpressing *paxB* have no trouble moving in a folate gradient, they have a difficult time chemotaxing up the gradient.

Overexpression of *paxB* Causes Decreased Endocytosis

Polymerization of actin is required for endocytosis. To examine whether PaxB is involved in this process, we looked at endocytosis and exocytosis by examining the uptake and release of FITC-dextran in wild-type and PaxBOE cells. We found that cells overexpressing *paxB* had a decreased rate of endocytosis, being about 2.5 times less than that of wild-type cells (Fig. 10A). Interestingly, while the overall amount of fluid exocytosed in cells overexpressing *paxB* was less than that in wild-type cells, the rate of exocytosis was approximately the same as in wild-type cells (Fig. 10B). Taken together, these results suggest that overexpression of *paxB* disrupts proper endocytosis without greatly affecting the rate of exocytosis.

Discussion

PaxB is required for development, as *paxB*⁻ cells arrest at the mound stage (Bukharova et al. 2005). It was suggested that the developmental arrest could be due to adhesion defects, as PaxB may play a role in the movement of prestalk cells, which form the organizing tip of the mound (Bukharova et al. 2005; Vasiev and Weijer 1999). This is similar to the developmental arrest seen in paxillin knockout mice, which is also due to defective cell migration (Hagel et al. 2002). In agreement with this, we found that overexpression of the *paxB* gene arrests development at the mound stage, indicating that normal expression of *paxB* is required for development past the mound stage. This problem is resolved in chimeric mounds. We found that the tips of the chimeric mounds are composed of wild-type cells (data not shown). This suggests that the reason why chimeras can form fruiting bodies is because unlike PaxBOE cells, the wild-type cells are able to move to the tip of the mound and possibly create the signals for further development, thus explaining the non-cell autonomous defect in the PaxBOE cells.

Therefore, while PaxBOE cells cannot move to the tip and signal further differentiation, they can still respond to those signals.

The chimera experiments also lend insight into the role of PaxB in the sorting of cells in the fruiting body. In chimeras with wild-type cells, PaxBOE cells predominantly sorted to the cups of the spore mass and the stalk, structures derived from prestalk cells. Given that PaxB is more strongly produced in prestalk cells, it is not surprising that overproduction of PaxB appears to push cells along the prestalk pathway. Prestalk and prespore cell-types differentiate in a spatially independent manner throughout the mound (Fosnaugh 1991; Williams et al. 1989; Williams 2006), but completely sort out by the time the tipped mound is formed. Thus, cell movement must occur for cells to sort. Since cell movement depends on regulated cell-cell and cell-substrate adhesion, it is not surprising that PaxB influences differentiation as it is involved in these two processes. The overexpressing cells cannot sort out correctly, therefore, they cannot differentiate properly. In this light, the defect in spore viability exhibited by PaxBOE cells is most likely due to the cells not differentiating into spores, rather than *paxB* playing a direct role in spore viability.

It is clear that actin-based adhesion is an integral part of *Dictyostelium discoideum* development. It is important for initial stream formation, creation of the mound, morphogenesis, and cell type differentiation (Siu et al. 2004). Aggregating cells differ from growth-phase cells by their increased cohesion (Secko et al. 2006). The overexpression of *paxB* increases cell-cell cohesion, indicating that *paxB* plays a positive role in cell-cell cohesion. This interpretation is supported by previous findings that PaxB is localized to sites of cell-cell contact (Bukharova et al. 2005). Our finding that addition of EDTA completely negates the effects of overexpressing *paxB* suggests that PaxB-mediated cell-cell cohesion is Ca^{2+} or Mg^{2+} dependent. One putative PaxB interacting protein is the *D. discoideum* homologue of mammalian cadherin, DdCAD-1. DdCAD-1 is a Ca^{2+} dependent adhesion molecule, and like PaxB, is enriched in regions of cell-cell contact, is involved in cell-cell cohesion and influences cell sorting (Desbarats et al. 1994; Lin et al. 2006; Secko et al. 2006; Sesaki and Siu 1996; Wong et al. 2002; Yang 1997). To date, a direct interaction between paxillin and cadherins has not been examined in any organism. However, a role for paxillin in cell-cell cohesion has been suggested, as paxillin interacts with proteins involved in cell-cell adherens junctions, such as β -catenin (Birukova et al. 2007; Palovuori and Eskelinen 2000). Given that β -catenin-containing adherens junctions exist in *Dictyostelium* (Grimson et al. 2000), and that DdCAD-1 has been extensively studied, we are poised to examine this possible undiscovered interaction. Simple immunoprecipitation experiments can delineate the potential interaction of PaxB with DdCAD-1, advancing our understanding of their functions in cell-cell cohesion.

In addition to adhesion, the actin cytoskeleton regulates endocytosis, motility, and chemotaxis (Hall et al. 1988; Hecter et al. 1997). Consistent with PaxB regulating actin, we found that cells overexpressing *paxB* have decreased endocytosis. Additionally, we saw that overexpressing *paxB* decreases motility in starved cells and decreases the efficiency of chemotaxis in vegetative cells. This is not to say that PaxB is directly involved in all of these processes. The more likely explanation is that PaxB regulates cell-cell cohesion through the regulation of actin. Thus, when *paxB* is overexpressed, there is an increase in cell-cell cohesion, most likely due to increased F-actin associated with cell-cell cohesion. By necessity, this limits the F-actin available for other processes. Hence, the defects we observed in cell-substrate adhesion, motility, chemotaxis and pinocytosis. This is not surprising as there is considerable evidence that these processes are in competition with each other. It is well established that leading edges compete with each other (Segall and Gerisch 1989). Maniak et al. (1995) provided data suggesting that there is also competition between pseudopod extensions and phagocytic cup formation, stemming from competition for shared proteins, such as the actin associated protein

coronin. In addition, cells lacking *rasS*, have increased motility, and consequently decreased endocytosis (Chubb et al. 2000). Thus, it appears that the cell must actively distribute its ability to polymerize actin to several different processes. Perhaps the defects observed in the PaxBOE cells are due to the inappropriate recruitment of F-actin for the process of cell-cell cohesion, to the detriment of other actin-based processes

This work leads to several conclusions of particular interest to the *D. discoideum* and paxillin fields. An intracellular signaling network links local cellular adhesion events to the more general regulation of cellular physiology. To date, more than 50 mammalian proteins have been reported to be associated with extracellular matrix adhesion (Gebbie et al. 2004). Paxillin interacts with a number of these proteins (Brown and Turner 2004). Investigating how and when these proteins associate is essential to comprehending how the cell effectively utilizes paxillin's adapter function to properly regulate adhesion and other actin-based processes. However, given the number of proteins involved, such an investigation is daunting. Thus, a simpler model organism is required to delineate how signal transduction involving paxillin occurs, is pivotal to understanding such a complex system.

Given that we have established that PaxB parallels many of the functions of paxillin, *D. discoideum* may be able to simplify the study of such a tangled process by providing a simpler system with fewer interacting proteins. Finding the proteins that interact with PaxB will give insight into how these signaling networks are regulated, and ultimately how PaxB is involved in the normal function and physiology of the cell. With *D. discoideum*'s genetic tractability and simple differentiation and development, we are poised to gain a greater understanding of the regulation of paxillin and its role in adhesion, actin assembly and cellular physiology.

Methods

Cell culture and transformation

Dictyostelium discoideum wild-type (Ax2) cells were grown in axenic HL5 medium (0.5% (w,v) yeast extract, 0.5% proteose peptone, 0.5% thiotone peptone, 1% dextrose, 4.7 mM Na₂HPO₄, 2.5 mM KH₂PO₄, pH 6.5) on a 180 rpm shaking platform at 22 °C (Sussman 1987). HR30 (Ax2 expressing β-galactosidase) with 10 µg/ml blasticidin. Tet-7 (Ax2 containing the endogenous MB35 vector) cells were grown in HL5 supplemented with 20 µg/ml G418. PaxBOE (*paxB* overexpressing) cells were grown in HL5 supplemented with 10 µg/ml blasticidin and 20 µg/ml G418. For development, cells at mid-log phase ($2-5 \times 10^6$ cells/ml) were washed with PBM (20 mM KH₂PO₄, 10 µM CaCl₂, 1 mM MgCl₂, pH 6.1 with KOH), plated on filter pads at 1×10^7 cells/pad and incubated at 22 °C following the procedure of Bishop et al. (2002).

The MB38 vector containing the *paxB* gene was transformed into Tet-7 cells by electroporation as described previously (Heikoop 1998; Howard et al. 1988). Transformed cells were selected on GYP plates containing 20 µg/ml G418 and 10 µg/ml blasticidin in association with a G418 resistant strain of *Escherichia coli*. Clones were grown on HL5 medium containing 20 µg/ml G418 and 10 µg/ml blasticidin and analyzed for expression of the desired product.

Construction of PaxBOE plasmid

The PCR amplification of *paxB* was prepared from genomic DNA using a proof-reading polymerase (Roche Expand High Fidelity PCR System, Indianapolis, IN) following manufacture's protocol. The 5' PCR primer was (5' GCGCATGCATGGCAACAAAAGGATTAAATATG), which introduced an SphI site. The 3' primer was (3' GCGACGTCTTAAGCGAATAATTTATTATGACAA), which introduced an AatII site. The PCR product was digested, gel purified with UltraClean DNA Purification

Kit (MO BIO Bedford, MA) and ligated into the MB38 vector, which had been similarly digested. The MB38-*paxB* construct was transformed into Tet-7 cells and positive transformants confirmed by Northern and Western blot analysis.

Northern Blot and Western Blot analyses

For Northern blots, RNA was prepared using Trizol Reagent (GIBCO BRL, Carlsbad, CA), according to manufacturer's protocol. Samples containing 10 µg of RNA were separated on one percent agarose gel (0.6% formaldehyde, 10mM MOPS, pH 7.5), and blotted to Hybond-N+ (Amersham Bioscience, Piscataway, NJ). A probe specific for the endogenous *paxB* gene was labeled with ³²P-dATP according to manufacturer's protocol (Invitrogen, Carlsbad, CA), and hybridization was performed as described by Engler-Blum et al. (1993).

The synthetic peptide GCVDALKDKKWHEPEHFV from amino acids 524 to 538 of PaxB was used to immunize a rabbit at Biosynthesis, Inc. (Lewisville TX). Serum was collected 8 weeks after the second injection and purified using the above peptide attached to agarose beads. Cells were collected and boiled for 3 minutes in SDS-PAGE sample buffer. Proteins were separated on 10% gel by SDS-PAGE, and electrophoretically transferred to Hybond-P membranes (Amersham Bioscience, Piscataway, NJ), immunoblotted with a peptide purified α-PaxB antibody, and visualized by enhanced chemiluminescent substrate for HRP detection (PIERCE, Rockford, IL)

Chimeras and β-galactosidase staining

Chimeras were created and stained as previously described by Jermyn and Williams (Jermyn and Williams 1991) with some adjustments. 1.0×10^7 cells were collected from HR30 strain and PaxBOE strain. Chimeras consisted of 20 percent HR30 cells and 80 percent PaxBOE cells. For β-galactosidase staining, cells were developed on white filter pads, 0.8 µm pore size (Millipore, Billerica, MA). Developed chimeras were stained for early culminant and fruiting body structures. Cells were fixed with glutaraldehyde solution (25% glutaraldehyde, 4% Triton X-100 in Z Buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄)), followed by staining with X-gal solution (5 mM K₃[Fe(CN)₆], 0.4 mg/ml X-gal, and 0.5% Tween 20 in Z Buffer), and incubated at 37 °C overnight. Images were taken with a dissecting microscope utilizing SPOT Advanced program with SPOT insight color camera, (Diagnostics Instruments, USA).

Cell-cell cohesion assay

The cell-cell cohesion assay was done as previously described by Secko et al. (2006) with slight modification. Exponentially grown cells were harvested and washed three times with PBM buffer (20 mM KH₂PO₄, 10 µM CaCl₂, 1 mM MgCl₂, a key player with KOH) and resuspended to 2×10^7 cells/ml. Two milliliters were transferred into a plastic 50 ml centrifuge tube (Corning 430828), and shaken horizontally (175 rpm) at 22 °C for four hours. 2.5×10^6 cells were collected, vigorously vortexed for 15 seconds to disperse aggregates, and placed on a platform shaker (160 rpm) at room temperature to allow aggregates to reform. Cells were collected at specific times and single and duplex cells were counted using a hemacytometer. To visualize aggregation, approximately 2.5×10^4 cells were incubated in PBM in tissue culture dishes under gentle agitation (60 rpm) for three hours. Cell agglomerates were viewed using a Nikon Eclipse TS 100 inverted microscope and photographed with a Nikon E 995 camera.

Cell-substrate adhesion assay

The cell-substrate adhesion assay was done as previously described by Chen and Katz (2000) with slight modification. Exponentially grown cells were harvested and washed three times with PBM buffer and resuspended to 1.0×10^6 cells/ml in the same buffer. Four milliliters

of this suspension was incubated in 50 ml glass cell culture flasks on a gyratory shaker at 120 rpm for 10 minutes at room temperature. The cells were incubated 2 hours further without shaking to allow them to adhere. The flasks were then agitated gently for 3 minutes at 60 rpm and the supernatants were transferred to a test tube. Non-adherent cells in each supernatant were counted using a hemacytometer. The paired t-test was used to determine statistical significance.

Spore ratio and spore viability assays

The spore ratio was assayed with chimeras consisting of 20 percent HR30 cells and 80 percent PaxBOE cells or Ax2 cells as previously described, (Bishop et al. 2002) with some adjustments. Chimeras were allowed to develop for 36 hours, followed by collection of spore mass using one milliliter PBM buffer. Spores were fixed and stained with X-Gal as described earlier, vigorously vortexed, and incubated at 37 °C for 24 hours. Spores were washed three times using Z Buffer and the number of blue colored spores (HR30) and unstained spores (PaxBOE or Ax2) were counted using a hemacytometer. The paired t-test was used to determine statistical significance. The spore viability was assayed as previously described by Dynes et al. (1994) and Bishop et al. (2002). The cells were plated on filter pads at 1.0×10^7 cells/ml and allowed to develop for 36 hours. The spores were collected with one milliliter PBM buffer and detergent (10 mM EDTA, 0.1 % Nonidet P-40), and serially diluted directly into *Klebsiella aerogenes* suspension and plated on SM/5 agar plates. The number of plaques formed was counted and scored as viable spores.

Cell motility assay

Cell motility was assayed as previously described by Lim et al. (2005) with some adjustments. Vegetative and starved cells were seeded at low density, ($\sim 2 \times 10^4$ cells/cm²), on plastic dishes in HL5 media or PBM buffer, and allowed to adhere for one hour. A time lapse movie was compiled by capturing an image of the cells every minute for 30 minutes using an inverted Nikon TE 200 Eclipse microscope using a Metafluor Image System viewed through a 40X objective. The individual amoeba tracks were traced and the distance traveled for 30 minutes was measured using the Image J software. The paired t-test was used to determine statistical significance.

Chemotaxis assay

Under agarose chemotaxis assays were performed as described in Woznica and Knecht (2006). Briefly vegetative cells were grown to log phase, collected and resuspended at 1×10^6 cells/ml. A 0.1 ml sample of the cells was placed in a trough 5 mm away from a trough with a 0.1 mM solution of folate. Cells were imaged and tracked as they moved up the folate gradient, under the agarose using Image J software as described above. Directionality is defined as the ration of (the absolute distance travel)/(the total path length). Thus, a value of 1 represents a straight path with no deviations, and decreasing values represent less efficient chemotaxis. The paired t-test was used to determine statistical significance.

Endocytosis and exocytosis assays

Endocytosis and exocytosis assays were performed as described in Brazill et al. (2001)

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Figure 1. Overexpression of the *paxB* gene and protein overproduction

Northern blot and Western blot analysis of PaxB in wild-type and PaxBOE overexpressing cells. Total mRNA, extracted from vegetative cells in the absence and presence of tetracycline was probed with a 900 bp fragment of the endogenous *paxB* gene, and with *IG-7* gene as a control. Western blot analysis of the PaxB protein extracted from vegetative cells, and resolved by SDS-PAGE was probed with a peptide purified antibody for α -PaxB.

Figure 2. Development of wild-type and PaxBOE cells

Logarithmically growing cells were washed free of growth medium and plated for development on non-nutrient agar for 24 hours. (A) Wild-type cells. (B) PaxBOE cells. (C) Chimeras containing 20% wild-type and 80% PaxBOE cells. Bars indicate 0.5 mm.

Figure 3. Localization of wild-type and PaxBOE cells in chimeras

Wild-type cells carrying the *actin15::lacZ* reporter construct (HR30) were mixed in a 20:80 ratio with (A) wild-type cells or (B) PaxBOE cells and developed on filters. Fruiting bodies were stained at 24 hours of development. Bars indicate 0.5 mm

Figure 4. Spore ratio analysis of wild-type and PaxBOE cells in chimeras

Wild-type cells carrying the *actin15::lacZ* reporter construct mixed in a 50:50 ratio with wild-type cells or PaxBOE cells were developed for 36 hours on filters. Spores were collected and counted as described in materials and method. The percentage of spores developing from wild-type and PaxBOE cells was calculated and plotted. Values represent the mean of four independent experiments $\pm$ SEM, $p < 0.005$.

Figure 5. Cell-cell cohesion is increased in PaxBOE cells

Representative photographs of (A) wild-type and (B) PaxBOE overexpressing cell agglutinants that formed after 3 hours of starvation in suspension. Bars indicate 0.4 mm. Photographs are from one experiment with similar results from three independent experiments.

Figure 6. PaxB mediated cell-cell cohesion is calcium dependent

Wild-type and PaxBOE cells were starved for four hours, separated by vortexing, and allowed to readhere in the absence or presence of 10 mM EDTA. Values represent the mean of three independent experiments $\pm$ SEM.

Figure 7. Adhesion of wild-type and PaxBOE cells to the underlying substrate

Adhesion of vegetative wild-type and PaxBOE overexpressing cells was measured under mild agitation. Values represent the mean of three independent experiments $\pm$ SEM, $p < 0.005$.

Figure 8. Average speed of wild-type and PaxBOE cells

The average speed of vegetative and starved cells was measured in $\mu\text{m}/\text{min}$. Values represent the mean of three independent experiments $\pm$ SEM. For starved cells, $p < 0.05$.

Figure 9. Chemotaxis to folate

Vegetative wild-type and PaxBOE cells were subjected to the under agarose chemotaxis assay and (A) the speed and (B) the directionality of the cells were measured. Values represent the average of at least 4 different experiments with at least 20 different cells monitored per experiment. Error bars represent the SEM. For directionality, $p < 0.005$. (C) A sample tracking of cells from one set of experiments, with cells moving from right to left. The black triangle represents increasing concentration of folate.

Figure 10. Endocytosis and exocytosis

(**A**) Wild-type and PaxBOE cells were collected and resuspended in HL5 containing FITC-dextran. At the times indicated, cells were removed, washed and lysed. The fluorescence of the lysate was then measured. (**B**) Wild-type and PaxBOE cells were allowed to internalize FITC dextran for 3 hours. They were then washed and resuspended in fresh HL5. At the times indicated, cells were removed, washed and lysed. The fluorescence of the lysate was then measured. Values represent the mean of three independent experiments $\pm$ SEM

Table 1**Spore viability of wild-type and PaxBOE cells**

1×10^7 wild-type and PaxBOE cells were starved on filters and allowed to develop for 36 hours. The developed structures were collected and assayed for cell and spore viability. Data shown are from one experiment with similar results obtained from three other experiments.

Cell line	Viable cells ($\times 10^4$)	Viable spores ($\times 10^4$)
Wild-type	775	349
PaxBOE	509	0.042