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Bukharova, T.; Weijer, G.; Bosgraaf, L.; Dormann, D.; van Haastert, Petrus; Weijer, C.J.; Bukahrova, T

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Paxillin is required for cell-substrate adhesion, cell sorting and slug migration during *Dictyostelium* development

Tanya Bukahrova[1], Gertrud Weijer[1], Leonard Bosgraaf[2], Dirk Dormann[1], Peter J. van Haastert[2] and Cornelis J. Weijer[1,*]

[1]Wellcome Trust Biocentre, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, UK
[2]Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
*Author for correspondence (e-mail: c.j.weijer@dundee.ac.uk)

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Summary

Paxillin is a key regulatory component of focal adhesion sites, implicated in controlling cell-substrate interactions and cell movement. We analyse the function of a *Dictyostelium discoideum* paxillin homologue, PaxB, which contains four highly conserved LD and four LIM domains, but lacks two characteristic tyrosine residues, that form the core of vertebrate SH2-binding domains. PaxB is expressed during growth and all stages of development, but expression peaks during slug formation. Using a *paxB-gfp* knockin strain we show the existence of focal adhesions and characterise their dynamics. During multicellular development PaxB is not only found in focal adhesions at the cell-substrate interface, but also in the tips of filopodial structures predominantly located at the trailing ends of cells. *paxB*$^{-}$ strains are less adhesive to the substrate, they can aggregate but multicellular development from the mound stage onwards is severely impeded. *paxB*$^{-}$ strains are defective in proper cell type proportioning, cell sorting, slug migration and form-defective fruiting bodies. Mutation of a conserved JNK phosphorylation site, implicated in the control of cell migration, does not have any major effects on cell sorting, slug migration or morphogenesis in *Dictyostelium*. PaxB does not appear to function redundantly with its closest relative Lim2 (*paxA*), which when deleted also results in a mound arrest phenotype. However, analysis of *paxA*$^{-}$ and *paxB*$^{-}$ single and double null mutants suggest that PaxB may act upstream of Lim2.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/18/4295/DC1

Key words: Paxillin, Cell motility, Chemotaxis, Sorting, Morphogenesis, Signalling

Introduction

During cell movement cells have to gain traction from the substrate. This involves cycles of making and breaking cell contacts with the extracellular matrix, while cells moving in tissues may also have to make and break contacts with other cells. In higher organisms the contacts with the extracellular matrix are regulated by several different adhesion mechanism, but interactions mediated by integrins, heterodimeric receptor proteins are very important (Hynes, 2002). Integrins consisting of an α and β transmembrane chain interact through their extracellular domain with extracellular matrix molecules, especially fibronectin, laminin and vitronectin. Binding of integrins to the extracellular matrix results in clustering and assembly of intracellular focal adhesion complexes, structures linking the extracellular receptors to the actin cytoskeleton. Integrins may also modulate cell-cell contacts involving homophilic interactions between molecules of the cadherin superfamily (Yano et al., 2004). It has been shown that moving cells can respond to the mechanical properties of their environment and that focal adhesion contacts may thus be involved in sensing the rigidity of the environment (Giannone et al., 2004; Lo et al., 2000). Focal adhesion plaques are assemblies of large molecular complexes linking adhesion molecules such as the integrins to the actin cytoskeleton via linker molecules such as talin, vinculin, α-actinin and paxillin. Paxillin is the major phosphotyrosine protein in focal adhesions (Turner, 1991) and appears to be an essential regulatory component of the molecular clutch that couples integrin molecules to the actin cytoskeleton (Giannone et al., 2004; Smilenov et al., 1999). Paxillin does not contain any known enzymatic activity, but is proposed to function as an adapter molecule that interacts with linker molecules, such as Vinculin and Talin, as well as many other regulatory proteins. Paxillin contains several important interaction domains. It contains five leucine-rich LD motifs and four double zinc-finger LIM domains (Brown and Turner, 2004; Turner, 2000). In mammalian cells there are several paxillin proteins, at least four splice variants (α, β, γ, δ) of the paxillin gene. The β and γ splice variants contain insertions between LD domains 4 and 5, and paxillin δ contains an alternative start site just before LD domain 2. There are two members of the hydrogen peroxide inducible clone5 (Hic5) protein, which contains four

LD and four LIM domains. There also exists a leukocyte-specific paxillin variant leupaxin that also contains four LD and four LIM domains. The LD domains 1, 2 and 4 have all been shown to interact with a number of proteins. Notably LD1 interacts with vinculin, actopaxin and the integrin-linked kinase (ILK) and the papilloma virus E6 protein. LD2 binds vinculin and focal adhesion kinase (FAK), while LD4 binds actopaxin, FAK, the Arf Gap's p95PKL/Git2/Git1 and pak3 and possibly clathrin. The Lim domains 2 and 3 are important for the targeting of paxillin to the focal adhesion sites and may mediate interactions with tubulin, while LIM domains 3 and 4 may mediate interactions with the protein tyrosine phosphates PTP-PEST (reviewed in Brown and Turner, 2004).

These interactions between the integrins and intracellular components of the focal adhesion complex are dynamically regulated through especially focal adhesion kinase and members of the Src family of tyrosine kinases (Schlaepfer and Hunter, 1998). Paxillin is heavily phosphorylated on tyrosine residues at positions 31 and 118 presumably through focal adhesion kinase and Scr kinases. These conserved tyrosine residues Y31 and Y118 form the core of SH2-binding motives. Y31 seems to be phosphorylated mainly in response to growth factor signalling, while Y118 is tyrosine phosphorylated in response to adhesion to the extracellular matrix (Schaller and Schaefer, 2001). However, paxillin is also heavily tyrosine phosphorylated after activation of serpentine receptors with bioactive lipids such as LPA, or with mitogenic neuropeptides such as bombesin as shown in Swiss3T3 and HEK293 cells (Needham and Rozengurt, 1998; Zachary et al., 1993). It is becoming increasingly evident that also serine-threonine protein kinases are involved in the phoshorylation of adaptor molecules of the paxillin family. Recently it has been shown to be the target of JNK kinase signalling pathways and it has been claimed that this interaction is critical for migration (Huang et al., 2004; Huang et al., 2003).

In mice paxillin is first expressed in extra-embryonic mesoderm, but after the formation of the primitive streak expression is found especially in mesodermal tissues. The knockout of paxillin in mice has shown it to be essential for the development of mesodermal structures, such as heart and somites and homozygous knockout mouse die at E9.5 (Hagel et al., 2002). Fibroblasts derived from these mice show abnormal focal adhesions, reduced cell migration and defects in the localisation of focal adhesion kinase and reduced activation of p42/44 MAPK. Knockout experiments have shown that Erk activation requires paxillin function in embryonic stem cells derived from mouse paxillin knockouts and that paxillin null cells show reduced cell spreading on fibronectin and reduced phosphorylation of focal adhesion kinase (Liu et al., 2002; Wade et al., 2002). These experiments show that paxillin plays an important role in the coordination of the behaviour of cell movement and cell matrix interaction during development and adult life.

A Paxillin-like protein has been identified in yeast where it seems to play a role in regulation of cell polarity (Gao et al., 2004) suggesting that this may be an important evolutionary conserved function. Cell polarity during cell movement has been studied extensively during chemotactic movement occurring during the development of the social amoebae *Dictyostelium discoideum* (Devreotes and Janetopoulos, 2003; Iijima et al., 2002). *Dictyostelium* development results from movement of differentiating cells. In their natural habitat *Dictyostelium* cells live as individual amoebae in the soil that feed on bacteria and divide by binary fission. However, under starvation conditions the cells enter a multicellular developmental cycle, in which up to a few hundred thousand cells aggregate to form a fruiting body, consisting of a stalk supporting a mass of spores. During the early stages of development the cells move around on almost any substrate, but during the mound stages of development the cells also start to secrete a complex extracellular matrix and motility becomes strongly dependent on this extra cellar matrix (Morrison et al., 1994; Ti et al., 1995). During late aggregation and mound formation novel adhesion molecules of the lagC family start to be expressed and their expression has been shown to be essential for development (Dynes et al., 1994b; Kibler et al., 2003; Wong et al., 1995). So far there has not been any clear evidence for focal adhesion sites in *Dictyostelium*, although recently the dynamics of actin spots possibly involved in cell substrate contacts have been described (Bretschneider et al., 2004). Actin-rich membrane fragments are left behind by migrating cells (Uchida and Yumura, 2004). *Dictyostelium* contains no known focal adhesion kinase, but it contains two talin homologues (talA and talB) that have been shown to be important for multicellular development (Kreitmeier et al., 1995; Niewöhner et al., 1997; Tsujioka et al., 1999; Tsujioka et al., 2004). Deletion of *talB* results in mound arrest. It has been shown that deletion of Lim2 (or *paxA*), a protein containing five Lim domains typical of the paxillin family but lacking clearly recognisable LD domains is required for later development, since when deleted it results in an arrest at the mound stage (Chien et al., 2000). This phenotype is rather similar to the talB^{null} mutant and several other mutants with defects in the actin-myosin cytoskeleton such as the α-actinin/gelation factor double null mutant and the myosin II^{-} mutant (Lo et al., 2000; Rivero et al., 1996). Since the original description of *paxA* it has become clear that *Dictyostelium* contains a gene *paxB,* coding for a protein PaxB with high homology to vertebrate paxillins, which contains four Lim and four LD domains. We now investigate the localisation and function of PaxB during *Dictyostelium* development.

Materials and Methods

Strains and developmental conditions

Strains used in this study were: the parental axenic strains AX2 and AX3; *paxB*$^{-}$, *limB*$^{-}$; *limB*$^{-}$*paxB*$^{-}$; *paxB*$^{-}$[*A15/gfp*]; *paxB*$^{-}$[*A15/paxB-gfp*]; *limB*$^{-}$[*A15/paxB-gfp*]; *paxB*$^{-}$ *limB*$^{-}$ [*A15/paxB-gfp*]; *paxB*$^{-}$[*A15/paxB-gfp*]; *paxB*$^{-}$[*D19/paxB-gfp*]; *paxB*$^{-}$[*ecmA/paxB-gfp*]; *paxB*$^{-}$[*ecmB/paxB-gfp*]; *paxB*$^{-}$[*A15/paxB-lacZ*]; *AX2*[*paxB/lacZ*]. The S65TGFP variant was used in all GFP constructs. Cells were grown in HL5 medium according to standard condition and all strains were kept a at density of 2-5×10^6 cells/ml (Sussman, 1987). Doubling time was 8-9 hours under these conditions. *paxB*$^{-}$ cells were kept under 5 μg/ml Blasticidin selection, *limB*$^{-}$ with 10 μg/ml Hygromycin selection, strains expressing GFP expression constructs were kept in 10 μg/ml G418. To initiate development, cells were harvested by centrifugation at 600 ***g*** for 3 minutes and washed twice in KK_2 buffer (20 mM KH_2PO_4/K_2HPO_4 pH 6.8) before plating on 1% agar plates in KK_2 at a density of 10^6 cells/cm^2. Development was examined at 22°C. Synergy experiments were performed by mixing wild type and mutant cells at various ratios followed by washing them once in deionised water and plating 10 μl drops of cell mixture at 10^8 cells/ml on 1% water agar plates.

Generation of gene disruption mutants

To create the paxillin knockout construct a full-length genomic fragment was amplified with the primers pax sense (CCA AAG AGT TCA ATA AAA GTA ACA GCA ACT G) and pax antisense (CCT TTA CAA TAT GGT TTA CCA TTA GCG G), cloned in BluescriptSKII and a BSRC resistance cassette was cloned blunt ended in a unique *Nhe*I site. After transformation random clones were tested by PCR for successful gene disruption, using the primers used to make the disruption construct and a number of successful integrants to be used in further experiments were confirmed to be knockouts by Southern blot analysis using the CDP-Star™ detection module (Amersham, Plc).

To create paxillin expression constructs, paxillin was amplified using the primers pax1 (ACAAAAGGATCCAATATGGATGAT) and pax2 (TTTATTGGATCCAAATAATTTATTATG) and cloned as a *Bam*HI fragment in a PB17S-rsGFP expression vector that contained a S65T rsGFP as a *Bam*HI/*Xho*I fragment. The full length *Bam*HI-*Xho*I fragment obtained by limited digest was cloned into the 56gal, 63gal and D19gal vectors from which the *lacZ* fragment had been removed by a *Bgl*II-*Xho*I double digest, this created the expression vectors 56paxGFP, 63paxGFP and D19PaxGFP. The paxillin GFP knockin was created by insertion of A15/paxB-gfp/A8T cassette obtained as a *Spe*I fragment in to a BluescriptSKII vector containing a blasticidin resistance cassette [A15/bsr] from PucBsrΔBam (Sutoh, 1993) as a *Bam*HI-*Hin*dIII fragment. A second genomic fragment was amplified using the primers pax3 (AAAAAGTCGACAAAAAAAATCAACAATAAATGGTAATTCC) and pax4 (AAAAGGTACCTATTACTTGTACATACACCA) and cloned into BluescriptSKIIas a *Sal*I/*Kpn*I fragment. The gene-replacement construct was amplified using the pax1 and pax4 primers described above and used for transformation. Transformations were performed by electroporation using a Bio-Rad genepulserII as described (Howard et al., 1988). Successful integrants were tested by Southern blot hybridisation using the CDP-Star™ detection module according to the manufacturer's instructions (Amersham).

The limB knockout construct was made from a genomic PCR fragment amplified with the primers AAAAAAGGAAAAAAAAACCCACACCCAC and GCAATCTTTCAACCCACAATAACCTTTAGA. This was cloned into a pCR-Blunt II-TOPO vector (Invitrogen). A hygromycin resistance cassette (obtained from Masashi Fukuzawa) was cloned blunt ended in the unique *Mfe*I site. The disruption construct was amplified by PCR using the primers described above, and used for transformation, after purification and concentration by ethanol precipitation. Independent disruptants were collected tested by PCR and disruption was confirmed by Southern blot.

To investigate the regulation of paxillin expression an 800 bp genomic fragment was amplified using the primers TCTAGAACCCATCAAGGCGTTTATTGG and ATCCATAATTAGATCTTTTTGGTGCCAT and cloned as a *Xba*I-*Bgl*II fragment into a *lacZ* expression vector pD19gal from which the D19 promoter had been removed (gift from J. G. Williams).

An ABD120-RFPmars expression vector was constructed by PCR amplification of the ABD120 fragment from the pDxactin neo (Pang et al., 1998) with the sense primer CTATACGGATCCGCTGCTGCTGCTAGATCCACA, containing a *Bam*HI site and antisense primer TTTGTTCTAATGCATCTCGAGCGGC containing a *Xho*I site. This was cloned into a PB17 Mars vector, which was constructed by cloning of a *Hin*dIII-*Bam*HI mRFPmars fragment from the 339-3:mRFPmars expression vector (Fischer et al., 2004) in PB17S (Manstein et al., 1995). For the generation of the paxillin-GFP ABD120Mars double transformant, this construct was co-transfected with the A15paxillin GFP construct in Ax2.

Paxillin antiserum and immunoblotting

Antibodies were generated against the CMNPLAGGSYTANN peptide that was coupled to KLH and injected into rabbit (Sigma-Genosys). Sera were affinity purified on peptide affinity-columns before use. The antibody recognised a 68 kDa recombinant protein on western blots. For developmental time-courses cells were allowed to develop on 1% KK_2 agar plates at a density of 5×10^6 cells/cm^2 for various times. Samples were collected and solubilised in Laemmli sample buffer, and 5×10^5 cell equivalents were subjected to 10% SDS-PAGE. Immunoblotting was performed on nitrocellulose membranes using affinity purified antibody against PaxB-specific C-terminal peptide. A sheep anti-rabbit horseradish peroxidase-coupled antibody (Promega) in 1:10,000 dilution was used for detection. Chemi-luminescence was recorded using a Xograph Imaging Systems, Ltd Compact X4.

Phototaxis experiments

Cell were harvested by centrifugation at 600 ***g*** 3 minutes and washed twice in water before placing 10 ul drops (10^8 cells/ml) on 1% water agar plates containing 1% charcoal. The plates were incubated in metal petridish storage containers (20 plates) containing a vertical 2 mm wide perforation along the length of the container. Individual agar plates were separated by black paper disks and the containers were incubated in constant subdued light for 48-64 hours at 22°C. The slime trails left behind the migrating slugs were blotted onto clear PVC discs and stained with Coomassie blue (10 mg/l in 40% methanol, 7% acetic acid) for 30 minutes, destained in 7% acetic acid and dried.

Histochemical staining for β-galactosidase activity

Slugs were developed on nitro-cellulose membranes and fixed for 15 minutes in 1% glutaraldehyde in Z-buffer (60 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4$, 2 mM $MgCl_2$). Samples were washed twice for 10 minutes in Z-buffer then incubated in staining solution (0.1% Xgal, 5 mM potassium ferrocyanide and 5 mM ferricyanide in Z-buffer) at 37°C (Dingermann et al., 1989).

Antibody staining

For prespore antibody staining cells were allowed to develop and, after various times of development, structures were re-suspended with dissociation solution (10 mg/ml cellulase, 2 mM EDTA, 20 mM KH_2PO_4, 20 mM K_2HPO_4 pH 6.8), gently dissociated by pipetting and fixed for 10 minutes with 60% methanol in KK_2 at room temperature (RT). Fixed cells were washed three times for 5 minutes in PBS, pH 7.4, containing 1% BSA, stained with an anti-prespore vesicle-specific antibody (Weijer and Durston, 1985) for 1 hour at RT, washed three times for 5 minutes with PBS, pH 7.4, and stained with a secondary goat anti-rabbit FITC-conjugated antibody. The proportion of prespore cells in the population was determined by counting cells containing more than three fluorescent vacuoles. For paxB immuno-staining cells were allowed to settle on cover slips, fixed in 4% formaldehyde in KK2 for 30 minutes and washed three times in KK2. To unmask the paxillin epitope the cells were washed for 30 minutes in 0.05% SDS in KK2 followed by three washes in KK2. Staining with affinity purified paxB antibody (as described above) at 1:100 dilution at 4°C overnight. This was followed by incubation with a FITC conjugated goat anti-rabbit antibody (Sigma 1:200 dilution in KK2) and three washes in KK2. The slides were covered with 80% glycerol in 20 mM Tris/HCl (pH 8.0) and observed in the Leica TCS SP2 confocal microscope.

Microscopy and image processing

The cellular distribution of paxillin was imaged using a Leica TCS SP2 confocal microscope with PL FLUOTAR 40×/1.40NA and 100×/1.40NA immersion objectives. Images were taken at 2-10 seconds intervals and in several instances Z sections were collected

at 1 μm intervals. Dark field waves were recorded and analysed using published procedures (Siegert and Weijer, 1989). Macroscopic images were taken with a Nikon SMZ 1500 fluorescence dissection-microscope equipped with a Nikon DXM 1200 digital camera. Images were analysed using custom written macros using Optimas 6.1 (Dormann et al., 2001). To determine the kinetics of contact site formation we measured the average fluorescence intensity in a 5×5 or 10×10 pixel window at the position of forming contact sites over time. The raw data were normalised to correct for differences in fluorescence intensity for example due to differences in expression levels. Around 40-50 spot measurements were obtained and processed in this way per strain, the curves were aligned so that the half-maximum rise in fluorescence occurred at t=0 seconds and finally averaged.

Measurement of cell-substrate adhesion

Cell substrate adhesion was measured in a substrate detachment assay (Fey et al., 2002). 1 ml of 5×10^5/ml cells were plated in HL5 in 3.5 cm tissue culture Petri dishes (TPP, Switzerland). The cells were grown overnight at 22°C and the following morning the dishes were shaken on a gyratory shaker with a 2.5 cm radius of gyration at 60 rpm. At various time intervals the number of detached cells was counted in a haemocytometer. The results were plotted as percentage of cells detached as a function of time. The total number of cells was determined at the end of the experiment after resuspension of all cells.

Results

The structure of the *Dictyostelium* paxillin

Analysis of the genome has shown that *Dictyostelium* contains a single gene that is highly homologous to that of mammalian paxillin. It contains four conserved LD domains and four highly conserved LIM domains (Fig. 1). The LIM domains contain seven conserved cysteine residues and a histidine. The arrangement followed by these conserved residues is C-x(2)-C-x(16,23)-H-x(2)-[CH]-x(2)-C-x(2)-C-x(16,21)-C-x(2,3)-[CHD]. The LIM domain binds two zinc ions and seems to act as an interface for protein-protein interactions (Bach, 2000). The LD motifs are specific to paxillin and are characterised by the consensus sequence LDXLLXXL and have been shown to act as interaction sites for several molecules (Tumbarello et al., 2002). It would appear that the PaxB LD domains correspond closely to the mammalian LD1, LD2, LD3 and LD5 domains of vertebrate paxillins, the spacing between the LD domains is, however, not conserved. Vertebrate paxillins contain two conserved SH2-binding sites centred around two tyrosine residues (Y31 and Y118 in human paxillin), which are heavily phosphorylated in vertebrates since they are targets for focal adhesion kinase and Scr kinases (Brown and Turner, 2004). It is clear from the sequence comparison between human paxillin α and *Dictyostelium* PaxB that these sites are not conserved in paxB. Mammalian paxillin also contains a proline-rich potential SH3-binding domain, which is also absent in *Dictyostelium* paxillin. Mammalian paxillin is also phosphorylated by serine threonine kinases on a number of different sites and most recently it has been shown that paxillin is phosphorylated on serine 178 by Jun kinase and that this phosphorylation plays an important role in the migration of fish keratinocytes and rat bladder tumour cells (NBTII) cells (Huang et al., 2004; Huang et al., 2003). Interestingly this site and some of the surrounding amino acids are conserved in PaxB. Two other sites serine 126 and serine 130, which are phosphorylated in a Raf-Mek-Erk-dependent fashion, are also conserved in *Dictyostelium* (Woodrow et al., 2003). However, mutation of these sites S126A and S130A in mammalian cells did not result in noticeable changes in interactions with other focal adhesion

Alignment of Human and *Dictyostelium* paxillin

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                          LD1                          (Y31)
Paxα  -------MDDLDALLADLESTTSHISKRPVFLSEETPYSYPTGNHT---------YQEIA  44
PaxB  MATKGLNMDDLDLLLADLGRPKSSIKVTATVQTTATPSSGKNFDNSDIQNEIQSIIEELD  60
            ***** *****  ..* *.  ... :  ** *  . :::          :*:

Paxα  VPPPVPPPPSSEALNGTILDPLDQWQPSGSRFIHQQPQSSSPVYGSSAKTSSVSNPQDSV 104
PaxB  QQPQTVQTISTPAPKNHNTTTTTASFSVSSQPAPQPPQQSQQIDGLDDLDELMESLNTSI 120
        * .  . *: * :.     .     . .*:   * **.*. : * .   . :.. : *:

                (Y118)                          LD2
Paxα  GSPCSRVGEEEHVYSFPNKQKSAEPSPTVMSTSLGSNLSELDRLLLELNAVQHNPPGFPA 164
PaxB  STALKAVPTTPEEHITHANSNSPPPSLHKNTSSTNS-ASSLSRPNNNPSVVSTPQPGKVT 179
      .:. . *    . :    :.:*. **    ::* .*  *.*.*   : ..*.   **  :

                (S178)                                          LD3
Paxα  DEANSSPPLPGALSPLYGVPETNSPLGGKAGPLTKEKPKRNGGRGLEDVRPSVESLLDEL 224
PaxB  STATITTKKQPALSKATLETTSGNNVYSSQPSQSQPQPYKVTAT---NSQPSSDDLDELL 236
      . *. :.     ***      . :.. : ..  . :: :* :  .    : :** :.* :

                                    LD4
Paxα  ESSVPSPVPAITVNQGEMSSPQRVTSTQQQTRISASSATRELDELMASLSDFKFMAQGKT 284
PaxB  KGLSPSTTTTTVPP-PVQRDQHQHHHQHQHHHHHNPNHNQTQTVTTQINIGRTNTPNNN 295
      :.  **...: **    :.  *:    *:* :   ..  .: : : :.:.  :  : .:.

                          LD5                          Lim1
Paxα  GSSSPPGGPPKPGSQLDSMLGSLQSDLNKLG-VATVAKGVCGACKKPIAGQVVTAMGKTW 343
PaxB  NNNNTNSPKVVHGDDLDNLLNNLTSQVKDIDSTGPTSRGTCGGCRKPIFGETIQAMGKFY 355
      ..... .     *.:**.:*..* *:::.:. ....:*.**.*:*** *:.: **** :

                                                        Lim2
Paxα  HPEHFVCTHCQEEIGSRNFFERDGQPYCEKDYHNLFSPRCYYCNGPILDKVVTALDRTWH 403
PaxB  HPEHFCCHNCQNPLGTKNYYEQESLPHCEKCYQELFCARCAHCDEPISDRCITALGKKWH 415
       ***** * :**: :*::*::*::. *:*** *::**..** :*: ** *: :***.:.**

                                                        Lim3
Paxα  PEHFFCAQCGAFFGPEGFHEKDGKAYCRKDYFDMFAPKCGGCARAILENYISALNTLWHP 463
PaxB  VHHFVCTQCLKPFEGGNFFERDGRPYCEADFYSTFAVRCGGCNSPIRGECINALGTQWHP 475
       .**.*:**   *   .*.*:**:.**. *::. ** :****  .*  : *.**.* ***

                                                        Lim4
Paxα  ECFVCRECFTPFVNGSFFEHDGQPYCEVHYHERRGSLCSGCQKPITGRCITAMAKKFHPE 523
PaxB  EHFVCQYCQKSFTNGQFFEFGGKPYCDVHYHQQAGSVCSGCGKAVSGRCVDALDKKWHPE 535
      * ***: * ..*.**.***..*:***:****:: **:**** *.::***: *: **:***

Paxα  HFVCAFCLKQLNKGTFKEQNDKPYCQNCFLKLFC 557
PaxB  HFVCAFCMNPLAGGSYTANNGKPYCKGCHNKLFA 569
      *******:: *  *::. :*.****:.*. ***.
```

Fig. 1. Comparison of *Dictyostelium* PaxB with Human paxillin α. PaxB contains four conserved LD domain and four highly conserved Lim domains. PaxB does not contain any of the conserved tyrosine phosphorylation sites Y31 and Y118, which are part of SH2-binding domains in human paxillin, nor does it contain the proline-rich SH3-binding domain. A potential JNK phosphorylation site S178 (S192 in paxB) is conserved as are possible Erk phosphorylation sites S141 and S143.

proteins nor in changes of the cellular localisation of these paxillin mutants. Comparison with other paxillin sequences shows that PaxB is most closely related to one of the vertebrate paxillins, Hic5, which also contains only four LD domains. Its closest homologue in the *Dictyostelium* genome is another Lim domain-containing protein known as paxA or Lim2 (http://dictybase.org). The PaxA protein contains five Lim domains, but no clearly discernable LD motifs and, therefore, it is questionable whether this is a bona fide member of the paxillin family.

PaxB expression peaks during the multicellular stages of development

paxB RNA is expressed during all stages of *Dictyostelium* development, but the strongest expression is observed during the mound and slug stages (not shown). This RNA expression pattern is reflected in the expression of the protein as determined by western blot analysis using a *Dictyostelium*-specific affinity purified paxillin polyclonal antibody (Fig. 2A). Paxillin is expressed at lower levels in vegetative cells, but expression then rises dramatically at the tipped mound stage, remains high at the slug stage, after which there is a gradual decline during culmination. Initial attempts to use the affinity purified polyclonal antibody to study possible differences in expression of paxillin in prespore and prestalk cells were not very successful. We therefore cloned a 800 bp *paxB* promoter fragment in a *lacZ* expression vector to study the expression of *paxB* in the various cell types during development. It appeared that expression is found in all cells at all stages of development, but that expression increases at the mound stage and is more prominent in prestalk cells than in prespore cells. During culmination expression was particularly strong in the upper and lower cup regions of the fruiting body (Fig. 2B). This expression pattern is reflected very well by in situ hybridisation patterns of paxillin expression observed during development (not shown) and expression of the paxillin protein in a *paxB-GFP* knockin strain (Fig. 2C).

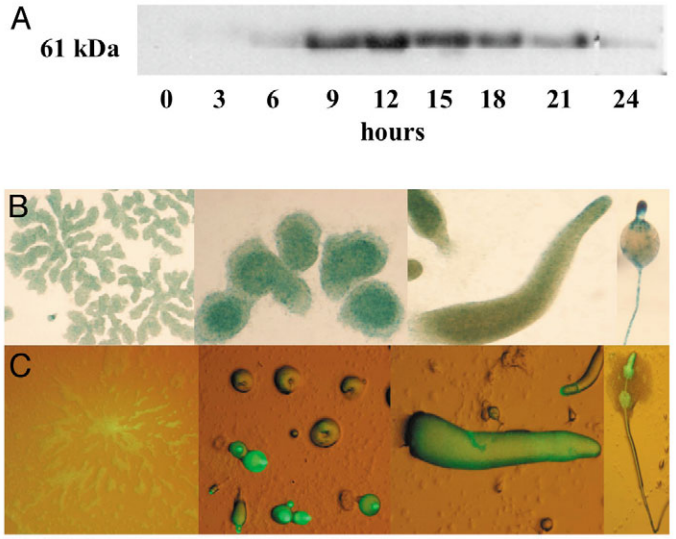

Fig. 2. Expression of PaxB during development. (A) PaxB expression during development as detected by western blot with a *Dictyostelium*-specific affinity purified anti-peptide antibody. Maximal expression is reached at 12-15 hours of development. The experiment shown is representative for results obtained in four independent time course experiments. (B) Expression of a paxB/*lacZ* expression construct showing expression of *paxB* during all stages of development. Note and the increased levels of expression in the tip of the slug and the upper and lower cup of culminates. (C) Expression of a C terminal PaxB-GFP knockin construct again showing expression from the vegetative stage onwards and increased expression in the slug and culminate tip.

Cellular localisation

Paxillin is an adapter protein in focal adhesion sites, and has been identified to play an important role in coupling cell-substrate adhesion receptors to the actin cytoskeleton (Giannone et al., 2004; Webb et al., 2004). So far there have been no reports of focal adhesion sites in *Dictyostelium* mostly since none of the classical components of focal adhesion sites such as focal adhesion kinase and integrins have been identified. The presence of PaxB, however, allows us to explore the existence of focal adhesion sites in more detail. The antibody directed against a C-terminal peptide could not be used successfully in the immunohistochemical localisation of PaxB, apparently the antigen-binding site was masked. It only became accessible after mild SDS treatment, which resulted in detectable staining in small foci localised in close apposition to the actin cytoskeleton (not shown). However, the harsh fixation procedures needed left some doubt about the validity of this cellular localisation of the protein. It also did not allow us to examine PaxB localisation in the later multicellular stages of development, since these structures did not survive the SDS treatment. To circumvent these problems we analysed the distribution of a PaxB-GFP fusion protein expressed under the control of a constitutive (actin15), and prespore or prestalk cell-specific promoters. Investigation of cells expressing Pax-GFP indicated that there may be focal adhesion sites present in vegetative and aggregation stage *Dictyostelium* cells, but due to the high cytoplasmic level of expression these were difficult to see except in cells expressing the lowest level of PaxB-GFP. However, to be able to study endogenous levels of paxillin expressed under the control of its own promoter, a *paxB* C-terminal GFP knockin strain was constructed as described in Materials and Methods. Western blot analysis showed that a GFP fusion protein of the correct size was produced in this strain and that expression closely matched that of the endogenous protein (data not shown).

In the knockin cells we observed a clear localisation of PaxB-GFP in small stationary spots located at the interface of the cells with the substrate (Fig. 3A-D). These structures were formed at the leading extending edge of the cells and stayed present as long as the cells were attached to the substrate (Fig. 3C,D). These structures look like authentic focal adhesion sites. Vegetative cells often protrude very fine filopodial structures and it was observed that the tips of these structures contained a lot of PaxB especially when they contacted the substrate (Fig. 3B,D). These structures persisted in all stages of development, i.e. aggregates, mounds and slugs (Fig. 3E,F). During migration in the slug stage it was clear that the PaxB-rich focal adhesion sites were formed at the front of the cells and then stayed present during the migration of the cells and that they were disassembled when they reached the trailing end

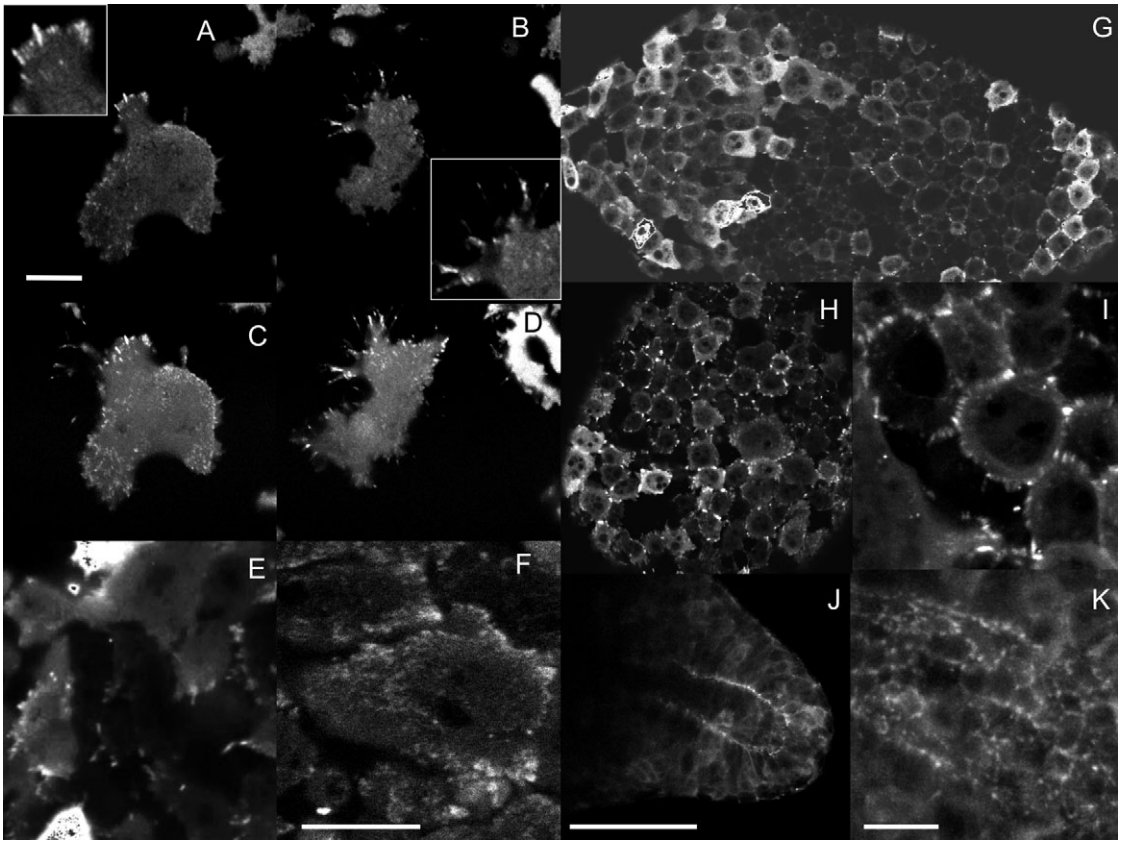

Fig. 3. Cellular localisation of paxB in a *paxB-gfp* knockin mutant. (A,B) Localisation of paxillin in a vegetative cell at 0 and 120 seconds. Note the localisation of PaxB in small spots at randomly distributed sites where the cell is in close contact with the substrate (see supplementary material Movie 1). (C,D) Cumulative PaxB distribution of the cell shown in A,B for 0-60 seconds (C) and 60-120 seconds (D). The cumulative distribution shows that many of the spots are stationary and also show more clearly the localisation of PaxB in the tips of the filopodia. Scalebar (A): 10 μm. (E) Localisation in retraction fibres at the back of cells in migrating slugs. (F) Localisation of PaxB in small focal adhesion spots localised at the contact area between the slime sheath and the substrate in the outermost cells of a slug. The slug shown in images E,F is migrating from left to right. These structures are also found in cells deeper in the slug as can be seen in the supplementary information provided (see supplementary material Movie 2). Scale bar in F, 10 μm. (G) Distribution of cell-cell contacts in an early culminant. These contacts are distributed around the cell (marked with arrows) and are most obviously detected in the small epithelial layer of cells surrounding the spore mass in the forming fruiting body as well as in the cells in the upper cup and lower cup (see supplementary material Movie 3). (H) Another culminant showing the presence of PaxB enriched cell-cell contacts (arrows) in the back of an early culminant and (I) a higher magnification image of a central part of the image shown in H. (J) Section through the forming stalk of an early culminant showing high levels of PaxB localisation at the contact area between cells and the stalk sheath. Scale bar, 40 μm. (K) Higher magnification of a maximum projection of a stack of 10 adjacent images of the cells in contact with the stalk tube showing the presence of special PaxB-rich areas between the cells contacting the stalk sheath. Scale bar, 10 μm.

of the cells. The behaviour of these spots was different from the actin-rich contact spots observed with a number of fluorescent actin-binding proteins, such as ABP120, Coronin and Arp3-GFP (Bretschneider et al., 2004; Weijer, 2003). These actin-rich spots had a shorter half-life and their size and shape was also different. We measured the kinetics of integration of PaxB-GFP in these sites and found that they were formed at a rate of 0.06 per second (Fig. 12), which is ~40 times faster than observed for the formation of focal adhesion sites in mouse embryonic fibroblasts (Webb et al., 2004), but still significantly slower than the rate for the formation of actin spots as measured by integration of the GFP-tagged actin-binding domain of ABP120 in localised spots at the cell substrate interface, which we determined to be 0.17 per second in accordance with other data in the literature (Bretschneider et al., 2004). Although these typical focal adhesion plaques stayed present during the later stages of development, another type of contact became more apparent, cell-cell contact. In slugs these contacts appeared at the trailing edge of the cells and were located throughout the slug tissue (Fig. 3E). In fruiting bodies they were especially numerous in the outer epithelial layer of cells overlaying the upper and lower cup and encapsulating the spore mass (Fig. 3G-I). These cell-cell contacts often appeared to form fine rows of inter-digitating filopodial structures with high levels of paxillin in the tips of these structures, which were distributed in groups of 2-4, more or less evenly around the periphery of the cells (Fig. 3I). Closer examination of these structures by 3D sectioning microscopy showed that these structures were the end of fine cell-cell contacts, which also appeared to be stationary in time. These contacts are not only found in the epithelial layer of cells in contact with the slime sheath but also in culminates between

the cells surrounding the stalk tube and the stalk tube sheath (Fig. 3J,K). Simultaneous observation of the paxillin localisation sites and reflection interference contrast images showed that paxillin adhesion sites where found only at sites where the cells were in close contact with the substrate. In many cases it was noticeable that the density of these spots was highest at the cell substrate contact boundary especially in the regions where new contacts are being made (Fig. 4).

It has previously been reported that cells make highly dynamic actin-enriched spots at the sites of cell substrate contact. To investigate whether these actin spots co-localised with the areas of paxillin enrichment we made a double transformant expressing PaxB-GFP and a red fluorescent actin-binding domain of ABP120. Observation of the actin and PaxB foci showed that the actin foci were much more dynamic and also in general did not coincide with the paxillin foci (Fig. 5) (see also below for quantitative data). The PaxB foci originated at the leading edge of the cell and stayed present during the time the surface was in contact with the substrate, while the actin foci could arise anywhere and be disassembled, while the surface was still in contact with the substrate. This indicates that these PaxB and actin foci may serve different functions.

paxB⁻ cells are defective in cell-substrate adhesion

To investigate the functional role of PaxB for the *Dictyostelium* multicellular development we constructed a number of independent mutants in the Ax2 and Ax3 backgrounds. The *paxB* gene was deleted by homologous recombination. These strains all grew with indistinguishable kinetics in shaking suspension. Ax2 cells grew with 9.0±1 hours doubling time measured at six time points over 60 hours, in two independent experiments. The *paxB* mutants had a doubling time of 10.3±0.9 hours averaged over three *paxB*⁻ strains and the same three *paxB*⁻ strains expressing PaxB-GFP. A further characterisation of the *paxB*⁻ cells showed that cells were less adhesive to various substrates (Fig. 6). We measured the adhesion of vegetative stage cells to plastic substrates and found that the knockout strains were considerably less adhesive when exposed to moderate conditions of shear stress, and this effect could be completely rescued by expression of the *paxB* under the A15 promoter in the *paxB*⁻ background.

paxB⁻ cells reveal that PaxB plays a role in cell migration during the multicellular stages of development

In all knockouts we observed that the initial phases of development were essentially comparable to that of the parental strains. Cells aggregated normally, made optical density waves that were similar to the parent strains, formed streams and flat mounds of normal morphology with approximately normal timing (data not shown). However when the null mutants reached the mound stage, in many cases further development of the mounds was arrested, i.e. they did not proceed to form tips (Fig. 7A,C). This phenotype was especially obvious when the cells were plated on agar plates containing buffer and at lower densities, where almost all the mounds arrested their development, even at prolonged incubation. At higher densities, especially on water agar plates, some fruiting bodies formed, but these were generally oddly shaped with big basal mounds of cells (Fig. 7D) and small spore masses, while wild-type fruiting bodies form long slender stalks and well-defined spore masses (Fig. 7B). This phenotype indicates that paxillin could have an important function in the process of cell movement especially of the cells that sort to form the tip, the structure that controls the movement of all other cells during later development.

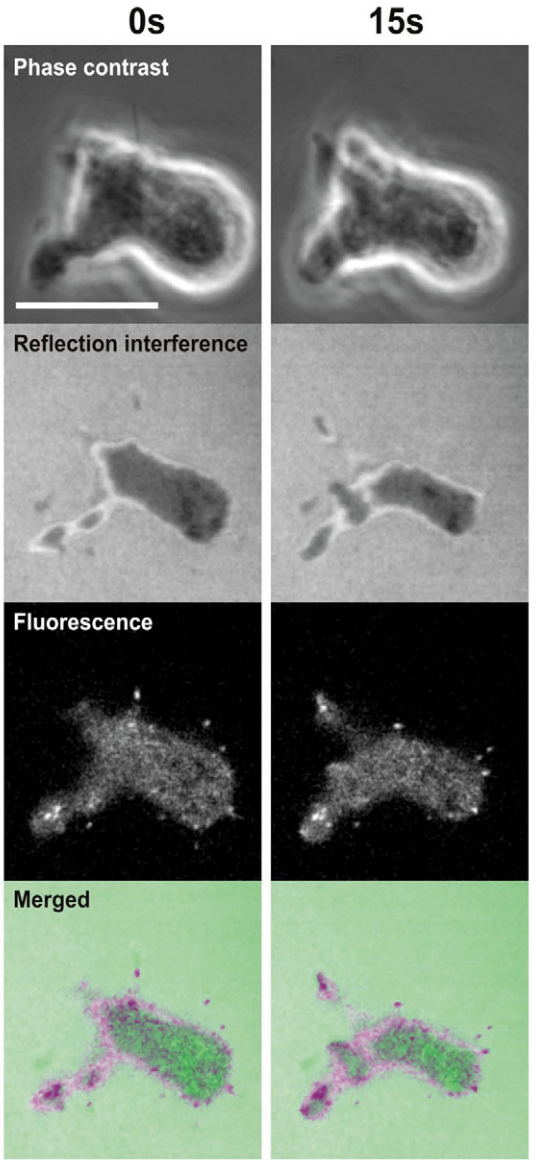

Fig. 4. PaxB-GFP foci co-localise with cell-substrate contact areas. Brightfield, interference reflection contrast and fluorescence images were recorded simultaneously on a confocal microscope. Dark areas in the reflection interference contrast image surrounded by brighter interference patterns correspond to parts of the cell in close proximity to the substratum, the small very dark structures in the adhesion area are part of the contractile vacuole network. As the cell extends a pseudopod that makes contact with the substratum (15 second timepoint) PaxB-GFP localises to contact sites in this new adhesion area. In the merged fluorescence and interference contrast images at the bottom, PaxB-GFP appears purple and the contact area in dark green. Scale bar, 10 μm. See also supplementary material, Movie 4 for dynamics of paxillin in relation to the contact area in vegetative stage cells.

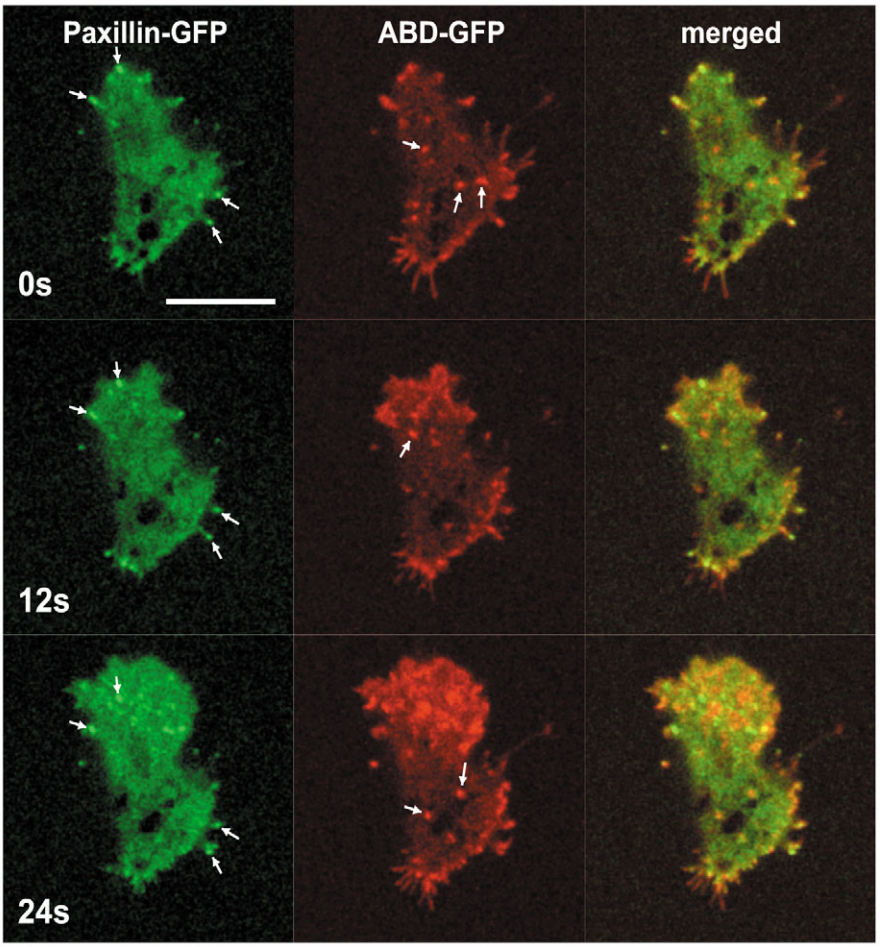

Fig. 5. Co-localisation of PaxB-GFP and ABD-mRFPmars. Confocal time series of vegetative wild-type Ax2 cell expressing PaxB-GFP and ABD-mRFPmars. Time is indicated in seconds. (Left panel) PaxB-GFP (green) localises to long lived stationary contact sites at the cell/substratum interface as indicated by the arrows that mark the same contact sites at different time points. (Centre panel) ABD-mRFPmars (red) accumulates at very short-lived contact sites as indicated by the arrows. (Right panel) Merged image showing that PaxB-GFP and ABD-mRFPmars localise to different contact sites. Scale bar, 10 μm. See also supplementary material, Movie 5 for dynamics of mRFPactin and PaxB-GFP in vegetative stage cells.

To test a role for paxillin in cell movement we performed synergy experiments with wild-type cells (Fig. 8). In synergy experiments in which 5% *lacZ*-expressing wild-type cells were allowed to co-aggregate with *paxB*⁻ cells they formed random mixtures at first during early aggregation (Fig. 8A), however just before tip formation the wild-type cells started to aggregate into groups in the centre of the aggregates (Fig. 8B) after which they went on to form tips (Fig. 8C) and finally small culminates sitting on top of masses of *paxB*⁻ cells (Fig. 8D) indicating that the mutant phenotype is cell autonomous. In the reverse experiment where labelled *paxB*⁻ cells (50%) were allowed to co-aggregate with 50% wild-type cells the *paxB*⁻ cells populated the back of the slug and many of them were lost during subsequent migration indicating that they have a movement defect in competition with wild-type cells. When development was allowed to proceed to the culmination stage it was found that the *paxB*⁻ cells were preferentially found in the lower cup and the basal disk (Fig. 8G). This phenotype could only be partly rescued by expression of paxillin GFP fusion protein under the control of the actin15 promoter (Fig. 8G). These results clearly indicated that *paxB*⁻ cells are defective in cell migration in multicellular tissues. In the course of these experiments we noted that the few *paxB*⁻ slugs that formed were essentially incapable of migration. To test this more directly we performed slug migration experiments in which slugs are allowed to form on water agar and are enticed to migrate in the direction of a faint localised light source (Fig. 8H). Wild-type cells show a strong directed migration, which is essentially absent in *paxB*⁻ strains and can be rescued to a large extent, but not completely by expression of a paxillin GFP fusion protein under the control of an actin 15 promoter. We observed that transformation of the Ax3 knockout with a paxillin GFP

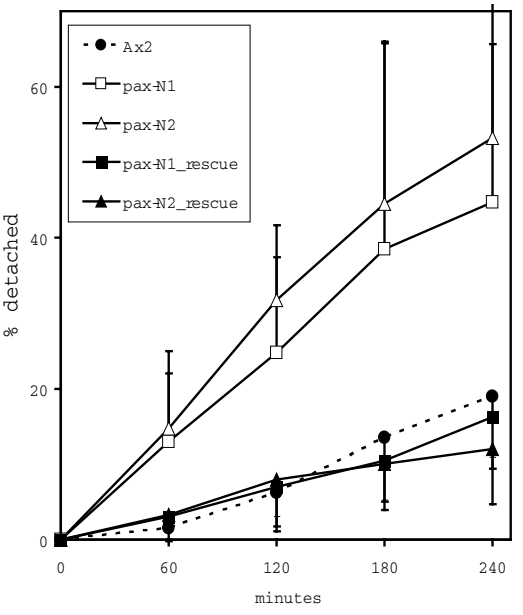

Fig. 6. Role for PaxB in cell-substrate adhesion. Adhesion of vegetative cells was measured under conditions of moderate shear stress as described in detail in Materials and Methods. It can be seen that two independent *paxB*⁻ strains (open symbols) show an increased tendency to be dislodged from the substrate when compared with an Ax2 control strain (stippled line). This defect can be rescued completely by re-introduction of *paxB-gfp* under the control of the Actin15 promoter in these strains (closed symbols). The data shown are the mean values and one-sided standard deviations of four independent experiments performed on four different days.

construct under the control of an actin 15 promoter could rescue the migration-less phenotype (Fig. 5H). However rather surprisingly, this construct gave only a partial rescue in the Ax2 strain (not shown). Investigation of the expression levels of the paxillin GFP fusion protein showed that it was expressed at similar levels in all strains and the length of the fusion protein indicated that a full length protein was expressed in all strains at similar levels (data not shown).

Further investigations into the timing of the sorting out of *paxB* cells from wild-type cells showed that this occurred at the stage of slug formation (Fig. 9). Aggregation of *paxB*$^-$ cells was indistinguishable from wild-type cells (Fig. 9A), however as soon as all cells have entered the aggregate the wild-type cells sort out from the *paxB*$^-$ cells. Their movement is more vigorous and becomes directed towards the forming tip (Fig. 9C). Quantitative analysis of the movement of the cells showed that the *paxB*$^-$ cells moved at 1.09±0.18 μm/minute, while wild-type cells moved at 1.60±0.26 μm/minute, i.e. ~60% faster than *paxB*$^-$ cells during this crucial stage of development.

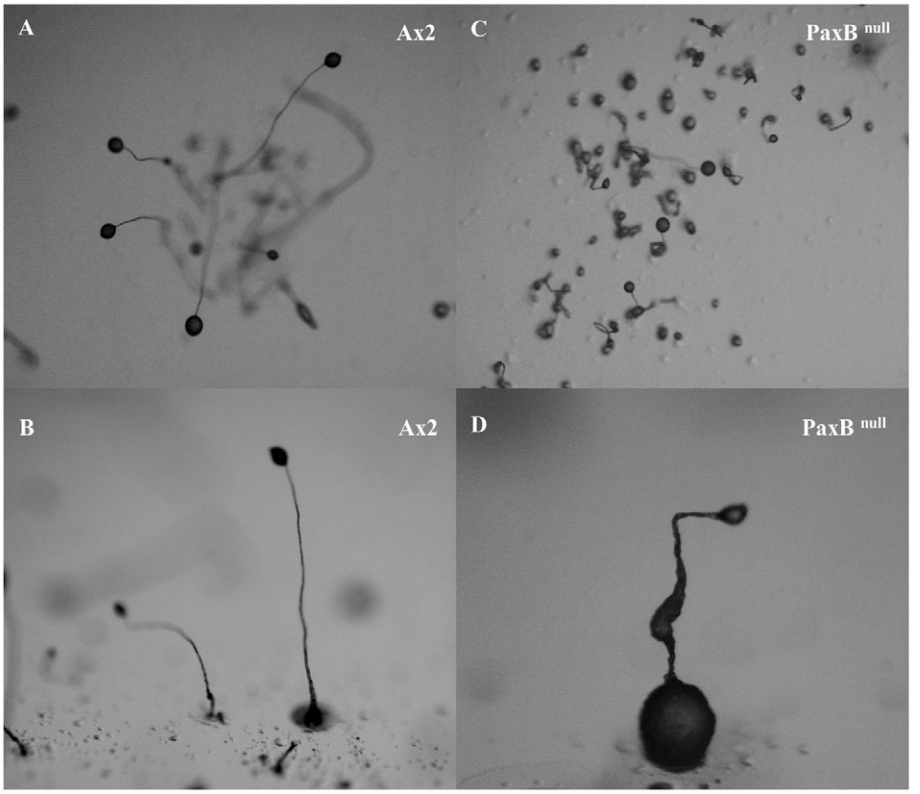

Fig. 7. Characterisation of paxB knockout phenotypes. Comparison of Ax2 and an Ax2 paxB knockout strain (A,C) Ax2 and *paxB*$^-$ in top view after 24 hours of development on agar. In Ax2 all cells develop to fruiting bodies, while in the *paxB*$^-$ strains many cells do not develop into fruiting bodies but arrest at the mound stage, or when they continue development later development is grossly abnormal. (B,D) Ax2 and *paxB*$^-$ knockout strain in side view after development on water agar for 24 hours. Note the differences in scale between images in B and D. Ax2 form fruiting bodies with slender stalks and well define basal disks while the *paxB*$^-$ mutant often form small fruiting bodies on a large mass of cells that is not lifted off the substrate.

PaxB is needed in both prestalk and prespore cells for effective slug migration and culmination

To define the role of paxillin and possible cell-type-specific requirement for development and slug migration in more detail we tried to complement the paxillin strain with the PaxB-GFP fusion protein under the control of prestalk and prespore cell-type-specific promoters (Fig. 10). In *paxB* knockouts in Ax2 and Ax3 backgrounds expression of PaxB under the control of the prestalk-specific ecmA and ecmB promoters and the prespore-specific psA promoter resulted in partial rescue of the migration defective phenotype. In all experiments the best rescue was achieved by expression of PaxB under the prestalk-specific ecmA promoter, followed by expression under the control of the ecmB and psA promoters. This is measured as completion of fruiting body formation and slug migration in response to light. The best rescue was obtained by expression of PaxB-GFP under the control of the actin15 promoter but in no case was development completely rescued. These experiments show that both prestalk and prespore cells need to express paxillin to complete development efficiently and, importantly, to allow slugs to migrate.

Ser192 phosphorylation does not affect cell and or slug migration

Paxillin is one of the most heavily phosphorylated proteins in mammalian cells and phosphorylation is thought to play a key role in its function. Paxillin is normally phosphorylated on several conserved tyrosine residues Y31 and Y118, which appear to be absent from the *Dictyostelium* paxillin. In vertebrates, paxillin is one of the major tyrosine phosphorylated proteins, however paxB is not a major tyrosine phosphorylated protein in *Dictyostelium* as determined by immunopreciptation of PaxP followed by detection of phosphotyrosine with a phosphotyrosine-specific antibody G4E10 (data not shown). It has recently been proposed that phosphorylation on serine 178 in human paxillin is necessary for cell migration and that this phosphorylation may be performed by Jun kinase. This phosphorylation site and surrounding amino acids are conserved in *Dictyostelium* and we therefore tested the importance of this site by making non-phosphorylatable and phosphomimetic mutants (S192A and S192D) to investigate its role in the control of cell migration, especially in slugs. We expressed these PaxB mutants in the *paxB*$^-$ strain under the control of the actin15 promoter and assessed the effects of these mutants for their ability to complement normal development compared with wild-type PaxB expressed under the same promoter. Furthermore we investigated the formation of focal adhesion sites in these

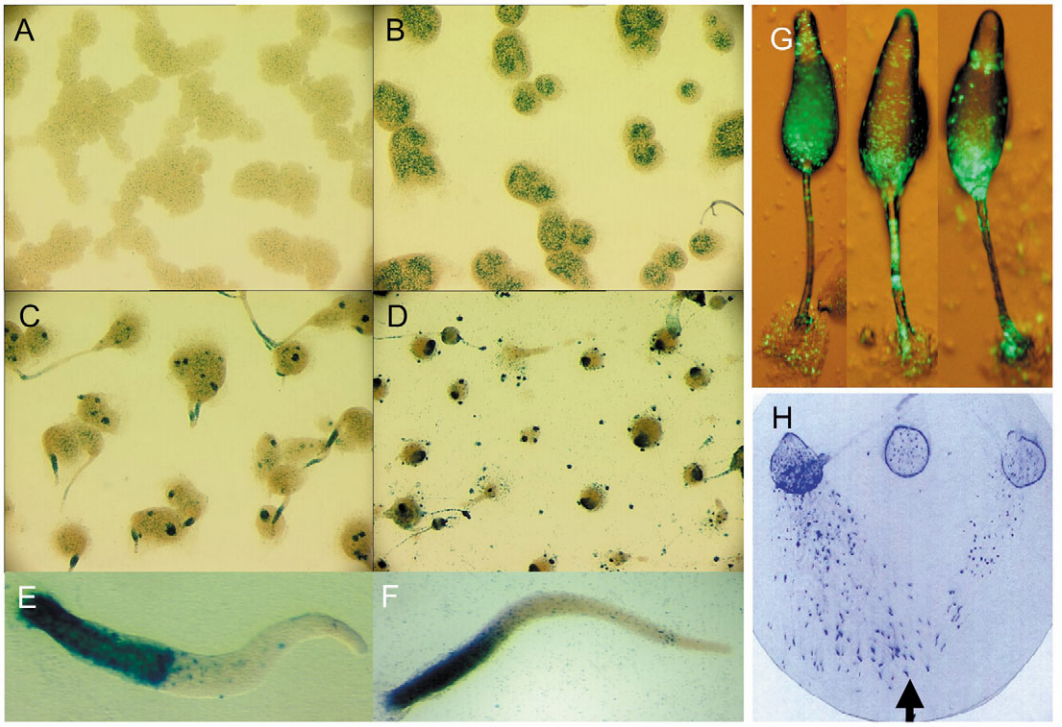

Fig. 8. Role of PaxB in cell sorting and slug movement. To assess whether *paxB*$^{-}$ strains are defective in sorting we let 5% Ax2 cells expressing the paxB/*lacZ* construct synergize with paxB^{-} cells. As can be seen during aggregation (A) the cells are randomly intermingled but at the late aggregate stage (B) the Ax2 cells start to sort to the centre of the cell masses until they are found in the tips of forming slugs (C) and small culminates (D). This clearly shows that *paxB*$^{-}$ cells are defective in cell sorting. (E,F) Sorting of 50% *paxB*$^{-}$ cells in Ax2 and of another Ax3 paxB knockout mutant in Ax3. As can be seen the majority of the *paxB*$^{-}$ cells both in Ax2 and Ax3 sort to the back of the slug indicating that they may be defective in moving in cell masses. (G) a panel showing three culminants from synergy experiments where Ax2 cell synergised with GFP expressing Ax2 cells (left culminant), PaxB-GFP expressing cells synergised with Ax2 (middle culminant) and *paxB*$^{-}$ cells expressing a PaxB-GFP fusion protein under the control of a constitutive Actin15 promoter. As can be seen Ax2 cells do not sort in Ax2, while *paxB*$^{-}$ cells are confined mostly to the lower cup and basal disk when allowed to develop in an Ax2 environment and that expression of *paxB* under the control of an string actin15 promoter does rescue development somewhat, i.e. now cells are also found in upper cup, but few cells are found in the tip or in the spore mass. (H) Migration assay comparing the migration of slugs towards a localised light source indicated by an arrow. This experiment compares the migration of Ax2 cells (left), *paxB*$^{-}$ cells (middle) and *paxB*$^{-}$[*A15/paxB-gfp*] cells (right). Ax2 cells show a strong directed migration towards the light. The *paxB*$^{-}$ cells are completely unable to migrate while the *paxB*$^{-}$[*A15/paxBgfp*] cells show a substantial rescue of migration ability, however pooling many experiments shows that migration is not as efficient as that of wild-type cells, implying that proper level of expression of PaxB may be important.

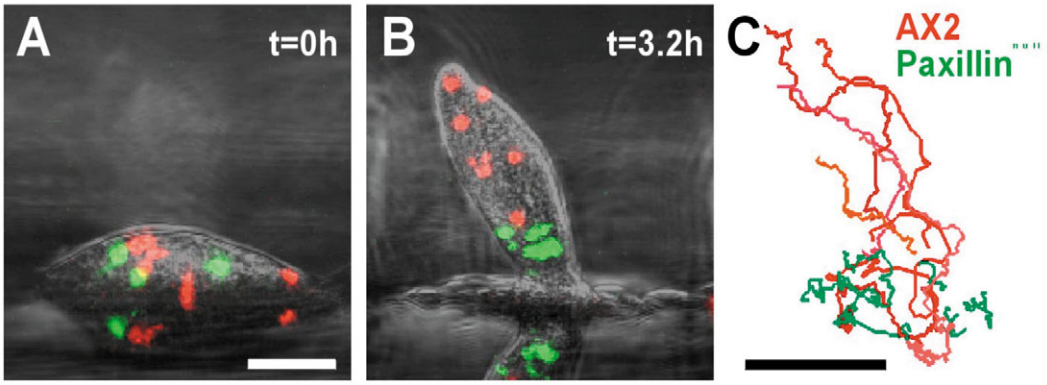

Fig. 9. *paxB*$^{-}$ cells show reduced motility in synergy experiments. (A) Side view of an Ax2 mound containing 2% EGFP labelled paxB^{null} cells (green) and 2% mRFPmars expressing Ax2 cells (red). Scale bar, 50 μm. (B) As the mound transforms into a slug the initially uniformly dispersed paxB^{null} cells are restricted to the posterior of the forming slug. (C) The corresponding cell traces of paxB^{null} cells (green, n=7 cells) and Ax2 cells (red, n=7) indicate reduced motility and directionality of paxB^{null} cells during slug formation. Scale bar, 50 μm. See also supplementary material, Movie 6 for dynamics of cell sorting.

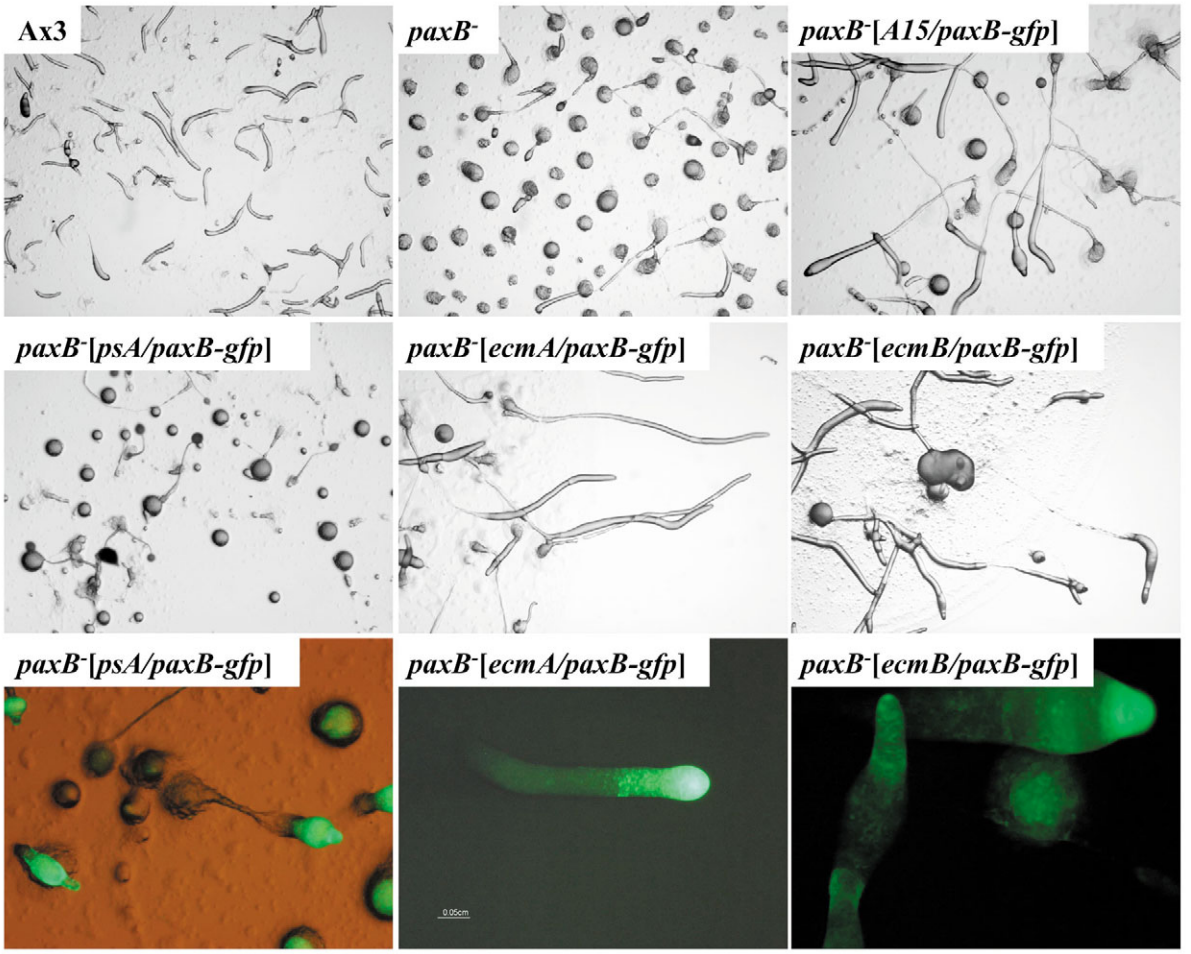

Fig. 10. PaxB expression is needed both in prespore and prestalk cells to rescue development. To assess the requirement of paxB for development we expressed the paxB GFP construct under the control of the actin15 promoter, the prestalk-specific ecmA and ecmB promoters, and the prespore-specific psA promoter. The outcome of these experiments was that expression of paxB under the control of the A15 promoter gave a fairly good but not complete rescue, followed by expression under the ecmA promoter, the ecmB promoter and the psA promoter. This was also reflected in the rescue of migration of slugs formed (not shown). The lower row of three images shows from left to right the expression of the PaxB-GFP constructs at the slug stage under the control of the psA, ecmA and ecmB promoters in the *paxB*⁻ strain.

mutants. Slug migration and cell sorting of *paxB*⁻ strains complemented with PaxB(S178A)-GFP and paxB(S178D)-GFP were not noticeable different from those expressing PaxB, indicating that phosphorylation of this site is not important in the control of cell movement in *Dictyostelium* (Fig. 11). To determine the effect of these mutations more quantitatively we measured the rate of PaxB(S192A)-GFP and PaxB(S192D)-GFP integration in the focal adhesion sites in the slugs compared to that of wild type (Fig. 12). These measurements showed that there were no statistical differences between wild type and *paxB* mutants in the rate of their incorporation in focal adhesion sites.

Effects of PaxB on cell differentiation

Since the fruiting bodies that formed were generally rather odd in their morphology we decided to characterise the differentiation of the cells in these structures in more detail. We measured the kinetics of prespore cell formation and we transformed the *paxB*⁻ strains with a variety of cell-type-specific reporter-gene constructs to assess the differentiation of prespore and prestalk cell types. The kinetics of prespore differentiation during development in wild type and *paxB*⁻ strains showed that in the *paxB*⁻ strain prespore cell differentiation started at the same time as in wild-type strains, but did not reach the same level as the wild type and during later development the number of prespore cells decreases more rapidly in the *paxB*⁻ mutant than in wild-type strains (Fig. 13A). Analysis of the expression of the prestalk reporter *lacZ* gene constructs showed that there is an over-expression of prestalk-specific genes in the cells at the base of the culminants (Fig. 13B,C). This could be the result of a failure of the cells to proceed along their normal differentiation path or alternatively it might mean that the cells do take up their correct position in the organism resulting in incorrect exposure to differentiation signals. To see whether the effects were cell autonomous we performed synergy experiments in which *paxB*⁻ cells transformed with various prespore and prestalk reporter gene constructs were allowed to synergise with wild-type cells. The results showed that both the prespore and

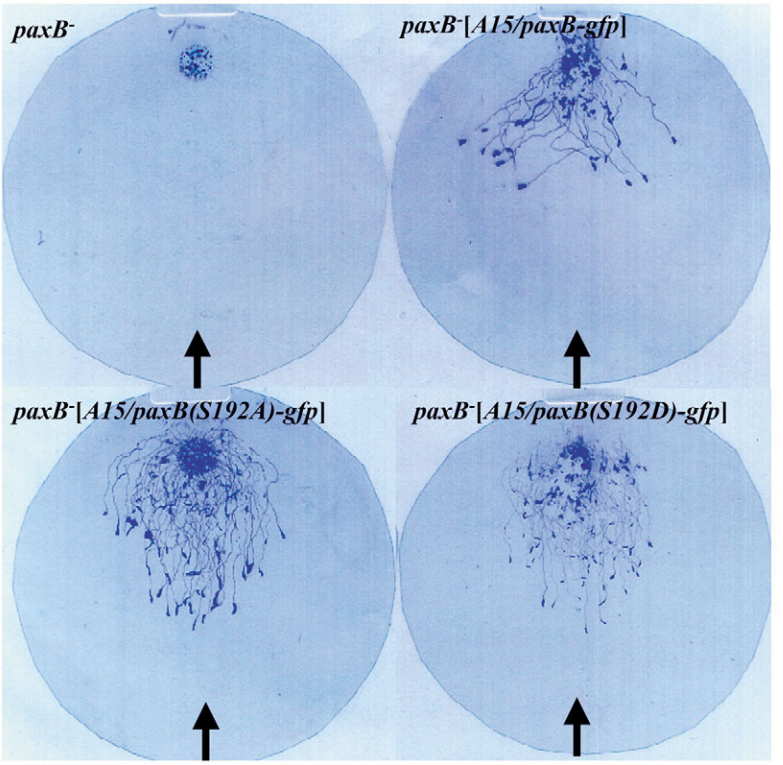

Fig. 11. PaxB phosphorylation mutants do not affect slug migration. Comparison of the migration of the paxB⁻ mutant with the *paxB⁻*[*A15/paxB-gfp*] mutant expressing the wild type PaxB under the control of the actin 15promoter with that PaxB phosphorylation mutants *paxB⁻*[*A15/paxB(S192A)-gfp*] and *paxB⁻*[*A15/paxB(S192D)-gfp*]. It can be seen that there is no significant difference in the rescue by the wild type and mutant PaxB forms.

prestalk cells occupied the back of the prespore and prestalk zones respectively, showing that the defects are cell autonomous and that they most likely reflect the inability of the cells to move (data not shown).

PaxB and Lim2 have nonredundant roles in development

The fact that the phenotype of the *paxB⁻* mutant is somewhat variable, i.e. dependent on the exact developmental conditions and genetic background led us to believe that there might be another closely related protein that could possibly partly substitute for the loss of PaxB function and that smaller changes in expression of this protein might result in variable penetrance of the mutant phenotype. The closest gene related to paxillin in *Dictyostelium* is another LIM domain protein Lim2, containing five Lim domains. Deletion of this protein has been shown to cause an arrest at the mound stage (Chien et al., 2000) a phenotype very reminiscent of the *paxB⁻* phenotype described here. To investigate a possible redundancy in the function of Lim2 and PaxB we investigated whether overexpression of paxB in *limB⁻* cells resulted in a rescue of the mound arrest phenotype. We first recreated the *limB⁻* mutant in an Ax2 background and confirmed the mound arrest phenotype previously observed in Ax3 (Chien et al., 2000). Transfection of this *limB⁻* strain expressing A15/paxB-gfp could not rescue the mound arrest phenotype and cells overexpressing PaxB did not sort from *limB⁻* cell expressing wild-type levels of PaxB (Fig. 14A,B). Interestingly these *limB⁻[A15/paxB-gfp]* cells still formed focal adhesion sites suggesting that the mound arrest in *limB⁻* cells was not due to the absence of focal adhesion sites (data not shown). Furthermore, we created a *paxB⁻/limB⁻* mutant which showed essentially the same phenotype as the *limB⁻* knockout and was arrested at the tipped mound stage. Sorting experiments between the *paxB⁻/limB⁻* and *limB⁻* strains showed that there was little sorting suggesting that the double mutant has essentially the same phenotype as the *limB⁻* mutant (data not shown). Also the *lim2⁻/paxB⁻* double mutant did not sort from the *lim2⁻*[*A15/paxB-gfp*] mutants. Interestingly the *paxB⁻* cells could sort in *limB⁻* cells to the centre of the mound, but could not rescue their development. This indicates that PaxB and LimB do not function in a partly redundant manner, but that both functions are needed for proper development and Lim2 may function downstream of PaxB.

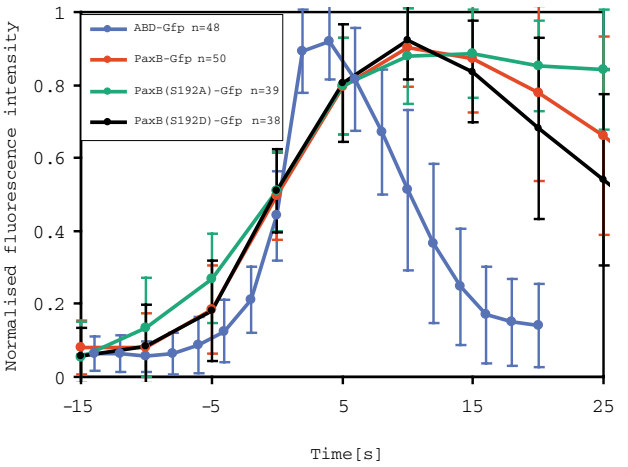

Fig. 12. Role of phosphorylation sites on focal adhesion kinetics. We measured the rate of formation of focal adhesion sites in *Dictyostelium* strains transformed with the A15/pax-gfppaxB-GFP construct and compared this with the kinetics of formation of actin-rich spots and *paxB⁻* mutant mutants in which the potential JNK phosphorylation site has been mutated to alanine (S192A) or aspartate (S192D) respectively (see Materials and Methods for details on measurements). Curves were aligned so that the half-maximal increase in fluorescence intensity occurred at *t*=0 seconds, the number of adhesion sites measured per strain is indicated in the legend. The results clearly show that the kinetics of the focal adhesion sites differ from that of the actin spots. Their rate of formation is slower and they persist longer than the actin spots. There was no significant difference between the rate of formation between wild type and mutant paxillin. The total lifetime of the focal adhesion sites is more difficult to determine, since most sites disassemble when they reach the end of the cells. The lifetime and possibly rate of disassembly are therefore mostly determined by the movement speed of the cells.

Discussion

The cellular localisation of PaxB during development

Focal adhesion sites have been well-documented structures in

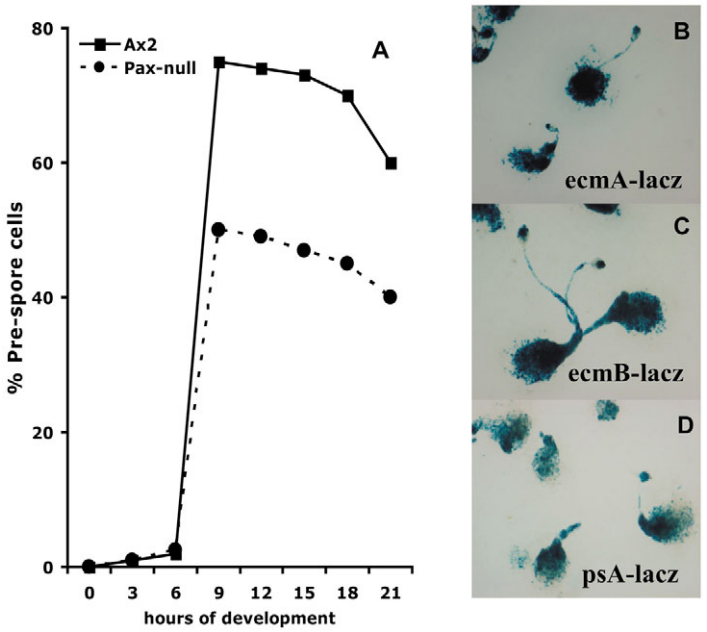

Fig. 13. Effect of paxillin on celltype differentiation. To assess the effect of paxillin loss on celltype-specific gene expression we measured the kinetics of prespore cell formation by using a prespore vesicle-specific polyclonal antibody as function of time. As can be seen for a typical experiment the number prespore cells starts to increase very dramatically at the mound stage. The time of the appearance of prespore cells is the same in the wild type and in the *paxB*$^{-}$ strain but levels do not exceed 50% in the *paxB*$^{-}$ knockout strain after which they start to decline, suggesting that many of the cells may differentiate into prestalk cells. This impression is confirmed by the transformation of the *paxB*$^{-}$ strains with *lacZ* expression constructs under the control of the prestalk-specific ecmA, ecmB and the prespore-specific PsA promoters. This shows that many of the cells, especially in the base of the structures express prestalk-specific markers.

mammalian cells, however they have not been described in detail before in simple eukaryotes, such as *Dictyostelium* or yeast. Recently a paxillin-like gene was found to play a role in growth orientation in yeast, which does not make focal adhesion sites, pointing to a role for paxillin in setting up polarity (Gao et al., 2004). Here we show that *Dictyostelium* cells make many PaxB-enriched sites during the process of cell migration. They are made at the front of the cells and remain stationary, while the cells move over them before they are rapidly disassembled in the retracting back of the cells (Fig. 3). These are all the characteristics of classical focal adhesion sites. The PaxB accumulations are found at areas of close cell-substrate contact as evident from the reflection interference measurements (Fig. 4). Investigation of the dynamics of their formation has indicated that they are formed at a rate rather faster than those observed in mammalian cells, which may be related to the fast rate of movement of *Dictyostelium* cells, especially in slugs where cells can easily move at a rate exceeding 50 μm/minute, which is roughly 20 times faster than most mammalian cells. The appearance and dynamics of these sites is different from the small actin-rich spots that have been described before in *Dictyostelium*, which appear and disappear at the ventral side of cells in areas of cell contact (Fig. 5). These spots have a approximately threefold faster assembly dynamics than the PaxB sites (Fig. 8). Furthermore their lifetime is considerably shorter and therefore these actin-rich sites presumably represent distinct structures that are regulated separately. The focal adhesion sites persist during the later stages of development, where the cells are in contact with the extracellular matrix. Due to the small size of these structures we have not been able to observe whether they exist between cells. Already in vegetative cells it can be seen that PaxB accumulates at the tips of fine filopodial structures that touch the substrate. In later development these filopodial structures are found mostly at the back of the migrating cells. It appears that when a cell moves forwards it holds on to cells behind it and the cell-cell contact sites form 'retraction' fibres. These structures become very prominent during the slug and culmination stages of development. In the

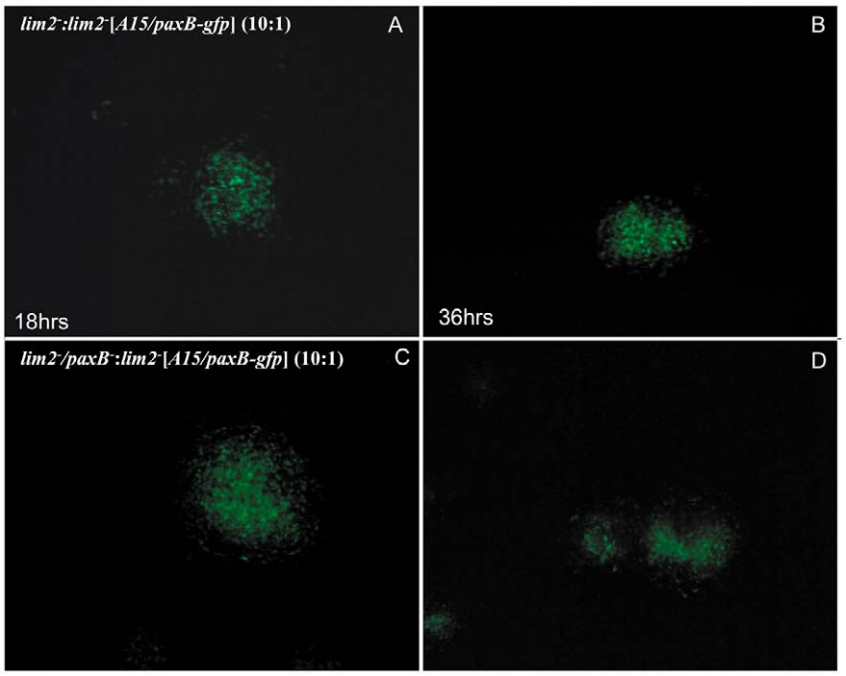

Fig. 14. Failure of complementation of Lim2 with paxB. To asses whether PaxB functions partly redundantly with its closest homologue Lim2 we performed synergy experiments between *lim2*$^{-}$ and cells of a strain overexpressing a PaxB-GFP fusion protein under the control of the strong actin15 promoter in the *lim2*$^{-}$ background. After 18 and 36 hours the cells are still stuck in the mound stage and the distribution of the lim2^{-} cells over-expressing PaxB-GFP is random within the mound, showing that over expression of PaxB cannot rescue the *lim2*$^{-}$ phenotype. When we tested sorting between a *lim2*$^{-}$/*paxB*$^{-}$ and lim2^{-} strain expressing PaxB-GFP we also found no evidence for further development and cell sorting after 18 and 36 hours of development, suggesting that lim2 and paxillin may act in the same process, with PaxB possibly upstream of Lim2. In both experiments the PaxB-GFP expressing cells were 10% of total cells.

culmination stage they appear to be more evenly distributed around the periphery of the cell, which may be caused by the fact that in these stages there is relatively little cell movement in these cells. It is interesting to speculate that the development defect observed in the *paxB*$^{-}$ mutant is caused by the failure of these retraction fibres to form, which then would suggest that they play an important role in polarised cell movement.

The role of PaxB in cell substrate adhesion

Dictyostelium cells have no clear integrin-like adhesion molecules but contain a number of adhesion receptors that appear to be responsible for cell substrate adhesion. During the vegetative stage these are the SAD receptors (Fey et al., 2002). SAD receptors are large transmembrane proteins with an extended extracellular domain, and mutation of individual members results in a loss of cell-substrate adhesion. They could act as potential initiators for the formation of the focal adhesion sites between the cells and the extracellular matrix. Less is known about their occurrence and role in later development. The phenotypes of the null mutants would suggest that the role of PaxB becomes more important when the cells enter the multicellular stages of development and have to move over the slime sheath and each other. This is also the stage where the cells start to secrete their own extracellular matrix in large amounts and migration of cells becomes very dependent on the exact substrates on which the cells move. Dissociated slug cells cannot move very effectively on glass or agar any more but they can move on cellulose and especially well on their own slime sheath (D.D. and C.J.W., unpublished observations), indicating the presence of special cell-matrix interactions most likely mediated by distinct receptors from those used during the earlier stages of development. Again it is not definitively known which molecules are responsible for these contacts, although it appears likely that they may be members of the *lagC* family, since deletion of *lagC* results in a mound arrest phenotype they are thought to be important in mediating cell-cell contacts and they are expressed for the first time during later aggregation (Dynes et al., 1994; Kibler et al., 2003; Sukumaran et al., 1998). Interestingly, we also observed a strong enrichment of PaxB in the cells that make contact with the stalk tube forming a layer of cell surrounding this structure, suggesting a role for special cell-cell contacts or cell-matrix interactions in these locations.

The role of PaxB in cell sorting and migration

We have shown that PaxB is expressed throughout development, but especially strongly during the mound and slug stages of development, i.e. stages where there is a lot of differential cell movement. It has been reported that paxillin is necessary for polarised cell migration, however that does not seem to be the case in the *Dictyostelium paxB*$^{-}$ mutant. The null cells migrate quite normally during the early stages of development, when the cells adhere to a variety of substrates. It seems likely that PaxB function becomes more important once the cells start to secrete their own extracellular matrix in the multicellular stages of development. During the later stages the cells have to move under and over each other, presumably involving new cell-cell and cell substrate interactions. Deletion of *paxB* resulted in severe defects in later development. *PaxB*$^{-}$ cells have difficulty in proceeding from the mound to the tipped mound stage (Fig. 9). Interestingly, this defect is dependent on the size of the mounds. Small mounds are less likely to make tips and stalks than larger mounds. The reason for this behaviour is unknown, but could reflect some kind of cooperative behaviour of the cells in the process of tip formation or alternatively could reflect defects in differentiation of 'tip' competent cells, which when this is an infrequent event would be less likely in small mounds than in larger mounds. The synergy experiments between *paxB*$^{-}$ cells and wild-type cells showed that *paxB*$^{-}$ cells are preferentially found in the back of the slug (Fig. 8) and that this sorting occurs at the end of the mound stage (Fig. 9) when the wild-type cells sort out to form the tip. It is worth noting that in the tipped mound stage the cell migration rates are much lower than during aggregation and during the subsequent slug migration stage. This is typical for the Ax2 strain, that does not show significant rotational cell movement during this stage of development and all effort seems to be concentrated on tip formation. The Ax3 parent strain is characterised by extensive rotational movement at this stage of development (Sternfeld, 1995). However, the rescue experiments where paxillin was expressed from prestalk- and prespore-specific promoters, showed that paxillin expression needs to be expressed in both prespore and prestalk cells to be able to achieve a complete rescue of development, especially slug migration, showing that all cells in the slug actively contribute to the migration of the slug.

PaxB is expressed somewhat stronger in prestalk than in prespore cells. Synergy experiments of wild-type cells in a *paxB*$^{-}$ background show that wild-type cells preferentially accumulate in the upper and lower cup of the fruiting body (Fig. 8D,G). It is thought that upper cup cells are especially important in the elevation of the spore mass from the substrate and it may be that these cells need to make especially strong cell-cell and possibly cell matrix contacts (Tsujioka et al., 1999). However, the rescue experiments where *paxB* was expressed from prestalk- and prespore-specific promoters showed that *paxB* expression needs to be expressed in prespore and prestalk cells to be able to achieve a complete rescue of development, especially slug migration, showing that all cells in the slug actively contribute to the migration of the slug.

The phenotype of the *paxB*$^{-}$ mutant is in many ways rather similar to that of the talin (*talB*$^{-}$) mutant (Tsujioka et al., 1999) and it is to be expected that these proteins may be involved in the same complex. It has not been reported that talin localises in small contact sites as we have observed for PaxB, but as with the PaxB this may require the generation of a low copy plasmid or a talB-GFP knockin strain. More recently it was shown that TalB is required for the force generation of slugs and that slugs lacking TalB generate less force per unit of volume than wild-type cells (Tsujioka et al., 2004). We are now investigating whether PaxB is also involved in the generation of traction forces in slugs.

Role of PaxB in differentiation

Paxillin is preferentially expressed in the upper and lower cup of the later structures and wild-type cells accumulate in the upper and lower cup in synergy experiment with *paxB*$^{-}$ cells. PaxB was also shown to be required for the proper differentiation of cells in prespore and prestalk cells. *PaxB*$^{-}$ mutants showed a reduced number of prespore cells and an

increased number of prestalk cells, indicating that PaxB plays a role in proper cell differentiation. It has been described in other systems that paxillin may shuttle through the nucleus where it may be involved in the transport of transcription factors, such as Stat3 and it has been shown to interact and activate steroid receptors, especially Hic5 and affect transcription by acting as binding partners and co-activators for steroid receptors. Our observations with the paxillin GFP knockin strain certainly confirm the presence of paxillin in the nucleus but there appears to be no particular enrichment there. From the experiments described here it cannot not be conclusively concluded whether the effect of PaxB is a direct or indirect effect on differentiation. It could be that PaxB affects the differentiation of cells and as a result of this, morphogenesis is affected, however it could equally well be argued that *paxB*$^{-}$ cells are defective in movement resulting in cells differentiating initially in prestalk and prespore cells, but then they are exposed to the wrong signals and change their differentiation. A hint for this process is seen in the time course of the differentiation of prespore cells which seems to indicate that initially more prespore cells differentiate, but that at later stages of development these cells lose their prespore differentiation state again (Fig. 9A).

Signalling pathways controlling PaxB function

In vertebrates paxillin is highly phosphorylated in conserved tyrosine residues Y31 and Y118 (Brown and Turner, 2004). These residues are not conserved in *Dictyostelium*, nor are any of the other known tyrosine phoshorylated sites. This is not unexpected since *Dictyostelium* does not contain obvious focal adhesion kinases or Src related kinase homologues. The absence of a clear LD4 domain in PaxB, which in vertebrate paxillin is a major interaction site with Fak/PYK2 appears to support the assertion that tyrosine phosphorylation is not likely to be a major regulator of PaxB function. However some of the sites phosphorylated by serine/threonine kinases are conserved in PaxB. Ser178 that is phosphorylated by Jun kinase in mammalian cells has been implicated in the control of cell migration (Huang et al., 2004; Huang et al., 2003). Phosphorylation site mutants in the corresponding site in paxB (S192), i.e. mutants that could not be phosphorylated (S192A) or that should mimic the constitutive phosphorylated form (S192D) did not show any differences in their ability to rescue development when compared with *paxB*$^{-}$. Both forms could rescue the *paxB*$^{-}$ mutant phenotype with respect to ability to complete development and slug migration equally well. Mutation of these sites also did not influence the rate of assembly in the characteristic PaxB spots although they could affect the interaction with other proteins not rate limiting in the assembly of the complexes. There are however a number of other protein kinases especially of the MAP kinase family that have been implicated in chemotaxis (Gaskins et al., 1996; Kosaka et al., 1998; Kosaka and Pears, 1997; Maeda and Firtel, 1997; Wang et al., 1998). A first step towards establishing whether phosphorylation plays a role in paxillin regulation will be to determine, which sites are phosphorylated in vivo after the cells contact different substrates and after stimulation with chemo-attractants such as cAMP. Finally in vertebrates paxillin interacts with a number of other proteins, especially vinculin and actopaxin through the LD1 and LD4 domains. The *Dictyostelium* genome contains a sequence distantly related to meta-vinculin. So far this protein has not yet been characterised and it remains to be seen whether it will interact with PaxB.

PaxB does not function redundantly with Lim2

The *paxB*$^{-}$ mutant has a phenotype that is shared by many other cytoskeletal mutants, especially the *talB*$^{-}$ and *lim2*$^{-}$ mutants, but also mutants in the actin-myosin cytoskeleton, such as the α-actinin/gelation factor double null mutant and the *myosinII*$^{-}$ mutant, namely a mound arrest phenotype. This shows that the cytoskeletal organisation is particularly important for later development. Our experiments have shown that there is no clear genetic interaction between the PaxB and Lim2 function. Expression of PaxB-GFP in the *lim2*$^{-}$ strain could not complement its function, while the assembly of PaxB spots was normal in the *lim2*$^{-}$ background showing that Lim2 does not play a role in assembly of these structures. Synergy experiments between the *limB*$^{-}$ and *limB*$^{-}$*/paxB*$^{-}$ double mutants showed that their functions do not appear to be additive. Therefore these molecules could act in the same pathway with PaxB acting possibly upstream of LimB.

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References

Bach, I. (2000). The LIM domain: regulation by association. *Mech. Dev.* **91**, 5-17.

Bretschneider, T., Diez, S., Anderson, K., Heuser, J., Clarke, M., Muller-Taubenberger, A., Kohler, J. and Gerisch, G. (2004). Dynamic actin patterns and Arp2/3 assembly at the substrate-attached surface of motile cells. *Curr. Biol.* **14**, 1-10.

Brown, M. C. and Turner, C. E. (2004). Paxillin: adapting to change. *Physiol. Rev.* **84**, 1315-1339.

Chien, S., Chung, C. Y., Sukumaran, S., Osborne, N., Lee, S., Ellsworth, C., McNally, J. G. and Firtel, R. A. (2000). The *Dictyostelium* LIM domain-containing protein LIM2 is essential for proper chemotaxis and morphogenesis. *Mol. Biol. Cell* **11**, 1275-1291.

Devreotes, P. and Janetopoulos, C. (2003). Eukaryotic chemotaxis: Distinctions between directional sensing and polarization. *J. Biol. Chem.* **2**, 20445-20448.

Dingermann, T., Reindl, N., Werner, H., Hildebrandt, M., Nellen, W., Harwood, A., Williams, J. and Nerke, K. (1989). Optimization and in situ detection of Escherichia coli beta-galactosidase gene expression in *Dictyostelium* discoideum. *Gene* **85**, 353-362.

Dormann, D., Kim, J. Y., Devreotes, P. N. and Weijer, C. J. (2001). cAMP receptor affinity controls wave dynamics, geometry and morphogenesis in *Dictyostelium*. *J. Cell Sci.* **114**, 2513-2523.

Dynes, J. L., Clark, A. M., Shaulsky, G., Kuspa, A., Loomis, W. F. and Firtel, R. A. (1994). LagC is required for cell-cell interactions that are essential for cell-type differentiation in *Dictyostelium*. *Genes Dev.* **8**, 948-958.

Fey, P., Stephens, S., Titus, M. A. and Chisholm, R. L. (2002). SadA, a novel adhesion receptor in *Dictyostelium*. *J. Cell Biol.* **159**, 1109-1119.

Fischer, M., Haase, I., Simmeth, E., Gerisch, G. and Muller-Taubenberger, A. (2004). A brilliant monomeric red fluorescent protein to visualize cytoskeleton dynamics in *Dictyostelium*. *FEBS Lett.* **577**, 227-232.

Gao, X. D., Caviston, J. P., Tcheperegine, S. E. and Bi, E. (2004). Pxl1p, a paxillin-like protein in Saccharomyces cerevisiae, may coordinate Cdc42p and Rho1p functions during polarized growth. *Mol. Biol. Cell* **15**, 3977-3985.

Gaskins, C., Clark, A. M., Aubry, L., Segall, J. E. and Firtel, R. A. (1996).

The *Dictyostelium* MAP kinase ERK2 regulates multiple, independent developmental pathways. *Genes Dev.* **10**, 118-128.

Giannone, G., Dubin-Thaler, B. J., Dobereiner, H. G., Kieffer, N., Bresnick, A. R. and Sheetz, M. P. (2004). Periodic lamellipodial contractions correlate with rearward actin waves. *Cell* **116**, 431-443.

Hagel, M., George, E. L., Kim, A., Tamimi, R., Opitz, S. L., Turner, C. E., Imamoto, A. and Thomas, S. M. (2002). The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol. Cell. Biol.* **22**, 901-915.

Howard, P. K., Ahern, K. G. and Firtel, R. A. (1988). Establishment of a transient expression system for Dictyostelium discoideum. *Nucl. Acids Res.* **16**, 2613-2623.

Huang, C., Rajfur, Z., Borchers, C., Schaller, M. D. and Jacobson, K. (2003). JNK phosphorylates paxillin and regulates cell migration. *Nature* **424**, 219-223.

Huang, C., Jacobson, K. and Schaller, M. D. (2004). A role for JNK-paxillin signaling in cell migration. *Cell Cycle* **3**, 4-6.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.

Iijima, M., Huang, Y. E. and Devreotes, P. (2002). Temporal and spatial regulation of chemotaxis. *Dev. Cell* **3**, 469-478.

Kibler, K., Svetz, J., Nguyen, T. L., Shaw, C. and Shaulsky, G. (2003). A cell-adhesion pathway regulates intercellular communication during *Dictyostelium* development. *Dev. Biol.* **264**, 506-521.

Kosaka, C. and Pears, C. J. (1997). Chemoattractants induce tyrosine phosphorylation of ERK2 in *Dictyostelium discoideum* by diverse signalling pathways. *Biochem. J.* **324**, 347-352.

Kosaka, C., Khosla, M., Weeks, G. and Pears, C. (1998). Negative influence of rasg on chemoattractant-induced erk2 phosphorylation in *Dictyostelium*. *Biochim. Biophys. Acta Mol. Cell Res.* **1402**, 1-5.

Kreitmeier, M., Gerisch, G., Heizer, C. and Müller-Tautenberger, A. (1995). A talin homologue of *Dictyostelium* rapidly assembles at the leading edge of cells in response to chemoattractant. *J. Cell Biol.* **129**, 179-188.

Liu, S., Kiosses, W. B., Rose, D. M., Slepak, M., Salgia, R., Griffin, J. D., Turner, C. E., Schwartz, M. A. and Ginsberg, M. H. (2002). A fragment of paxillin binds the alpha 4 integrin cytoplasmic domain (tail) and selectively inhibits alpha 4-mediated cell migration. *J. Biol. Chem.* **277**, 20887-20894.

Lo, C. M., Wang, H. B., Dembo, M. and Wang, Y. L. (2000). Cell movement is guided by the rigidity of the substrate. *Biophys. J.* **79**, 144-152.

Maeda, M. and Firtel, R. A. R. A. (1997). Activation of the mitogen activated protein kinase erk2 by the chemoattractant folic acid in *Dictyostelium*. *J. Biol. Chem.* **272**, 23690-23695.

Manstein, D. J., Schuster, H. P., Morandini, P. and Hunt, D. M. (1995). Cloning vectors for the production of proteins in *Dictyostelium discoideum*. *Gene* **162**, 129-134.

Morrison, A., Blanton, R. L., Grimson, M., Fuchs, M., Williams, K. and Williams, J. (1994). Disruption of the gene encoding the EcmA, extracellular matrix protein of *Dictyostelium* alters slug morphology. *Dev. Biol.* **163**, 457-466.

Needham, L. K. and Rozengurt, E. (1998). Galpha12 and Galpha13 stimulate Rho-dependent tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130 Crk-associated substrate. *J. Biol. Chem.* **273**, 14626-14632.

Niewöhner, J., Weber, I., Maniak, M., Muller-Taubenberger, A. and Gerisch, G. R. A. (1997). Talin null cells of *Dictyostelium* are strongly defective in adhesion to particle and substrate surfaces and slightly impaired in cytokinesis. *J. Cell Biol.* **138**, 349-361.

Pang, K. M., Lee, E. and Knecht, D. A. (1998). Use of a fusion protein between GFP and an anti-binding domain to visualize transient filamentous-actin structures. *Curr. Biol.* **8**, 405-408.

Rivero, F., Koppel, B., Peracino, B., Bozzaro, S., Siegert, F., Weijer, C. J., Schleicher, M., Albrecht, R. and Noegel, A. A. (1996). The role of the cortical cytoskeleton: F-actin crosslinking proteins protect against osmotic stress, ensure cell size, cell shape and motility, and contribute to phagocytosis and development. *J. Cell Sci.* **109**, 2679-2691.

Schaller, M. D. and Schaefer, E. M. (2001). Multiple stimuli induce tyrosine phosphorylation of the Crk-binding sites of paxillin. *Biochem. J.* **360**, 57-66.

Schlaepfer, D. D. and Hunter, T. (1998). Integrin signalling and tyrosine phosphorylation: just the FAKs? *Trends Cell Biol.* **8**, 151-157.

Siegert, F. and Weijer, C. (1989). Digital image processing of optical density wave propagation in *Dictyostelium discoideum* and analysis of the effects of caffeine and ammonia. *J. Cell Sci.* **93**, 325-335.

Smilenov, L. B., Mikhailov, A., Pelham, R. J., Marcantonio, E. E. and Gundersen, G. G. (1999). Focal adhesion motility revealed in stationary fibroblasts. *Science* **286**, 1172-1174.

Sternfeld, J. (1995). Spiral and concentric waves organize multicellular *Dictyostelium* mounds. *Curr. Biol.* **5**, 937-943.

Sukumaran, S., Brown, J. M., Firtel, R. A. and McNally, J. G. (1998). Lagc-null and gbf-null cells define key steps in the morphogenesis of *Dictyostelium* mounds. *Dev. Biol.* **200**, 16-26.

Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* **28**, 9-29.

Sutoh, K. (1993). A transformation vector for *Dictyostelium discoideum* with a new selectable marker Bsr. *Plasmid* **30**, 150-154.

Ti, Z. C., Wilkins, M. R., Vardy, P. H., Gooley, A. A. and Williams, K. L. (1995). Glycoprotein complexes interacting with cellulose in the cell print zones of the *Dictyostelium discoideum* extracellular-matrix. *Dev. Biol.* **168**, 332-341.

Tsujioka, M., Machesky, L. M., Cole, S. L., Yahata, K. and Inouye, K. (1999). A unique talin homologue with a villin headpiece-like domain is required for multicellular morphogenesis in *Dictyostelium*. *Curr. Biol.* **9**, 389-392.

Tsujioka, M., Yoshida, K. and Inouye, K. (2004). Talin B is required for force transmission in morphogenesis of *Dictyostelium*. *EMBO J.* **23**, 2216-2225.

Tumbarello, D. A., Brown, M. C. and Turner, C. E. (2002). The paxillin LD motifs. *FEBS Lett.* **513**, 114-118.

Turner, C. E. (1991). Paxillin is a major phosphotyrosine-containing protein during embryonic development. *J. Cell Biol.* **115**, 201-207.

Turner, C. E. (2000). Paxillin interactions. *J. Cell Sci.* **113**, 4139-4140.

Uchida, K. and Yumura, S. (2004). Dynamics of novel feet of *Dictyostelium* cells during migration. *J. Cell Sci.* **117**, 1443-1455.

Wade, R., Bohl, J. and Vande Pol, S. (2002). Paxillin null embryonic stem cells are impaired in cell spreading and tyrosine phosphorylation of focal adhesion kinase. *Oncogene* **21**, 96-107.

Wang, Y., Liu, J. and Segall, J. E. (1998). MAP kinase function in amoeboid chemotaxis. *J. Cell Sci.* **111**, 373-383.

Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T. and Horwitz, A. F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell. Biol.* **6**, 154-161.

Weijer, C. J. (2003). Visualizing signals moving in cells. *Science* **300**, 96-100.

Weijer, C. J. and Durston, A. J. (1985). Influence of cAMP and hydrolysis products on cell type regulation in Dictyostelium discoideum. *J. Embryol. Exp. Morph.* **86**, 19-37.

Wong, L. L., Dynes, J. and Firtel, R. A. (1995). Functional-analysis of lagc, a putative transmembrane protein essential for cellular-differentiation and multicellular development in *Dictyostelium*. *Dev. Biol.* **170**, 757-757.

Woodrow, M. A., Woods, D., Cherwinski, H. M., Stokoe, D. and McMahon, M. (2003). Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf→MEK→ERK pathway. *Exp. Cell Res.* **287**, 325-338.

Yano, H., Mazaki, Y., Kurokawa, K., Hanks, S. K., Matsuda, M. and Sabe, H. (2004). Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. *J. Cell Biol.* **166**, 283-295.

Zachary, I., Sinnett-Smith, J., Turner, C. E. and Rozengurt, E. (1993). Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *J. Biol. Chem.* **268**, 22060-22065.