An ezrin-rich, rigid uropod-like structure directs movement of amoeboid blebbing cells

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Summary

Melanoma cells can switch between an elongated mesenchymal-type and a rounded amoeboid-type migration mode. The rounded ‘amoeboid’ form of cell movement is driven by actomyosin contractility resulting in membrane blebbing. Unlike elongated A375 melanoma cells, rounded A375 cells do not display any obvious morphological front–back polarisation, although polarisation is thought to be a prerequisite for cell movement. We show that blebbing A375 cells are polarised, with ezrin (a linker between the plasma membrane and actin cytoskeleton), F-actin, myosin light chain, plasma membrane, phosphatidylinositol (4,5)-bisphosphate and β1-integrin accumulating at the cell rear in a uropod-like structure. This structure does not have the typical protruding shape of classical leukocyte uropods, but, as for those structures, it is regulated by protein kinase C. We show that the ezrin-rich uropod-like structure (ERULS) is an inherent feature of polarised A375 cells and not a consequence of cell migration, and is necessary for cell invasion. Furthermore, we demonstrate that membrane blebbing is reduced at this site, leading to a model in which the rigid ezrin-containing structure determines the direction of a moving cell through localised inhibition of membrane blebbing.

Key words: Ezrin, Amoeboid, Membrane blebbing, Migration, Uropod

Introduction

Cells adopt different morphologies and modes of movement depending on various factors, such as adhesion (Friedl et al., 1998; Renkawitz et al., 2009; Sroka et al., 2002), contractility (Leader et al., 1983; Polte et al., 2004), Rho-family GTPase signalling (Kolyada et al., 2003; Sanz-Moreno et al., 2008) and the composition and rigidity of the extracellular matrix (ECM) (Pelham and Wang, 1997; Young and Herman, 1985). Changes in any of these factors can induce switches in cellular morphology and the mode of migration. This plasticity allows cells to continue to migrate in response to changes in their environment and might facilitate the movement of metastasising cancer cells through heterogeneous physical and chemical environments (Wolf et al., 2003a).

Single migrating cells can adopt either a mesenchymal-type elongated mode of movement, which requires degradation of the ECM by proteases, or a rounded mode of movement, where cells move by membrane blebbing (Sahai and Marshall, 2003) or by squeezing through pores in the ECM (Wolf et al., 2003a). Non-mesenchymal types of cancer cell movement are often referred to as being amoeboid-like but are clearly different from the amoeboid movement of leukocytes or Dictyostelium, where cells adhere to the substrate, form actin-dependent lamellipodia, filopodia or pseudopods in the direction of movement and project a uropod at the cell rear. This uropod contains membrane receptors, such as integrins, intercellular adhesion molecules (ICAMs), hyaluronic acid receptor CD44 or P-selectin glycoprotein ligand-1 (PSGL-1), signalling molecules, such as ezrin-radixin-moesin (ERM) family proteins, protein kinase A (PKA) and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)2], and organelles, such as the Golgi complex and the microtubule organising centre (MTOC) (Sanchez-Madrid and Serrador, 2009). Uropods are associated with multiple processes necessary for amoeboid cell movement, such as rear retraction, receptor recycling, trafficking and secretion (Sanchez-Madrid and Serrador, 2009).

Different subtypes of amoeboid movement have been described recently (Friedl and Wolf, 2009; Lammermann and Sixt, 2009), and in some of these types no adhesion zones, which mediate interactions with the substrate, are formed at the front of the cell (Renkawitz et al., 2009). Leukocytes are able to adopt an integrin-independent type of movement, suggesting little or no dependence on adhesion (Friedl et al., 1998; Lammermann et al., 2008; Malawista et al., 2000; Woolf et al., 2007). One type of non-adhesive amoeboid movement is characterised by the formation of membrane blebs. This was first described during embryonic development (Rebhun, 1963; Trinkaus, 1973; Trinkaus and Lentz, 1967) and has been observed in Dictyostelium (Langridge and Kay, 2006; Yoshida and Soldati, 2006) and cancer cells (Cunningham et al., 1992; Keller and Zimmermann, 1986). Cells with blebbing amoeboid movement lack apparent polarisation and have high actomyosin contractility (Friedl and Wolf, 2009; Lammermann and Sixt, 2009) generated by signalling through RhoA–Rho-associated protein kinase (ROCK) and Cdc42–myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (Gadea et al., 2008; Sahai and Marshall, 2003). Blebbing is initiated by a rupture of the submembrane actin cortex (Paluch et al., 2005) or a detachment of the plasma membrane from the cortex (Charras et al., 2005; Dai and Sheetz, 1999; Maugis et al., 2010). Cytoplasm is pushed through such ruptures to form membrane blebs (Cunningham, 1995; Paluch et al., 2006) that grow until actin-binding proteins, such as those of the ERM family, are assembled in the blob, which in turn recruit actin and actin-bundling proteins to the blob membrane to form a new cortex. Finally, myosin II is recruited and drives retraction of the blob (Charras et al., 2006).
In confined spaces, blebbing cells can move forward without adhesion by a process termed ‘chimneying’ (Hawkins et al., 2009; Malawista and de Boisfleury Chevance, 1997). In fibrillar three-dimensional matrices, such as collagen gels, blebs can expand into pores in the matrix, causing the squeezing phenotype observed in amoeboid lymphocytes and cancer cells in collagen gels (Haston and Shields, 1984; Wolf et al., 2003a). Cells that form small blebs can use them to ‘elbow’ their way through the meshwork of the ECM (Friedell and Wolf, 2009). The site of bleb formation determines the direction of cell movement (Keller and Bebie, 1996). However, some cells, such as A375 melanoma cells, form multiple blebs over the membrane and would therefore not be able to move directionally. However, A375 melanoma cells are able to invade into a collagen matrix and move in vitro and in vivo (Sahai and Marshall, 2003; Sanz-Moreno et al., 2008). As asymmetry is a prerequisite of cell movement (Petrie et al., 2009), this suggests some form of cellular polarisation. To understand cell movement driven by multiple blebs, we investigated polarisation in blebbing ‘amoeboid’ A375 melanoma cells. We show that A375 cells form an ezrin-rich uropod-like structure (which we call the ERULS) at their cell rear, and that this is required for invasion into a three-dimensional matrix. We present a model whereby accumulation of ezrin, membrane and actin in this structure reflects strong connections between the plasma membrane and the submembrane cortex, leading to the formation of a rigid structure that inhibits blebbing at the back of the cell. This leads to net movement of the cell in the direction opposite to the uropod-like structure. Our model explains how cells can move without a defined front and only a defined rear.

**Results**

**Polarisation of blebbing amoeboid cells**

60% of motile A375P melanoma cells have a rounded morphology and exhibit high blebbing activity, but they lack an obvious morphological front–back axis (Fig. 1A, supplementary material Movie 1). To investigate the polarisation of these rounded cells, we re-examined the localisation of ezrin and β1-integrin (Sahai and Marshall, 2003). A375P cells were seeded on top of a thick pliable collagen-I matrix, where they adopt the same morphologies and types of movement as they do inside a three-dimensional collagen-I matrix (Sanz-Moreno et al., 2008), and were imaged using confocal microscopy (Fig. 1B). Co-staining of cells with antibodies against ezrin and β1-integrin showed that ezrin and β1-integrin localised at the plasma membrane and were highly enriched in a spot or cap at one end of the cell (Fig. 1B). Immunostaining for phosphorylated ERM proteins demonstrated that ezrin was in an active phosphorylated state in this spot (Fig. 1C). Ectopically expressed ezrin fused to mCherry (or other fluorescent proteins) had the same subcellular distribution as endogenous ezrin and colocalised with phosphorylated ezrin (Fig. 1D). To confirm that this spot represents an accumulation of ezrin and not simply an accumulation of plasma membrane (Dewitt et al., 2009), a membrane marker (YFP–Mem) was coexpressed and imaged together with ezrin–CFP. Fig. 1E shows that the plasma membrane was condensed (i.e. enriched) at the site of ezrin localisation; however, a ratio image of the ezrin and membrane shows that ezrin was accumulated at this spot in relation to the membrane as a whole. This result was confirmed using different ezrin- and membrane-labeling constructs (supplementary material Fig. S1). As ezrin binds to PtdIns(4,5)P₂ through its N-terminal FERM domain (Niggli et al., 1995), we tested for PtdIns(4,5)P₂ accumulation using RFP-fused to the pleckstrin homology (PH) domain of phospholipase C delta (RFP–PLCδ-PH) (Varnai and Balla, 1998). Cells coexpressing RFP–PLCδ-PH, YFP–Mem and ezrin–CFP had PtdIns(4,5)P₂ accumulated in this spot (Fig. 1F). Fig. 1G shows pairwise intensity plots. Because the C-terminus of ezrin binds to the actin cytoskeleton, the accumulation of actin at the spot was analyzed in A375P cells expressing ezrin–GFP and stained with Texas-Red–phalloidin (Fig. 1H). All the round cells showed a strong submembrane cortical actin staining that was increased at the site of the ezrin spot. To identify other components of the ezrin-rich spot, we showed that GFP-labelled myosin light chain (MLC) colocalises with ezrin (Fig. 1I), and phosphorylated MLC colocalises with β1-integrin (Fig. 1J).

**Ezrin accumulates at the cell rear**

Ezrin, actin and myosin play a role in the formation and function of uropods in leukocytes (Sanchez-Madrid and Serrador, 2009). To test whether the ezrin spot might be similar to a uropod, we stained A375P cells for the uropod markers CD44 and PSGL-1 (Fig. 1I,K,L). CD44 (Fig. 1K) and PSGL-1 (Fig. 1L) were concentrated at the spot, which means that the ezrin spot is localised at the side opposite to the uropod-like structure. To ascertain whether blebbing ‘amoeboid’ cells in other melanoma cell lines have an ezrin spot at the rear, during persistent movement (Fig. 2; supplementary material Movies 2 and 3). In all 22 cells analysed, ezrin was localised at the rear; we never observed a moving cell with ezrin localised at the front or side. To show the relationship between the direction of cell movement and the localisation of the ezrin spot, we produced ‘quiver’ plots of single cells that show the migration path of a cell depicted as vectors connecting the ezrin spot and the cell centre at each time point (Fig. 2B). Overall, during periods of persistent movement the vectors point in the general direction of cell movement at that time, which means that the ezrin spot is localised at the side opposite to the direction of movement. To ascertain whether blebbing ‘amoeboid’ cells in other melanoma cell lines have an ezrin spot at the rear, localisation of ezrin–GFP was also examined in WM3629 and WM1361 cells moving inside a collagen matrix. As for A375 cells, both of these cell lines can adopt an elongated or rounded shape in three-dimensional gels, so we specifically selected round cells for imaging. In WM3629 (supplementary material Movie 4) and WM1361 cells (supplementary material Movie 5), ezrin was localised at the cell rear. We conclude that ezrin localisation at the cell rear is a general phenomenon observed in moving cells with a rounded blebbing morphology. This localisation at the rear of the cell, together with the molecular composition, suggests that the ezrin-containing spot in blebbing tumour cells represents a uropod-like structure, which we call the ERULS.

**ROCK and novel PKC isoforms regulate the ERULS**

ROCK is necessary for uropod function in leukocytes (Smith et al., 2003; Worthylake and Burridge, 2003), and inhibition of ROCK
disrupts uropod morphology, although some cells retain a polar ezrin cap (Lee et al., 2004). To assess regulation of the ERULS by ROCK, we treated A375 cells expressing ezrin–GFP, and seeded onto a thick collagen matrix, with the ROCK inhibitor H-1152 (Tamura et al., 2005) (Fig. 3A). The effect on the ERULS was evaluated by quantifying the fraction of the cells with a rounded morphology that contained a localised ezrin–GFP spot. Given that the A375P cell line contains both cells with a rounded morphology and cells with Rac-dependent protrusions (Sanz-Moreno et al., 2008), only round cells without protrusions were scored in this experiment. On average 40–50% of rounded cells had polarised ezrin localisation (Fig. 3A). Following inhibition of ROCK, the fraction of round cells displaying an ezrin spot or cap was strongly decreased, from 47±5% to 15±6% (Fig. 3A). Treatment with H-1152, for more than 20 minutes, resulted in cells forming protrusions and switching to an elongated morphology (Sanz-Moreno et al., 2008).

Protein kinase C (PKC) isoforms have been shown to regulate ezrin and uropods (Ng et al., 2001; Niggli et al., 1996; Ren et al., 2009; Rossy et al., 2007; Simons et al., 1998; Wald et al., 2008). The PKC family comprises the conventional types, activated by diacylglycerol (DAG) in a Ca\(^{2+}\)-dependent manner, novel types, activated by DAG but Ca\(^{2+}\)-independent, and the atypical types, which do not require DAG or Ca\(^{2+}\) for activation (Mellor and Parker, 1998). In migrating Walker 256 carcinosarcoma cells, uropods are negatively regulated by PKC (Niggli et al., 1996; Rossy et al., 2007). We therefore examined whether the localisation of the ezrin spot was affected by the DAG analogue phorbol 12-
myristate 13-acetate (PMA), which activates classical- and novel-type PKCs, 1,2-bis-(o-aminophenoxo)ethane-N,N',N''-N'''-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), a Ca\(^{2+}\) chelator, or the PKC inhibitors bisindolylmaleimide I (BimI), Ro32-0432 or Gö 6976. A375P cells expressing ezrin–GFP were seeded on top of a thick collagen matrix and treated with PMA, BimI, Ro32-0432, Gö 6976 and/or BAPTA-AM and the fraction of cells with polarised ezrin localisation was quantified (Fig. 3B,C). Treatment with PMA significantly decreased the number of cells with localised ezrin, from 40±5% to 5±3%, whereas treatment with the general PKC inhibitors BimI or Ro32-0432 increased the fraction of cells with an ezrin spot to 62±17% and 70±16%, respectively (Fig. 3C). Interestingly, at higher concentrations, BimI also increased the number of ezrin spots per cell (Fig. 3B). Pretreatment of cells with Ro32-0432 or BimI negated the effect of PMA. Treatment with Gö 6976, a specific inhibitor of conventional Ca\(^{2+}\)-dependent PKCs, alone or in combination with PMA, did not increase the fraction of cells with localised ezrin. In addition, Ca\(^{2+}\) chelation, by pretreatment with BAPTA-AM, did not negate the effect of PMA. In order to validate these results, and to identify the specific PKC isoform responsible for ERULS regulation, polarisation of ezrin was assessed in cells upon knockdown of each novel PKC isoform (Fig. 3E,F). For each isoform knockdown, a pool of four small interfering RNAs (siRNAs) (Fig. 3E) and two individual siRNAs (Fig. 3F), targeting different sequences from those in the pool, were used. Knockdown of PKC\(\theta\) increased the number of cells with polarised ezrin (Fig. 3E,F), indicating that PKC\(\theta\) is the isoform responsible for the effects observed with the DAG and PKC inhibitors. Knockdown of PKC\(\delta\) or PKC\(\eta\) had no significant effect on ezrin polarisation (Fig. 3E,F). Interestingly, knockdown of PKC\(\varepsilon\) decreased the number of cells with polarised ezrin. These experiments demonstrate that the ERULS in A375P cells is regulated by the DAG-dependent and Ca\(^{2+}\)-independent novel-type PKCs, with PKC\(\varepsilon\) being a positive and PKC\(\theta\) being a negative regulator of the ERULS. Taken together, our observations indicate that the ERULS is similar to a uropod, despite the lack of a protruding morphology typical of uropods.

The Golgi is located in front of the nucleus in blebbing cells

In migrating leukocytes, the Golgi is located at the back of the cell, between the uropod and the nucleus (Biberfeld, 1971; Rosenstreich et al., 1972; Serrador et al., 1999), whereas in migrating fibroblasts the Golgi is located in the front of the cell, between the nucleus and the leading edge (Kupfer et al., 1982). In order to assess Golgi alignment in blebbing A375 cells, A375-M2 cells expressing ezrin–mCherry and the Golgi marker GalT–CFP were plated onto a thick collagen matrix, fixed after 2 hours and imaged (Fig. 4A). Cells were scored only if the Golgi, nucleus and ERULS were located in a line and in the same plane, so as to allow for analysis of two-dimensional images. Fig. 4B shows that the predominant alignment of A375-M2 cells on collagen clearly had the Golgi on the opposite side of the nucleus to the ERULS (90±3% of cells). The alignment of Golgi, nucleus and ERULS in blebbing melanoma cells with rounded amoeboid movement (Golgi in the front) is thus the opposite of that in classical amoeboid cells, such as leukocytes, that have the Golgi at the rear towards the uropod (Biberfeld, 1971; Rosenstreich et al., 1972; Serrador et al., 1999).

The ERULS is an inherent polarisation structure of rounded blebbing cells

Having shown that the ERULS is located at the back of moving A375-M2 cells, we wanted to discriminate whether its formation is a consequence of cell movement or whether it is an inherent feature of cell polarisation that precedes cell migration. Because A375-M2 cells in suspension have the same round and blebbing shape as that of blebbing cells on a pliable matrix, we compared cells that are round, blebbing and polarised when moving on a matrix (collagen) to when the cells were in suspension. The ERULS–Golgi–nuclear alignment was used as a marker of cell polarisation in A375-M2 cells. Cells were detached from a plastic cell culture dish, where they are polarised but cannot adopt the rounded shape. Cells were then fixed in suspension and imaged with confocal microscopy. Fig. 4B shows that in blebbing A375-
M2 cells, whether on top of collagen or in suspension, the Golgi, as visualised by GalT–CFP, was aligned in front of the nucleus, opposite to the ERULS (in 90±3% and 77±10% of cells, respectively). These experiments demonstrate that the ERULS is an inherent feature of polarisation in round blebbing cells, and its localisation is not a result of cell movement.

Fig. 3. Ezrin polarisation requires ROCK activity and is inhibited by novel-type protein kinase C. (A–D) A375 cells transfected with ezrin–GFP were seeded on top of a thick collagen-I matrix, treated with inhibitors and the fraction of cells with polarised localisation of ezrin quantified. Average values from four independent experiments (+s.d.) and P values from unpaired Student’s t-test are shown (A,C,D). (A) Results from cells treated with ROCK inhibitor H-1152 for 20 minutes compared with those in untreated cells (ctrl). (B) Typical images of untreated cells and cells treated with PMA or with BimI. (C) Results from untreated (ctrl) cells are compared with those from cells treated with PMA, and the PKC inhibitors BimI, Ro32-0432 (Ro) or Gö 6976 (Gö). PMA-treated cells are compared with cells pretreated with PKC inhibitors or BAPTA-AM. (D) Results from cells treated with BimI are compared with those in untreated cells and the respective DMSO controls. (E,F) Polarisation of ezrin in cells transfected with an siRNA pool (E) or single siRNAs (F) against novel PKC isoforms. The level of PKC isoform knockdown, measured by quantitative PCR, is given for each siRNA (lower panels). Average values from eight (E) and three (F) independent experiments (+s.d.) and P values from unpaired Student’s t-tests are shown.
Ezrin at the rear of blebbing cells

Ezrin mutants affect cell polarisation and invasion
To analyse the involvement of ezrin in the formation and function of the ERULS, we used mutants of ezrin. The phosphomimetic T567D mutant (TD) acts as a constitutively activated ezrin, whereas the non-phosphorylatable T567A mutant (TA) is constitutively inactive; the N-terminal mutant, comprising amino acids 1–310 (ezrin-N), contains the membrane-binding but not the actin-binding domain and is thought to act as a competitive inhibitor of endogenous ezrin. These mutants were expressed in A375 cells in order to assess the effects on polarisation and invasion (Fig. 6). Expression of the TA or the TD mutant increased the fraction of cells with ERULSs (Fig. 6A), suggesting that the ERULS is a dynamic structure that requires the turnover of ezrin. Interestingly, upon expression of the TD or TA mutant, a small fraction of cells (<5% in TD mutants, <2% in TA mutants) displayed two ezrin poles (Fig. 6B). Expression of ezrin-N decreased the fraction of cells with ERULSs (Fig. 6A), showing that actin binding by ezrin is necessary for the formation or maintenance of the ERULS. To investigate the effect of ezrin mutants on invasion, assays were performed with A375 cells stably expressing GFP fused to wild-type ezrin, or the TD, TA or ezrin-N mutants (Fig. 6C). Expression of the TD mutant inhibited invasion of A375 cells, consistent with the strongly inhibited blebbing (Fig. 6D) (Charras et al., 2006). Expression of the TA mutant, which neither inhibited blebbing nor ERULS formation, had no significant effect on invasion. Expression of ezrin-N inhibited invasion, as previously observed in A375-M2 cells invading into a Matrigel matrix (Sahai and Marshall, 2003). These results show that either decreasing blebbing or delocalisation of the ERULS inhibits amoeboid invasion.

Blebbing is decreased at the ERULS
To explore the role of the ERULS in blebbing movement, we looked at the relationship between sites of blebbing and the ERULS. Ezrin constitutes the link between the plasma membrane and the submembrane cortex; to form a bleb, this link first needs to be broken and then, for retraction of the bleb, needs to be reconnected (Charras et al., 2006). We hypothesised that the high concentration of ezrin at the ERULS might inhibit the formation of blebs by stabilising the link between the plasma membrane and the cortex. A375-M2 cells expressing cytoplasmic mCherry and ezrin–GFP were imaged on top of a thick collagen matrix (Sahai and Marshall, 2003). Blebbing was significantly decreased at the ERULS, with an average of 11±1.4 blebs per circumference outside the ERULS compared with 4±1.3 blebs per circumference at the ERULS. These findings clearly demonstrate an inverse relationship between blebbing and the ERULS and support the hypothesis that the ezrin link between the plasma membrane and the cortex inhibits blebbing at the ERULS.

Discussion
Cell polarisation is readily visible in migrating cells and is thought to be a prerequisite for cell migration because it determines the direction of movement. We investigated how melanoma cells with a round morphology and many small blebs over their surface can move without displaying morphological features of a front or a back. We show that blebbing melanoma cells form a uropod-like structure (ERULS) at the rear that is enriched in ezrin, β1-integrin, actin, MLC and PtdIns(4,5)P2 and regulated by novel isoforms of PKC. However, this structure does not protrude from the cell body in the manner typical of a leukocyte uropod and is not visible by

![Fig. 4. Alignment of the Golgi and ERULS.](image-url)
Fig. 5. Ezrin polarisation at the ERULS is necessary for invasion. The invasion of A375 cells into a collagen-I matrix shown as invasion indices (number of invading cells divided by the number of non-invading cells) for each experiment together with P values for each condition (A,C–E; compared with the results in the control by paired Student’s t-tests). (A) A375 cells transfected with a pool of non-targeting siRNAs (control), a pool of siRNAs targeting ezrin (ezrin pool) or two different individual siRNAs (ezrin 5 and ezrin 7). (B) The levels of ezrin expression measured by western blotting. (C) Experiments were performed as in A, but with the addition of 50 μM Rac inhibitor NSC23766 (RI). (D) Ezrin knockdown experiments were performed as in A, but in the A375-M2 cell line. (E) Invasion of untreated (control) A375 cells or A375 cells treated with 50 μM Rac inhibitor, 100 nM PMA or a combination of Rac inhibitor and PMA. (F) A375 cells expressing ezrin–GFP were seeded on top of a collagen-I matrix and treated with 50 μM Rac inhibitor, 100 nM PMA or a combination of Rac inhibitor and PMA. The number of cells with polarised ezrin localisation was quantified as in Fig. 3. Averages for three independent experiments (+s.d.) are shown, with P values from unpaired Student’s t-tests. (G,H) Bright-field (G) or confocal (H) images of cells treated with 50 μM Rac inhibitor, 100 nM PMA or a combination of Rac inhibitor and PMA. Cells in H are transfected with ezrin–GFP (red-to-white colour) and cytoplasmic mCherry (grey).
phase-contrast microscopy. We show that this structure is a feature of polarised round blebbing cells and is not a consequence of cell movement. Importantly, it is required for invasion of A375 cells, as inhibition of ezrin localisation, or inhibition of the ERULS, abrogates invasion.

We show that the actomyosin cortex typical of cells moving with a rounded morphology (Pinner and Sahai, 2008) is thicker at the ERULS. Additionally, plasma membrane and the actin–plasma-membrane linker protein ezrin are highly enriched at this structure. Therefore the ERULS is probably very rigid through strong connections to the plasma membrane and the cytoskeleton. Significantly, we find that membrane blebbing is reduced at this site. We propose that the strong actomyosin cortex and the accumulation of ezrin inhibit blebbing through preventing ruptures in the cortex or preventing detachment of the plasma membrane from the cortex (Charras et al., 2005; Dai and Sheetz, 1999; Maugis et al., 2010; Paluch et al., 2005). We show that the ERULS is enriched in the phosphorylated active form of ezrin, and expression of a constitutively active ezrin mutant (TD) strongly inhibits blebbing. Therefore it is probable that the local accumulation of active ezrin at the ERULS locally inhibits blebbing. This is consistent with the idea that, in migrating cells, mechanisms at the cell rear inhibit the formation of cell extensions to initiate front-back polarity. In movement driven by formation of actin-dependent protrusions, myosin-II-dependent contractility inhibits protrusion formation at the rear (Lo et al., 2004; Vicente-Manzanares et al., 2007), whereas, in blebbing-driven movement, the strong ezrin link between the rigid actomyosin cortex and the plasma membrane inhibits blebbing at the rear.

Our findings suggest a model of polarisation and migration of blebbing cells that differs from other established models of amoeboid cell migration in its generation of intrinsic directional movement and motile force (Charras and Paluch, 2008; Friedl and Weigelin, 2008; Lammermann and Sixt, 2009). In an adhesion-dependent amoeboid migration mode, amoeboid cells form pseudopods or lamellipodia at the front, and these in turn form adhesion sites with the substrate and determine the direction of movement (Fig. 8A) (Sanchez-Madrid and del Pozo, 1999). Detachment and retraction of the uropod results in movement of the cell in the direction of the site of adhesion. In leukocytes moving in a low-adhesion mode (Friedl et al., 1998; Wolf et al., 2003b), zebrafish primordial germ cells (Blaser et al., 2006) and in cancer cells (Keller and Bebie, 1996) that move by formation of large blebs, the situation is similar (Fig. 8B). The pseudopod or bleb can grow into a gap in the three-dimensional ECM, and retraction of the uropod squeezes the cell body through this gap, so that the direction of movement is determined by the site of pseudopod and bleb growth. However in amoeboid cells that form multiple small blebs, the direction of movement is not determined by the cell front, as there is no evidence that these cells have a defined front (Fig. 8C). Each bleb generates its own force, pulling the cell in the direction of bleb growth, by as yet unspecified interactions with the ECM. Inhibition of blebbing at the cell rear by the ERULS drives cell movement in the opposite direction. We consider a highly simplified model, in which the cell is treated as a rigid spherical body and the membrane is considered to be a surface (S) defined by all points \( \mathbf{x} \) where \( x_i = (x,y,z) \), and \( r \) is the cell radius, each of which is associated with a force \( \mathbf{f}(x,s,t) \) whose magnitude varies with time \( t \) but whose direction is always radial with respect to the cell centre. Neglecting friction and viscous drag forces, the resultant time-varying force on the cell is given by:

\[
\mathbf{f}(t) = \int_S \mathbf{f}(x,s,t) \, ds.
\]

This produces a change in velocity \( \Delta v \) of the cell in a time interval \( \Delta t \) given by the impulse divided by the mass \( m \) of the cell:

\[
\Delta v = \frac{1}{m} \int_{t}^{t+\Delta t} \mathbf{f}(t) \, dt.
\]
integrated in the above equations over a timescale that is long compared with the average bleb lifetime. Over short timescales the net force does not integrate to zero, and the cell makes small random motions about a mean position. The persistent absence of blebbing at the ERULS, for a time that is long compared with the bleb lifetime, creates an asymmetry of the time-integrated force-distribution around the cell. The net force that moves the cell forwards therefore acts in the direction opposite to the ERULS. The direction of movement is thus not determined by the cell front but only by the rear, in contrast with the mechanisms shown in Fig. 8A and 8B, where the direction of cell movement is determined by the cell front. According to our model in Fig. 8C, the driving force is created by the blebs, whereas the direction of movement is determined by the cell rear. The change in velocity is proportional to the persistence time of the ERULS and, so long as the ERULS is large compared with the average bleb size (and not too large), increases with the area of the ERULS.

In our model, the nature of the interaction of a bleb with the environment that generates the force has yet to be understood, but an important requirement is a mechanism that allows the force–time function for each bleb to be asymmetrical (i.e. more force needs to be generated during bleb retraction than in growth, so that the forces of a bleb pulling and pushing on the ECM do not cancel each other). This might happen because, during bleb growth, the plasma membrane lacks the supporting actin cortex, but bleb retraction requires the reassembly of this cortex (Charras et al., 2006; Charras et al., 2005). During bleb growth, the membrane might behave with a more viscous nature and it would then flow around ECM structures without pushing on them in any particular direction; during bleb retraction the membrane might have sufficient rigidity for the contractility brought about by myosin II recruitment to exert a traction force on the matrix.

We identify Golgi alignment in front of the nucleus in blebbing melanoma cells, which is the opposite of the alignment in migrating leukocytes. This difference in polarisation might reflect the difference in mechanisms shown in Fig. 8. The Golgi alignment of blebbing cells is independent of contact with substrate, as it is the same when cells are in suspension. The ERULS is therefore not a consequence of cell movement but part of the intrinsic polarity of the cell. Such pre-polarisation could facilitate extravasation of metastasising cells, an important step towards colonisation of a new tissue. A rounded morphology associated with high actomyosin contractility supports lung colonisation in a mouse model (Pinner and Sahai, 2008; Sanz-Moreno et al., 2008). A moesin-containing
structure, similar to the ERULS described here, was also shown recently to be required for initiation of invasion and early lung colonisation by melanoma cells (Esteche et al., 2009). Although these effects were strictly dependent on moesin, ezrin has been shown to be required for metastatic processes. Expression of the dominant-negative N-terminus of ezrin strongly inhibited migration and metastasis of human melanoma cells (Federici et al., 2009), and in many tumour types ezrin dysregulation has been implicated in tumour progression and metastasis (Elliott et al., 2004; Endo et al., 2009; Khamma et al., 2004; Makitie et al., 2001; Ren et al., 2009; Weng et al., 2005; Yu et al., 2004). Although ezrin and moesin are highly homologous and might act redundantly in many processes, they have specific cellular functions, undergo different regulation mechanisms and show different expression patterns (Ilani et al., 2007; Shaffer et al., 2009; Shcherbina et al., 1999). Therefore, it would interesting to assess the involvement of moesin in the movement of blebbing amoeboid cells in order to understand whether ezrin and moesin functions act redundantly of each other or whether ezrin is specifically important.

It is apparent that different types of cell movement should not be considered as strictly separated mechanisms but as continuous variations of migration modes that cells adopt in response to the balance of adhesion, contractility and physical properties of the matrix. It will therefore be necessary to understand all the different aspects of mechanisms and modes of migration that cells can adopt in order to design strategies that interfere with pathological cell migration. Given the relevance of blebbing amoeboid movement in melanoma progression (Pinner and Sahai, 2008; Sanz-Moreno et al., 2008), it will be of great interest to determine more about the underlying signalling mechanisms that control the formation and regulation of the ERULS and blebbing amoeboid movement.

Materials and Methods

Cell culture

A375P and A375-M2 cells were obtained from Richard Hynes (Howard Hughes Medical Institute, Massachusetts Institute of Technology, MA), and WM3529 and WM1361 from Richard Marais (Institute of Cancer Research, London, UK). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Stable A375P and A375-M2 cells expressing the mCherry sequence. RFP–PLC Mr was from the Odel’s laboratory (Smith et al., 2008). Rac inhibitor and PMA were added, together with the medium. The three-dimensional collagen invasion assay was performed as previously described (Smith et al., 2008). Rac inhibitor and PMA were added, together with the medium. Cells were incubated for 48 hours, fixed in 4% formaldehyde, stained with Hoechst 33258 (Molecular Probes-Invitrogen) and imaged on a Nikon eclipse TE 2000-S inverted wide-field microscope fitted with a motorised stage (Prior Scientific) using SimplePCI software (Compix) using a 20× objective. GFP was imaged using 490/20 nm excitation and 528/38 nm emission filters.

Live imaging of cells inside collagen

For live imaging of cell movement inside collagen, 15 µl slide VI chambers (IBIDI) were used. To increase cell viability and reproducibility of collagen fibre formation (Sung et al., 2009), chambers were precooled at 4°C and 1.7 mg/ml collagen was prepared and mixed with cells on ice. Each channel was filled with 60 µl of collagen containing 60,000 cells. Chambers were incubated for 1 hour at 4°C, 1 hour at room temperature and 1 hour at 37°C.

Immunoblotting and immunostaining

For western blots, an aliquot of cells used for the invasion assay was lysed [50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM MgCl2, 10% glycerol, 1% NP-40 and complete mini EDTA-free protease inhibitor cocktail (Roche),] fractionated by SDS-PAGE and transferred onto PVDF membranes. Western blotting was performed using the Odyssey imaging system (Li-Cor Biosciences), with primary rabbit anti-ezrin (1:5,000) and mouse anti-GAPDH (1:5,000) antibodies and secondary (1:10,000) IRDye-680-labelled anti-rabbit IgG and IRDye-800-labelled anti-mouse IgG antibodies. Band intensity was quantified using ImageJ (Rasband, 1997). For immunostaining, cells were seeded as described above, fixed with 4% paraformaldehyde, washed twice with PBS, twice with Tris buffer (100 mM Tris-HCl pH 7.4, 50 mM NaCl) and once with PBS. They were then permeabilised in 0.3% Triton-X100 in blocking buffer (4% BSA in PBS) for 20 minutes, washed with PBS and incubated with blocking buffer for 30 minutes. After washing twice, cells were incubated with primary antibody in blocking buffer for 12 hours at 4°C. Cells were then washed twice, and incubated with FITC- or Texas-Red-labelled secondary antibodies (1:50 in blocking buffer) for 2 hours. Samples were washed at least six times with PBS and then were mounted in Mowiol as described above.

Invasion assay

The three-dimensional collagen invasion assay was performed as previously described (Smith et al., 2008). Rac inhibitor and PMA were added, together with the medium. Cells were incubated for 48 hours, fixed in 4% paraformaldehyde, stained with Hoechst 33258 (Molecular Probes-Invitrogen) and imaged on a NCELL3000 (GE-Healthcare) at 0 μm (non-invading cells) and 50 μm (invading cells). Non-invading and invading cells were counted using the NCELL3000 software. The invasion index was calculated as the number of invading cells per number of non-invading cells. Each individual experiment was performed at least four times.

Confocal microscopy

Confocal images of fixed cells on top of collagen were taken on a Zeiss LSM710 inverted confocal microscope with a 63× objective using Zen 2009 software (Zeiss).

Image analysis and statistical analysis

The ratio images in Fig. 1 were calculated in ImageJ (Rasband, 1997) by dividing the mean intensity of the channel of the membrane (defined as the region between the membranes) by the mean intensity of the cell body. To quantify only the cell membrane, a mask was made in Image J using the membrane channel as a template. This mask was binarised and multiplied with each channel. The intensity plots in Fig. 1 were made in ImageJ. To calculate the fraction of cells containing localised ezrin-GFP, 180
the number of cells with localised ezrin-GFP was divided by the total number of cells. For each experiment, a minimum of 200 cells from a minimum of 30 images for condition, was counted. For quantification of blebbing, the number of blebs was counted manually, and the arc length of the uropod and the region outside the uropod was measured using ImageJ. Statistical analysis of these experiments was performed using GraphPad QuickCalc, a GraphPad online tool. All classifications, except for invasion assays, were statistically validated using unpaired Student’s t-tests. For invasion assays, paired Student’s t-tests were used because of expected inter-variability, probably resulting from differences in collagen preparation. MatLab (Mathworks) was used to generate the quiver plots of ezrin vectors. These vectors were created by manually selecting the centre of cell and the site of ezrin localisation for each frame.

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