

# Migration of human melanoma cells depends on extracellular pH and Na<sup>+</sup>/H<sup>+</sup> exchange

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Their glycolytic metabolism imposes an increased acid load upon tumour cells. The surplus protons are extruded by the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) which causes an extracellular acidification. It is not yet known by what mechanism extracellular pH (pH<sub>e</sub>) and NHE activity affect tumour cell migration and thus metastasis. We studied the impact of pH<sub>e</sub> and NHE activity on the motility of human melanoma (MV3) cells. Cells were seeded on/in collagen I matrices. Migration was monitored employing time lapse video microscopy and then quantified as the movement of the cell centre. Intracellular pH (pH<sub>i</sub>) was measured fluorometrically. Cell–matrix interactions were tested in cell adhesion assays and by the displacement of microbeads inside a collagen matrix. Migration depended on the integrin  $\alpha2\beta1$ . Cells reached their maximum motility at pH<sub>e</sub>  $\sim$ 7.0. They hardly migrated at pH<sub>e</sub> 6.6 or 7.5, when NHE was inhibited, or when NHE activity was stimulated by loading cells with propionic acid. These procedures also caused characteristic changes in cell morphology and pH<sub>i</sub>. The changes in pH<sub>i</sub>, however, did not account for the changes in morphology and migratory behaviour. Migration and morphology more likely correlate with the strength of cell–matrix interactions. Adhesion was the strongest at pH<sub>e</sub> 6.6. It weakened at basic pH<sub>e</sub>, upon NHE inhibition, or upon blockage of the integrin  $\alpha2\beta1$ . We propose that pH<sub>e</sub> and NHE activity affect migration of human melanoma cells by modulating cell–matrix interactions. Migration is hindered when the interaction is too strong (acidic pH<sub>e</sub>) or too weak (alkaline pH<sub>e</sub> or NHE inhibition).

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Cell migration away from the primary tumour is a major step in metastasis. This multistep process requires sequential interaction between the invasive cell and the surrounding extracellular matrix. It is based on (i) extracellular matrix proteolysis by matrix metalloproteinases (Egeblad & Werb, 2002), (ii) directed reorganization of the cytoskeleton (Mitchison & Cramer, 1996), (iii) activity of ion channels and transporters such as Na<sup>+</sup>/H<sup>+</sup> or Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers (Klein *et al.* 2000; Schwab, 2001; Denker & Barber, 2002*a,b*; Saadoun *et al.* 2005), and (iv) a coordinated formation and release of focal adhesion contacts to the extracellular matrix mediated by integrin receptor molecules (Beningo *et al.* 2001; Jalali *et al.* 2001; Webb *et al.* 2002).

Focal adhesion contacts are stable cell–substrate interactions evolving from focal complexes which are

characterized as small and transient cell interactions between the cell and the extracellular matrix (Friedl & Wolf, 2003). Focal contacts comprise integrins, the focal adhesion kinase (FAK), talin, vinculin, paxillin and other proteins attached to the actin filament network (Brakebusch & Fässler, 2003). The ubiquitously expressed Na<sup>+</sup>/H<sup>+</sup> exchanger isoform, NHE1, is also part of these focal contacts at which it may directly interact with integrins (Schwartz *et al.* 1991; Grinstein *et al.* 1993; Plopper *et al.* 1995). In fibroblasts, NHE1 anchors the actin cytoskeleton to the plasma membrane by its direct binding of ERM proteins (Denker *et al.* 2000; Denker & Barber, 2002*a*). A loss of this NHE1-dependent cytoskeletal anchoring impairs cell polarity and reduces the directionality in cell migration (Denker & Barber, 2002*b*). The transport activity of the NHE1 is stimulated

during cell adhesion (Schwartz, 1992; Schwartz & Lechene, 1992) and is necessary for the assembly of focal adhesions (Tominaga & Barber, 1998).

However, the primary task of the NHE1 is the regulation of intracellular pH ( $\text{pH}_i$ ) and cell volume. NHE1 activity is of importance for many tumour cells (Lagarde *et al.* 1988; Rotin *et al.* 1989; Tannock & Rotin, 1989; McLean *et al.* 2000). The insufficient and/or inefficient tumour vascularization leads to a diminished  $\text{O}_2$  supply so that the metabolism of tumour cells is mostly glycolytic (Gulledge & Dewhirst, 1996; Helmlinger *et al.* 2002). Thus, tumour cells are often challenged by an increased acid load (Griffiths, 1991). The excess protons are extruded into the extracellular space by transport proteins such as the NHE1 (Yamagata & Tannock, 1996; Wahl *et al.* 2002) which then causes an acidification of the extracellular space (Tannock & Rotin, 1989). The role of the extracellular pH ( $\text{pH}_e$ ) in tumour cell migration, however, has not yet been established. NHE activation enhances the invasiveness of human breast cell carcinoma cells (Reshkin *et al.* 2000) implying a possible involvement of NHE and extracellular pH ( $\text{pH}_e$ ) in metastasis and tumour malignancy. We therefore investigated whether  $\text{pH}_e$  and NHE activity modulate the motility of human melanoma cells *on* collagen matrices as well as *in* three-dimensional collagen lattices.

## Methods

### Cells and cell culture

The melanoma cell line MV3 (Van Muijen *et al.* 1991) was grown in bicarbonate-buffered RPMI 1640 (Sigma, Taufkirchen, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ . Confluent MV3 cells were either plated onto (two-dimensional migration, 2D; for further details see below) or incorporated into (three-dimensional migration, 3D) collagen matrices except where stated otherwise.

### Experimental solutions

Depending on the experimental set-up (experimental chamber airtight (5%  $\text{CO}_2$ , 2D experiments) or open (0.03%  $\text{CO}_2$ , 3D experiments)), experimental solutions and media were buffered with either  $\text{NaHCO}_3$  (2D) or with Hepes (3D). The amount of  $\text{NaHCO}_3$  to be added to the media was determined by the desired pH value.

Measurements of the intracellular pH were performed using Hepes-buffered Ringer solutions of different pH values containing ( $\text{mmol l}^{-1}$ ): 122.5 NaCl, 5.4 KCl, 0.8  $\text{MgCl}_2$ , 1.2  $\text{CaCl}_2$ , 1.0  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 5.0 glucose, 10 Hepes. The pH was adjusted by adding 1 M NaOH. Phosphate-buffered saline (PBS) solution was used as

solvent for the antibodies and as washing buffer as well. The PBS contained ( $\text{mmol l}^{-1}$ ): 137 NaCl, 2.7 KCl, 8.1  $\text{Na}_2\text{HPO}_4$ , 1.5  $\text{KH}_2\text{PO}_4$ .

When applicable the NHE inhibitors ethylisopropylamiloride (EIPA; final concentration:  $10 \mu\text{mol l}^{-1}$  in 0.05% ethanol) or HOE642 (Cariporide; final concentration:  $10 \mu\text{mol l}^{-1}$ ) were added. Rhodocetin, a component of snake venom that binds highly specifically to the integrin dimer  $\alpha 2\beta 1$  over a broad pH range (Eble & Tuckwell, 2003) was applied in a concentration of  $5 \mu\text{g ml}^{-1}$ .

### Antibodies

The monoclonal anti-hVin-1 (Sigma, Saint Louis, MO, USA) and the function blocking anti-human integrin  $\beta 1$  (4B7R) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Friedlander *et al.* 1996) were used for immunolabelling. Immunoblotting was performed with the anti-human integrin  $\beta 1$  from Chemicon International (Temecula, CA, USA). A Cy3-conjugated goat anti-mouse IgG or a horseradish peroxidase-conjugated goat anti-mouse IgG (both from Jackson Immuno-Research Laboratories, West Grove, PA, USA) served as the secondary antibodies.

### Preparation of collagen matrices

One hundred microlitres of  $10 \times$  RPMI<sup>-</sup> 1640 and  $100 \mu\text{l}$   $10 \times$  Hepes buffer (final Hepes concentration:  $10 \text{ mmol l}^{-1}$ ) were added to  $800 \mu\text{l}$  collagen G solution (Biochrom AG, Berlin, Germany). When cells were embedded in collagen lattices for three-dimensional (3D) experiments the different  $\text{pH}_e$  values were adjusted prior to the polymerization process by adding various amounts of 1 M NaOH. The cell density was kept at  $250\,000 \text{ cells ml}^{-1}$ . This cell-containing collagen solution polymerized within 3 h at 37°C in an open experimental chamber. When cell migration was observed on collagen matrices (2D) the pH of the cell free collagen mixture was always adjusted to pH 7.4 bearing in mind that the polymerization and self-assembly of collagen may be affected by the concentration of free protons (Williams *et al.* 1978; Freire & Coelho-Sampaio, 2000). An ensured reproducible collagen polymerization leading to a consistently fibrous matrix structure enabled us to exclude that the observed effects are based on pH-dependent differences in the matrix. The bottoms of culture flasks ( $12.5 \text{ cm}^2$ , Falcon) were covered with  $500 \mu\text{l}$  collagen solution. About  $100 \mu\text{l}$  of the same collagen solution were filled in a 1.5 ml Eppendorf tube in order to confirm the final pH of the gel after its polymerization by using a small pH electrode (Mettler, Toledo, Switzerland). The collagen was allowed to polymerize overnight at 37°C. Cells were seeded on this collagen matrix

and allowed to adapt to different pH values varying from 6.4 to 7.5 for 3 h prior to recording.

The tractive power exerted by cells pulling on collagen fibrils was visualized by incorporating fluorescent microspheres (FluoSpheres, 1  $\mu\text{m}$ , Molecular Probes, Inc., Eugene, OR, USA) into the collagen matrix at a concentration of  $4 \times 10^7$  beads  $\text{ml}^{-1}$  (Tamariz & Grinnell, 2002).

### Videomicroscopy and computer-assisted cell tracking

The culture flasks (2D) or experimental chambers (3D) were put in heated chambers on stages of inverted microscopes (ID03 and Axiovert25, Carl Zeiss, Inc., Göttingen, Germany). Cell migration was recorded for 5 h at 37°C using video cameras (Models XC-ST70CE and XC-77CE, Hamamatsu/Sony, Japan) and PC-vision frame grabber boards (Hamamatsu). Images were taken in 10 min intervals controlled by a high performance image control system (HiPic, Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany). The circumferences of the cells were labelled employing the AMIRA software (TGS Inc., San Diego, CA, USA). The cell contours then served as the basis for further analysis. Each cell that remained in the visual field during the experiment without dividing or colliding with another cell was evaluated. Parameters such as the cell area, the structural index (SI) and the migratory speed were analysed using self-made JAVA programs and the NIH ImageJ software (<http://rsb.info.nih.gov/ij/>). Migration was determined as the movement of the cell centre per time unit, the speed was estimated from the 10 min time interval applying a three point difference quotient and the cell area was measured as the number of pixels. The structural index (SI) represents the cell shape. SI was calculated as follows:

$$\text{SI} = (4\pi A)/p^2$$

where  $A$  is the area covered by the cell and  $p$  is the perimeter of  $A$  (Dunn & Brown, 1987, 1990). Values close to '1' correspond to a spherical cell shape whereas values close to '0' correspond to a spindle or a dendritic cell shape.

### Measuring the intracellular pH ( $\text{pH}_i$ )

$\text{pH}_i$  of MV3 cells was measured using video imaging techniques and the fluorescent pH indicator BCECF (Molecular Probes, OR, USA). Cells were treated as for migration experiments. They were resuspended in one of the Hepes-buffered experimental solutions containing EIPA or HOE642 where applicable. They were plated onto collagen coated coverslips and allowed to adapt for 3 h. Cells were then incubated in the respective experimental solution containing  $2 \mu\text{mol l}^{-1}$  BCECF-AM for 1–2 min. The coverslips were placed on the stage of

an inverted microscope (Axiovert TV 100; Carl Zeiss, Inc., Göttingen, Germany) and continuously superfused with prewarmed Hepes-buffered Ringer solution. The excitation wavelength alternated between 488 and 460 nm, respectively, while the emitted fluorescence was monitored at 500 nm using an ICCD camera (Atto Bioscience, Rockville, MD, USA). Filter change and data acquisition were controlled by Attofluor software (Atto Bioscience). Fluorescence intensities were measured in 10 s intervals and corrected for background fluorescence. At the end of each experiment, the  $\text{pH}_i$  measurements were calibrated by successively superfusing the MV3 cells with modified Ringer solutions of pH 7.5, 7.0 and 6.5 containing ( $\text{mmol l}^{-1}$ ): 125 KCl, 1  $\text{MgCl}_2$ , 1  $\text{CaCl}_2$ , 20 Hepes, and  $10 \mu\text{mol l}^{-1}$  nigericin (Sigma).

### Cell adhesion

MV3 cells were resuspended in serum free RPMI media ( $\text{RPMI}^-$ ) of different pH (pH 6.4 to pH 7.5) with or without  $10 \mu\text{mol l}^{-1}$  EIPA. Cells were then seeded in collagen ( $4 \text{ mg ml}^{-1}$ ) coated 24-well plates at a density of 30 000 cells per well. After 60 min the media including the non-adhesive cells were removed. The remaining cells were washed with cold PBS buffer, then fixed and counted. In addition to their number the cells' morphology given as structural index (SI) and cell area was evaluated as described above. The software MetaVue (Universal Imaging) was used for taking images and counting.

### Western blot of integrin $\beta 1$

Confluent cells were transferred into serum-free bicarbonate-buffered RPMI 1640 medium of various pH values (7.5, 7.0 and 6.6) and allowed to adapt for ~15 h. They were then washed with cold PBS and lysed at 4°C in lysis buffer containing ( $\text{mmol l}^{-1}$ ): 150 NaCl, 5.0 EDTA, 50 Tris-HCl (pH 7.5) and 1.0 Pefabloc SC Plus (Roche Molecular Biochemicals, Mannheim, Germany), 1% Triton X-100, and 0.2% (v/v) of a protease inhibitor cocktail (Sigma, P 8340). Lysates were scraped off 10 cm culture dishes (Falcon) and mixed with reducing sample buffer (4:1, v/v) containing  $500 \text{ mmol l}^{-1}$  Tris,  $100 \text{ mmol l}^{-1}$  dithiothreitol, 8.5% SDS, 27.5% sucrose and 0.03% bromophenol blue indicator. SDS-PAGE was performed using acrylamide gels (7.5%) and a Minigel System (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (~2  $\mu\text{g}$ ) were loaded. Electroblothing was performed at  $0.8 \text{ mA cm}^{-2}$  gel for 50 min. The nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) carrying the blotted proteins were bathed in 5% (w/v) milk in 0.1% (v/v) Tween in PBS for 1 h at room temperature and then washed with 0.1% Tween in PBS. Overnight incubation with the primary antibody

(1 : 600) at 4°C was followed by a 1 h incubation with a horseradish peroxidase-conjugated secondary antibody (1 : 50 000) at room temperature. Blots were developed using an ECL immunoblotting detection reagent kit (Amersham, Arlington Heights, IL, USA). In order to quantify  $\beta 1$  integrin expression, five blots were scanned and the number of pixels representing a marked band area was multiplied by the band's mean grey value calculated by the NIH ImageJ software.

### Immunofluorescence

Cells were treated with cold 1% Triton X-100 in PBS for 10 min prior to fixation. Without this prepermeabilization step vinculin could hardly be labelled as the epitope was not accessible enough to the antibody. Cells were then fixed by 3.5% paraformaldehyde in PBS. Non-specific binding sites were blocked using 100 mM glycine in PBS and 10% (v/v) goat normal serum in PBS. After staining the cells with the hVIN-1 mAb or the integrin  $\beta 1$  (4B7R) mAb for 1 h and a Cy3-conjugated IgG for another hour (in a dilution of 1 : 800 each), the slide preparations were fixed once again, washed in PBS and then covered with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Images were taken using an invert microscope (Axiovert200, Carl Zeiss, Inc., Göttingen, Germany), a digital camera (Model 9.0, RT-SE-Spot, Visitron Systems) and the MetaVue software.

### Statistics

All experiments were repeated three to six times. Data are presented as the mean values  $\pm$  s.e.m. The data were tested for significance employing Student's unpaired *t* test or analysis of variance (ANOVA) where applicable. The level of significance was set at  $P < 0.01$  except where otherwise stated.

## Results

### Cell migration depends on $\text{pH}_e$ and NHE activity

We calculated the average speed (given in  $\mu\text{m min}^{-1}$ ; Fig. 1A and C) and the mean translocation (= distance covered within 5 h; Fig. 1B, D and E) of migrating MV3 cells. Cells seeded on collagen (Fig. 1A) reached their maximum speed of  $0.68 \pm 0.03 \mu\text{m min}^{-1}$  at  $\text{pH}_e$  7.0 ( $n = 30$  cells from three to five trials in each case). An increase in  $\text{pH}_e$  to pH 7.5 or a decrease to pH 6.4 caused the migratory speed to decrease almost linearly to  $0.11 \pm 0.01 \mu\text{m min}^{-1}$  and  $0.24 \pm 0.02 \mu\text{m min}^{-1}$ , respectively. The cells covered  $109.9 \pm 15.1 \mu\text{m}$  at  $\text{pH}_e$  7.0, but only  $20.7 \pm 2.8 \mu\text{m}$  and  $27.2 \pm 5 \mu\text{m}$  at  $\text{pH}_e$  7.5 and  $\text{pH}_e$  6.6, respectively (Fig. 1B and E). NHE inhibition by EIPA or HOE642 decreased speed and translocation by up to 70% at  $\text{pH}_e$  7.0 ( $0.2 \pm 0.02 \mu\text{m min}^{-1}$ ,  $28 \pm 3.7 \mu\text{m}$

within 5 h). Extracellular acidification to  $\text{pH}_e$  6.8 did not amplify the inhibitory effect of NHE blockage on cell migration. However, migration was inhibited when cells were exposed to propionic acid ( $15 \text{ mmol l}^{-1}$ ) while the extracellular pH was kept constant at  $\text{pH}_e$  7.0. Protonated weak organic acids such as propionic acid permeate the plasma membrane and dissociate rapidly in the cytosol causing an initial intracellular acidification and an increase in NHE activity (Boron, 1983; Klein *et al.* 2000). After a 3 h exposure of MV3 cells to  $15 \text{ mmol l}^{-1}$  sodium propionate at a constant  $\text{pH}_e$  of 7.0 and a  $\text{pH}_i$  of  $7.1 \pm 0.05$  ( $n = 38$  cells from four trials, for details see below) the migratory speed on a 2D matrix was reduced to  $0.07 \pm 0.01 \mu\text{m min}^{-1}$  ( $n = 36$  cells from eight different trials). The translocation within 5 h was only  $18.5 \pm 2.77 \mu\text{m}$  (cf. Fig. 1A and B). Additional exposure of acidified cells to  $10 \mu\text{mol l}^{-1}$  HOE642 led to a further decrease in the migratory speed ( $0.03 \pm 0.004 \mu\text{m min}^{-1}$ ,  $n = 40$  cells from 4 different trials, cf. Fig. 1A and B) and the cells' translocation ( $6.45 \pm 0.9 \mu\text{m}$  ( $5 \text{ h}^{-1}$ )).

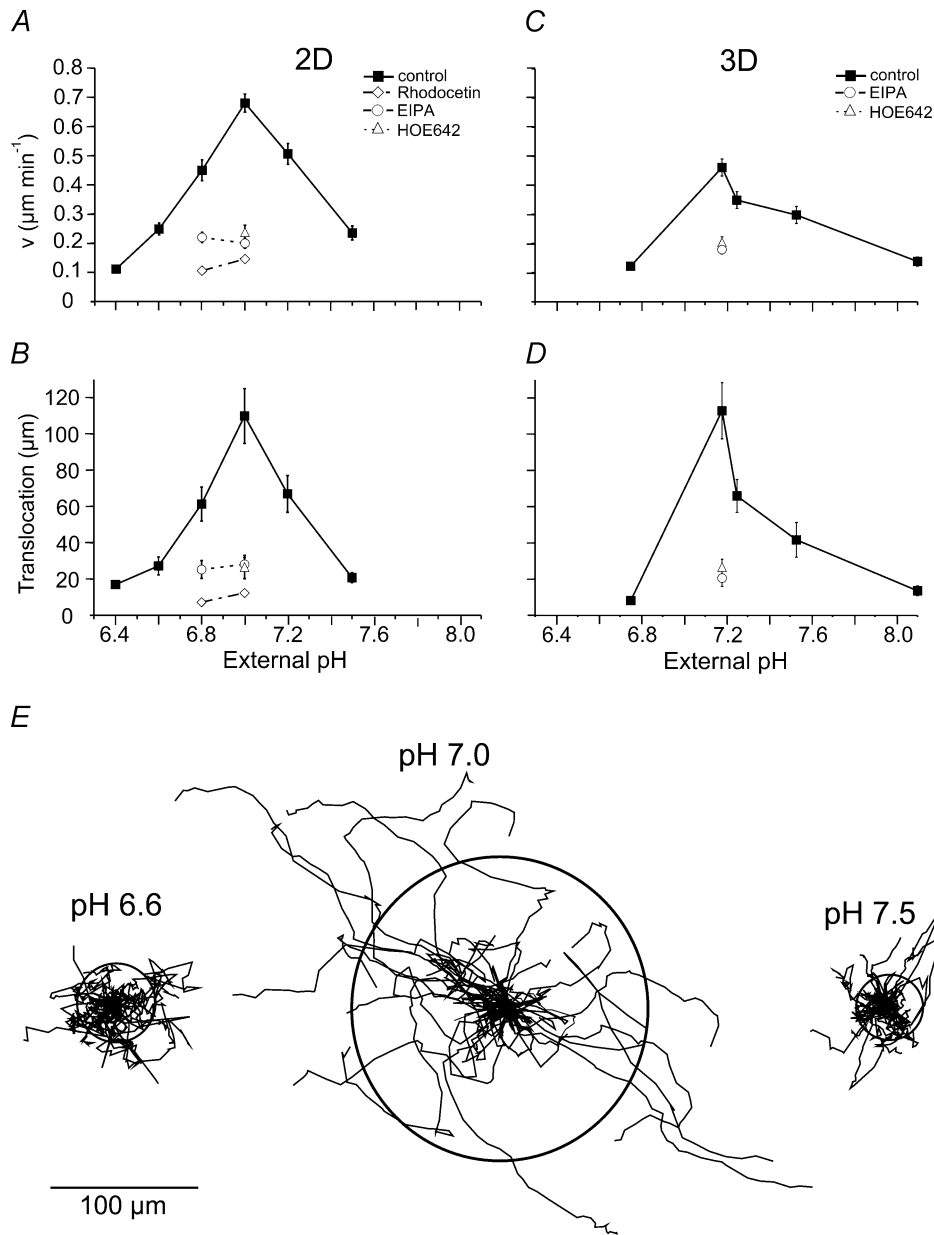
Cells embedded in three-dimensional collagen lattices (Fig. 1C and D) behaved similarly ( $n = 10$ – $20$  cells from three to five trials in each case). They reached their maximum speed and translocation at  $\text{pH}_e$  7.18:  $0.46 \pm 0.03 \mu\text{m min}^{-1}$  and  $112.9 \pm 15.5 \mu\text{m}$  ( $n = 16$ ; Supplemental material video 1). When  $\text{pH}_e$  was reduced to pH 6.75 (video 2) or increased to pH 8.1 (video 3) migratory speed and translocation decreased significantly (at  $\text{pH}_e$  6.75:  $0.12 \pm 0.01 \mu\text{m min}^{-1}$  and  $8.2 \pm 1.8 \mu\text{m}$  ( $n = 10$ ); at  $\text{pH}_e$  8.1:  $0.14 \pm 0.02 \mu\text{m min}^{-1}$  and  $13.6 \pm 2.7 \mu\text{m}$  ( $n = 15$ )). NHE inhibition by EIPA (video 4) or HOE642 (Cariporide; video 5) decreased speed and translocation by 55%:  $0.20 \pm 0.02 \mu\text{m min}^{-1}$  and  $20.6 \pm 4.6 \mu\text{m}$  (EIPA;  $n = 20$ ), and  $0.18 \pm 0.02 \mu\text{m min}^{-1}$  and  $26.7 \pm 4.3 \mu\text{m}$  (HOE642,  $n = 20$ ). HOE642 inhibits cell migration (Fig. 2A and B) at  $\text{pH}_e$  7.0 with an  $\text{IC}_{50}$  of  $\sim 5 \mu\text{mol l}^{-1}$  and affects cell morphology (Fig. 2C) in a dose-dependent manner indicating that NHE activity plays a major role in controlling cell migration and morphology. Most likely it is the isoform NHE1 as revealed by Western blot analysis (data not shown). Also, at the concentration used in this study HOE642 is unlikely to effectively inhibit the resistant NHE isoforms such as NHE3 (Scholz *et al.* 1995). The  $\text{IC}_{50}$  value for HOE642 effects on the human NHE3 is  $900 \mu\text{mol l}^{-1}$  (Schwark *et al.* 1998).

Speed and translocation were reduced to a similar extent when the integrin dimer  $\alpha 2\beta 1$  was specifically blocked by rhodocetin (Fig. 1). Moreover, Table 1 shows that, regardless of  $\text{pH}_e$ , cells seeded on poly L-lysine hardly migrated and that they did not spread but remained spherical. These observations confirm the finding by Maaser *et al.* (1999) that in MV3 cells the integrin  $\alpha 2\beta 1$  is indispensable for cell migration on collagen.

**Cell morphology is determined by  $pH_e$  and/or by NHE activity**

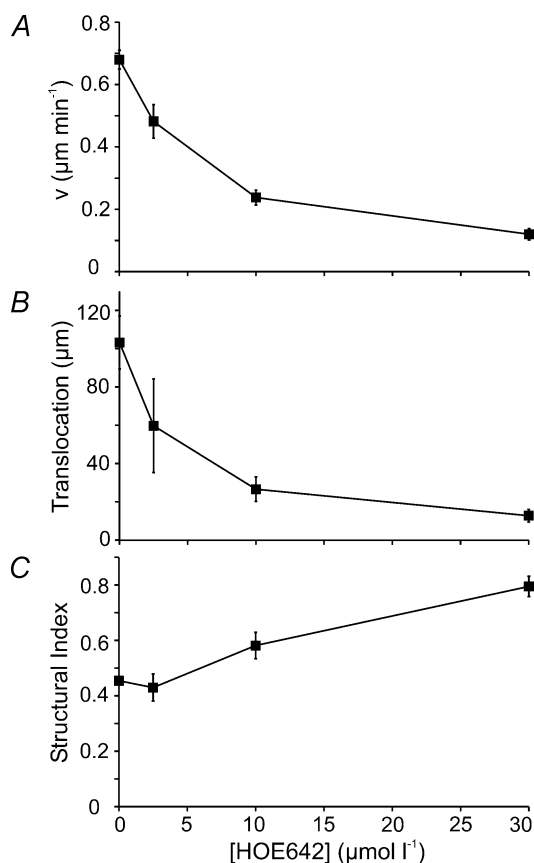
Since changes in motility and migratory behaviour might be accompanied by morphological modifications we checked whether  $pH_e$  and NHE activity also affected the cell morphology. As shown in Fig. 3, cells were dendritic

at  $pH_e$  6.6 or of ameboid shape at  $pH_e$  7.0 whereas they were spherical at  $pH_e$  7.5 (2D) or  $pH_e$  8.1 (3D) and in the presence of NHE inhibitors. These morphological properties were quantified by calculating the structural index (SI) and counting the lamellipodia per cell ( $n = 30$  cells from five trials in each case) 60 min after seeding



**Figure 1. Migratory speed, translocation and trajectories of MV3 cells depend on  $pH_e$**   
 Cells were either seeded on collagen matrices (2D: A, B and E) or incorporated in collagen lattices (3D: C and D; Supplemental material video 1; video 2; video 3) and recorded for 5 h. Migratory speed (A and C) and translocation within 5 h (B and D) under control conditions (■), after the application of EIPA (○; video 4) or HOE642 (△; video 5) and upon treatment with rhodocetin (grey diamonds).  $n$  varied between 30 (2D) and 10–20 (3D) cells taken from three to five different trials. E, trajectories of MV3 cells migrating on collagen (2D) at different  $pH_e$  values. Trajectories obtained under the same experimental conditions were standardized. They all begin at the same starting post represented by the centre of a circle. The radii of the circles represent the mean distances covered within 5 h.  $n = 30$  cells each, taken from three to five different trials.

onto collagen (Fig. 4). SI dropped from  $0.82 \pm 0.02$  to  $0.33 \pm 0.03$  as  $\text{pH}_e$  decreased from pH 7.5 to pH 6.6. It then rose to  $0.73 \pm 0.03$  as  $\text{pH}_e$  was further reduced to 6.4. In the presence of EIPA the entire graph is shifted to more acidic values. At  $\text{pH}_e$  6.4 EIPA-treated cells were less rounded than the control cells. The number of lamellipodia per cell increased from  $0.8 \pm 0.3 \text{ cell}^{-1}$  to  $4.6 \pm 0.5 \text{ cell}^{-1}$  as  $\text{pH}_e$  decreased from pH 7.5 to pH 6.6 (Fig. 4B). It decreased to  $2.0 \pm 0.3$  when  $\text{pH}_e$  was further reduced to 6.4. The application of EIPA caused a significant drop in the number of lamellipodia in cells that had been exposed to pH values varying between 6.6 and 7.5. Interestingly, in EIPA-treated cells the number of lamellipodia continuously rose from  $0 \pm 0$  to  $2.5 \pm 0.5 \text{ cell}^{-1}$  as  $\text{pH}_e$  decreased from pH 7.5 to pH 6.4. At pH 6.4 the number of lamellipodia was the same in EIPA-treated and in control cells ( $2.5 \pm 0.5 \text{ cell}^{-1}$  and  $2.0 \pm 0.3 \text{ cell}^{-1}$ , respectively). This finding suggests that the effect of NHE inhibition on the formation of lamellipodia can at least partially be compensated by lowering  $\text{pH}_e$ . Similar results were obtained when the morphology of MV3 cells was analysed under the same



**Figure 2.** HOE642 affects the migratory speed ( $v$ ), translocation and morphology (SI) of MV3 cells in a dose-dependent manner  $\text{pH}_e$  was 7.0 throughout.  $n = 20$ –30 cells from three to five different trials in each case.

conditions 180 min after seeding (data not shown). Sodium propionate ( $15 \text{ mmol l}^{-1}$ ) had an even more distinct effect on the morphology than had an acid  $\text{pH}_e$ . SI dropped to  $0.11 \pm 0.01$  and cells developed numerous long lamellipodia which they tended to shed (Fig. 3C). This effect could be reversed by the simultaneous application of HOE642 (SI =  $0.61 \pm 0.1$ ).

In the presence of rhodocetin cells developed none or only one lamellipodium and remained rather round (Figs 3C and 4). This suggests that the interaction between integrin  $\alpha 2\beta 1$  and the extracellular matrix is needed to form processes.

### $\text{pH}_e$ and NHE activity determine $\text{pH}_i$

The previous paragraphs showed that the extracellular pH and/or NHE activity have a strong impact on migration and morphology of MV3 cells. Thereupon we measured the resulting changes of the intracellular pH ( $\text{pH}_i$ ) in order to determine whether they could account for the altered migratory behaviour. The results are summarized in Fig. 5.  $\text{pH}_i$  decreased as  $\text{pH}_e$  decreased.  $\text{pH}_i$  was  $7.18 \pm 0.01$  ( $n = 58$ ) in cells exposed to  $\text{pH}_e$  7.5 while it was  $6.58 \pm 0.03$  ( $n = 59$ ) at  $\text{pH}_e$  6.4.  $\text{pH}_i$  of HOE642 treated cells was  $6.67 \pm 0.01$  ( $n = 45$ ) at  $\text{pH}_e$  7.2.  $\text{pH}_i$  of EIPA-treated cells was  $6.78 \pm 0.01$  ( $n = 21$ ) at  $\text{pH}_e$  7.2 and it significantly decreased further to  $6.71 \pm 0.01$  ( $n = 29$ ) at  $\text{pH}_e$  6.8. Thus, in contrast to migration, the effects of EIPA and extracellular acidification on  $\text{pH}_i$  are additive. After a 3 h exposure of MV3 cells to sodium propionate ( $15 \text{ mmol l}^{-1}$ ) at  $\text{pH}_e$  7.0,  $\text{pH}_i$  was  $7.1 \pm 0.05$  ( $n = 38$  cells from four trials). Additional exposure of acidified cells to  $10 \mu\text{mol l}^{-1}$  HOE642 led to a decrease in  $\text{pH}_i$  (pH  $6.69 \pm 0.04$ ,  $n = 30$  cells from three trials).

It is conspicuous that  $\text{pH}_i$ , migration and morphology of MV3 cells do not directly correlate with one another. An intracellular acidification caused by a low  $\text{pH}_e$  or by NHE inhibition is associated with completely different morphologies. Conversely, intracellular alkalinization by a high  $\text{pH}_e$  or intracellular acidification by NHE inhibition produce identical morphological changes. Similar discrepancies were found comparing control cells with propionate-treated cells. Thus, alterations of  $\text{pH}_i$  alone cannot explain the observed changes in migration and morphology.

### Adhesion to the extracellular matrix increases as $\text{pH}_e$ decreases

In this series of experiments we tested whether the observed changes in migration and morphology could be related to a  $\text{pH}_e$ - and/or NHE-mediated modulation of the cell–matrix interaction. To this end we performed adhesion assays at different  $\text{pH}_e$  values varying from 6.4

**Table 1. The migratory behaviour of cells seeded on poly L-lysine does not depend on pH<sub>e</sub>**

	pH 6.6	pH 6.8	pH 7.2	pH 7.5
$v$ ( $\mu\text{m min}^{-1}$ ) <sub>coll</sub>	0.25 ± 0.02 (30)	0.45 ± 0.036 (30)	0.51 ± 0.036 (30)	0.24 ± 0.024 (30)
$v$ ( $\mu\text{m min}^{-1}$ ) <sub>poly</sub>	0.06 ± 0.007 (29)*	0.04 ± 0.005 (16)*	0.04 ± 0.005 (23)*	0.05 ± 0.004 (19)*
Translocation ( $\mu\text{m}$ ) <sub>coll</sub>	27.2 ± 5 (30)	61.3 ± 9.4 (30)	66.9 ± 10.1 (30)	20.7 ± 2.8 (30)
Translocation ( $\mu\text{m}$ ) <sub>poly</sub>	4.39 ± 0.41 (29)*	5.8 ± 0.8 (16)*	3.49 ± 0.69 (23)*	7.07 ± 0.54 (19)*
Structural index (SI) <sub>coll</sub>	0.37 ± 0.02 (30)	0.34 ± 0.02 (30)	0.51 ± 0.02 (30)	0.73 ± 0.02 (30)
Structural index (SI) <sub>poly</sub>	0.86 ± 0.02 (29)*	0.84 ± 0.03 (16)*	0.87 ± 0.03 (23)*	0.88 ± 0.01 (19)*

The number in parentheses represents the number of analysed cells from three different trials for poly L-lysine (poly) and up to five different trials for collagen (coll). \* $P < 0.01$ ; poly L-lysine versus collagen.

to 7.5 in the presence and absence of EIPA (Fig. 6). The number of adhesive cells increased by 70% ( $P < 0.02$ ) from  $135 \pm 20$  cells/area ( $n = 6$  trials) to  $229 \pm 27$  cells/area ( $n = 6$ ) as pH<sub>e</sub> was lowered from pH 7.5 to pH 6.8. A further reduction of pH<sub>e</sub> below pH 6.8, however, led to a slight decrease in the number of adhesive cells ( $194 \pm 23$  cells/area;  $n = 6$ ). This decrease was not caused by acid-induced cell death or apoptosis since MV3 cells can easily be cultured at pH 6.6 for several months (data not shown). Inhibiting NHE with EIPA caused a significant drop in the number of adhesive cells by 44% at pH 6.4 up to 62% at pH 6.8. Interestingly, the adhesion of EIPA-treated cells continuously increased as pH<sub>e</sub> decreased. This indicates that an acidic pH<sub>e</sub> may compensate for the effect of NHE inhibition on cell adhesion. Cell adhesion could also be reduced by rhodocetin (Fig. 6). The number of adhesive cells upon rhodocetin treatment at pH<sub>e</sub> 6.8 or 7.0 resembled that of untreated cells at pH<sub>e</sub> 7.5.

The pH<sub>e</sub> dependence of the cell–matrix interaction could as well be demonstrated in another set of experiments. Cells incorporated into a collagen matrix and exposed to low pH<sub>e</sub> values (pH 6.8) pulled on the collagen matrix when they retracted their processes (Fig. 7). The cell-induced movement of the matrix was made visible by the displacement of microbeads that were incorporated in the matrix. Within 30 min the microbeads were displaced by  $1.53 \pm 0.27 \mu\text{m}$  at pH<sub>e</sub> 6.8 ( $n = 12$  beads from four different trials) as opposed to only  $0.15 \pm 0.05$  when cells were observed at pH<sub>e</sub> 7.5 ( $n = 15$ ). These observations indicate that the cell surface/collagen interaction is stronger at lower pH<sub>e</sub> values and weaker at higher pH<sub>e</sub> values.

### pH<sub>e</sub> does not stimulate the expression of integrin $\beta 1$

The integrin dimer  $\alpha 2\beta 1$  mediates cell adhesion, spreading and migration in MV3 cells (Friedl *et al.* 1998; Maaser *et al.* 1999; Friedl & Bröcker, 2000). We therefore tested whether the pH<sub>e</sub>-dependent strengthening of the cell–matrix interaction was due to a pH<sub>e</sub>-dependent up-regulation of the integrin  $\beta 1$ -expression. However, immunoblots

revealed no pH<sub>e</sub> induced differences in the overall expression of integrin  $\beta 1$  (Fig. 8). The intensity of the two integrin  $\beta 1$  bands at 110 and 130 kDa remained the same regardless of the pH<sub>e</sub> cells had been exposed to prior to cell lysis.

### Formation and distribution of focal adhesion sites are affected by pH<sub>e</sub>

The distribution of vinculin, a cytosolic component of the focal contacts, was also affected by pH<sub>e</sub> (Fig. 9A–C). Immunolocalization of the plasma membrane-associated vinculin revealed that it was more or less evenly distributed along the plasma membrane of cells exposed to pH<sub>e</sub> 7.5. At pH<sub>e</sub> 7.2 and 7.0 the vinculin was still rather evenly distributed but also appeared in a spotty fashion along the lamellipodia. At lower pH<sub>e</sub> values it accumulated in a typically patchy pattern at the leading edges, i.e. the tips, of the lamellipodia. The distribution of integrin  $\beta 1$  did not fully correspond to that of vinculin. The integrin  $\beta 1$  in lamellipodia of cells exposed to lower pH<sub>e</sub> at which stronger adhesion had been observed was hardly labelled (Fig. 9F). The incorporation of less integrin  $\beta 1$  into the plasma membrane appears to be an improbable cause for this phenomenon since the expression of integrin  $\beta 1$  does not depend on pH<sub>e</sub> (Fig. 8) and since the traction forces exerted on the collagen fibres are higher at low pH<sub>e</sub> (Fig. 7). We favour the explanation that the extracellular binding site for the function blocking antibody might be sterically blocked by collagen and therefore not accessible for the antibody.

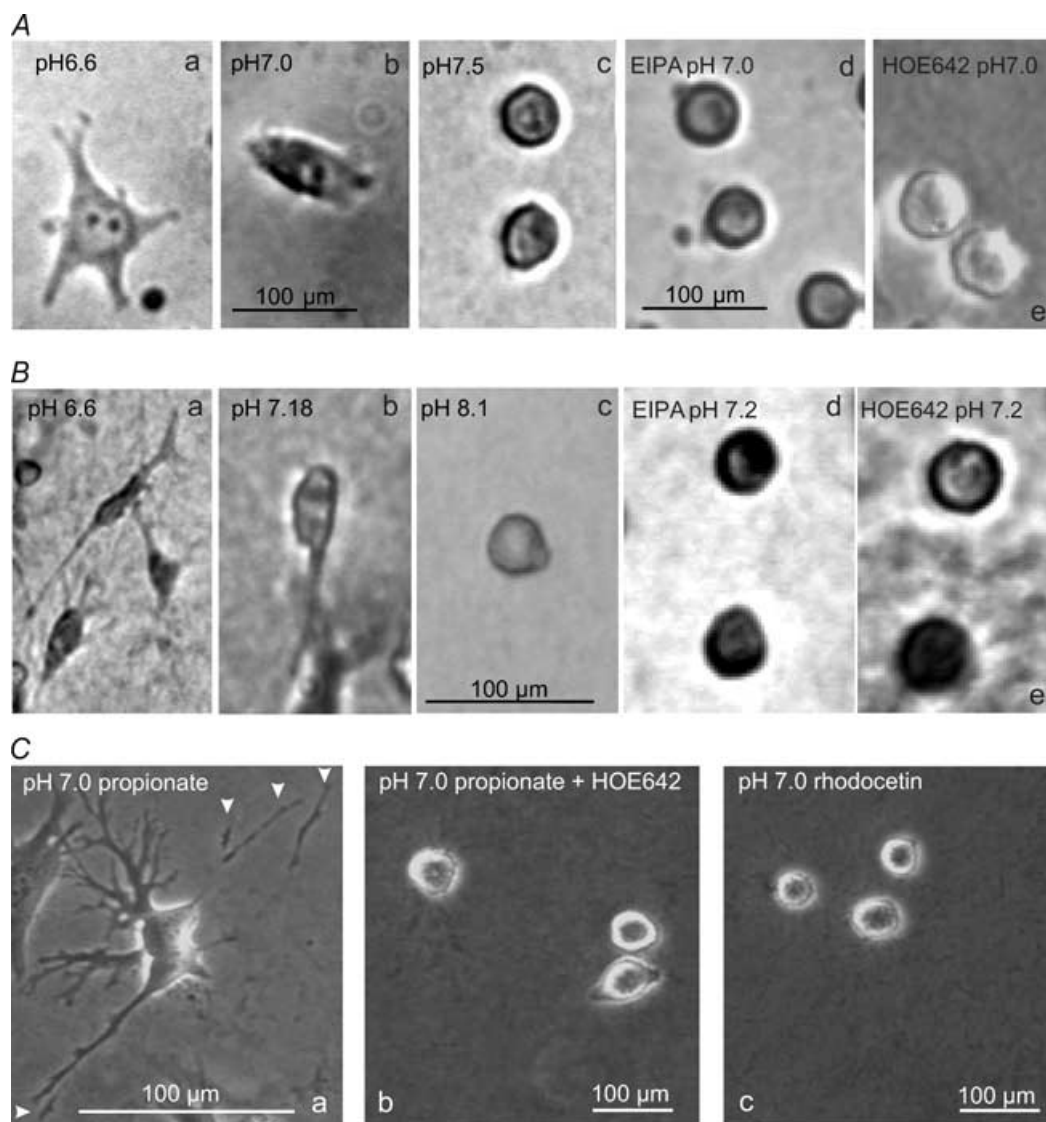
### Discussion

NHE activity plays a key role in cell migration. It is required for the migration of keratinocytes (Bereiter-Hahn & Voth, 1988), human leucocytes (Simchowitz & Cragoe, 1986; Ritter *et al.* 1998), neutrophil granulocytes (Rosengren *et al.* 1994), fibroblasts (Denker & Barber, 2002b) and renal epithelial (Madin–Darby canine kidney, MDCK-F) cells (Klein *et al.* 2000). NHE inhibition leads to a reduction in

the migration rate of MDCK-F cells (Klein *et al.* 2000) while NHE activation augments the motility and the invasiveness of human breast carcinoma cells (Reshkin *et al.* 2000). The present results are fully consistent with those findings showing a drastic impairment in cell motility of human melanoma cells in response to NHE inhibition by EIPA or HOE642.

Another key finding of the present study is that migration, morphology and adhesion or cell–matrix interactions mediated by integrin  $\alpha 2\beta 1$  depend on  $\text{pH}_e$ . Modifying the  $\text{pH}_e$  changed  $\text{pH}_i$  as well. However,  $\text{pH}_e$ -induced modifications of  $\text{pH}_i$  can be ruled out

as the only cause for the observed effects. Intracellular acidification was accompanied by either a round morphology and a low adhesion rate in the case of NHE inhibition or by a dendritic morphology and a higher adhesion rate upon exposure to a low  $\text{pH}_e$ . Conversely, cells were spherical regardless of whether  $\text{pH}_i$  was alkaline (in response to exposure to a high  $\text{pH}_e$ ) or acidic (because of NHE inhibition). Moreover, cells were dendritic with an almost normal  $\text{pH}_i$  in the presence of sodium propionate, whereas they became spherical with an acidic  $\text{pH}_i$  after additional exposure to HOE642. The question arises what factors other than  $\text{pH}_e$ -induced modifications of  $\text{pH}_i$



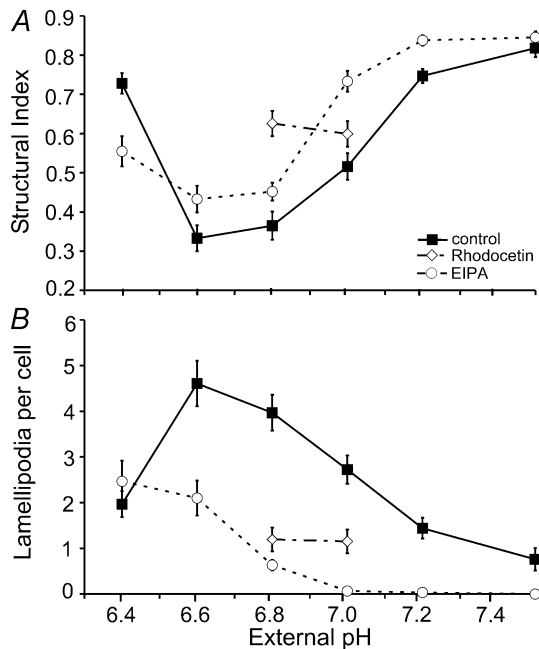
### Figure 3. Morphology of MV3 cells

A and B, cells seeded on collagen matrices (A) or incorporated into collagen lattices (B) after a 3 h exposure to different  $\text{pH}_e$  values (a–c; Supplemental material videos 1–3) or 3 h after the application of  $10 \mu\text{mol l}^{-1}$  EIPA (d; video 4) or  $10 \mu\text{mol l}^{-1}$  HOE642 (e; video 5) at  $\text{pH}_e$  7.2. C, cells seeded on collagen matrices were exposed to sodium propionate (a) or to sodium propionate plus HOE642 (b) or treated with rhodocetin (c). The arrow heads in Ca indicate shedding of cell processes.



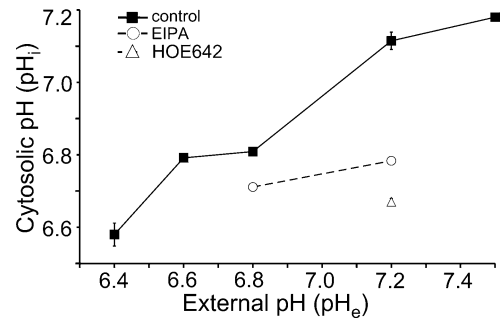
may be involved. Needless to say, NHE activity is one candidate. NHE blockage or alkaline  $pH_e$  and  $pH_i$  result in low or no NHE activity, which causes the cells to round up. Acidic  $pH_e$  and  $pH_i$  or stimulation of the NHE by loading the cells with propionic acid result in high NHE activity leading to a dendritic cell shape and a high adhesiveness. Thus, it is obvious that there is a correlation between the NHE activity of human melanoma cells, their migration, morphology and adhesion. These observations are in line with previous studies showing that (i) a loss of the NHE1-dependent cytoskeletal anchoring impairs cell polarity and reduces the directionality in cell migration (Denker & Barber, 2002b), (ii) the transport activity of the NHE1 is stimulated during cell adhesion (Schwartz, 1992; Schwartz & Lechene, 1992), and (iii) NHE1 activity is necessary for the assembly of focal adhesions (Tominaga & Barber, 1998).

However, the present results provide evidence that  $pH_e$  itself contributes to the observed effects. The results point to a modulation of the cells' adhesiveness, i.e. to a modulation of the interaction between integrin  $\alpha 2\beta 1$  and collagen I by  $pH_e$ . What is the experimental justification for this explanation? First, exposure of EIPA-treated cells



**Figure 4. Structural index (A) and formation of lamellipodia (B) in MV3 cells depend on  $pH_e$  and NHE activity**

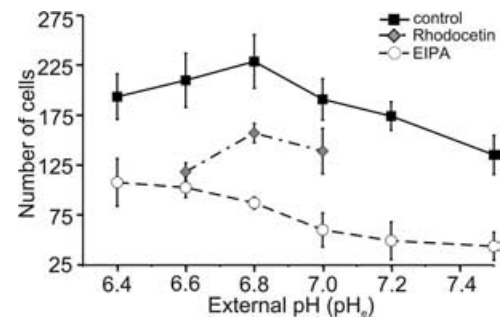
Cells were seeded on collagen and incubated in RPMI<sup>-</sup> media of different  $pH_e$  in the presence (○) or absence (■) of  $10 \mu\text{mol l}^{-1}$  EIPA or in the presence of  $5 \mu\text{g ml}^{-1}$  rhodocetin (grey diamonds). The parameters were determined at  $t = 60$  after seeding. The results shown in A and B were obtained from the same cells. Each data point represents the mean  $\pm$  s.e.m. obtained from  $n = 50$  cells from five different experiments (5 cells per area ( $2 \times 10^5 \mu\text{m}^2$ ), 2 areas per experiment).



**Figure 5. Cytosolic pH ( $pH_i$ ) of MV3 cells depends on the external pH ( $pH_e$ ) or NHE activity**

$pH_i$  was measured after a 3 h exposure to different  $pH_e$  values (■) or 3 h after the addition of  $10 \mu\text{mol l}^{-1}$  EIPA (at  $pH_e$  6.8 and 7.2; ○) and  $10 \mu\text{mol l}^{-1}$  HOE642 (at  $pH_e$  7.2; △).  $n$  varied between 21 and 106 cells from three to six trials.

to lower  $pH_e$  values leads to an increase in the cells' adhesiveness suggesting that the effects of NHE inhibition on cell adhesion can, at least partially, be neutralized by extracellular acidification (Fig. 6). Second, cells pull on the matrix at  $pH_e$  6.8 as shown by the displacement of beads incorporated in the matrix whereas they do not displace adjacent beads at  $pH_e$  7.5 indicating a rather tight cell surface–matrix interaction at lower  $pH_e$  values (Fig. 7). This experiment also demonstrates that extracellular acidification in the observed pH range does not reduce the contractility of MV3 cells. This is of interest since an acidic pH can exert effects on cell contractility by altering the structure, activity, or interaction of several actin-binding and cross-linking proteins such as the actin depolymerizing factor (Hawkins *et al.* 1993; Faff & Nolte, 2000), talin (Schmidt *et al.* 1993) or  $\alpha$ -actinin (Condeelis & Vahey, 1982). Third, the number of lamellipodia in EIPA-treated cells increases as  $pH_e$  decreases (Fig. 4). It

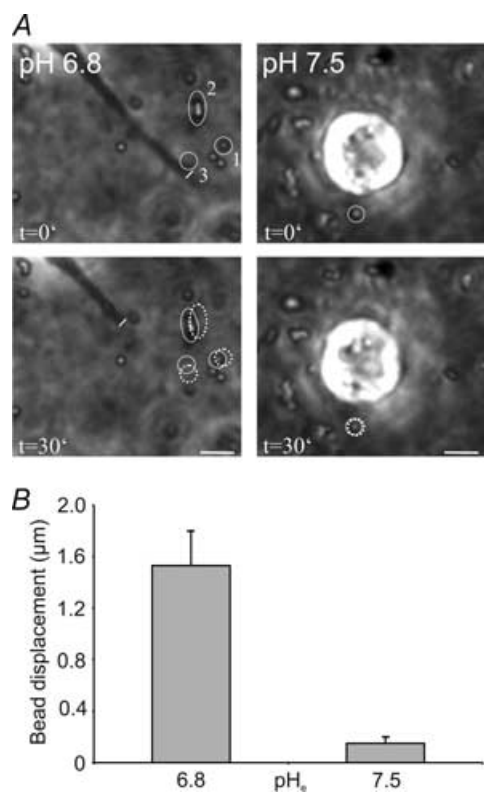


**Figure 6. Adhesion of MV3 cells to a collagen matrix depends on  $pH_e$  and NHE activity**

The adhesiveness is represented by the number of cells that remained stuck to the collagen matrix after washing with PBS 60 min after seeding. Cells were treated with rhodocetin (grey diamonds,  $n = 3$  trials, four areas per trial) or exposed to solutions of various  $pH_e$  with (○,  $n = 4$  trials, two areas per trial) or without (■,  $n = 6$  trials, two areas per trial)  $10 \mu\text{mol l}^{-1}$  EIPA.

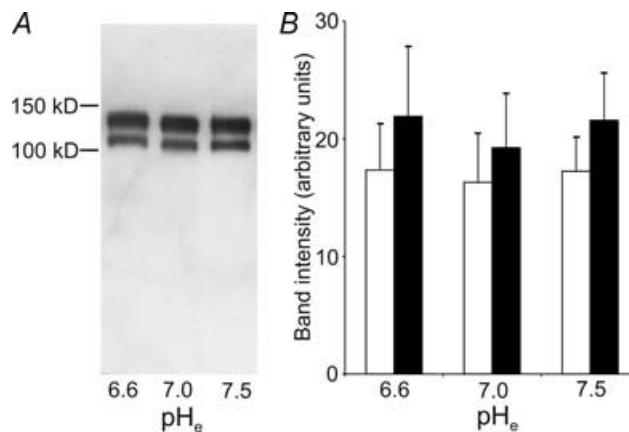
seems that at low  $pH_e$  values the lamellipodia cannot become detached from the matrix so that the affected cells remain stuck and hardly migrate while forming new processes that again can barely be retracted.

Fourth, although a rather soft finding, the present immunofluorescence stainings (Fig. 9) are in line with the previous arguments. Vinculin forming the cytoskeletal link between integrin  $\beta 1$ -bound talin and other cytosolic components of focal adhesions such as tensin, paxillin and  $\alpha$ -actinin (Zamir & Geiger, 2001) accumulates at the leading edges of lamellipodia at low  $pH_e$  while it seems to be evenly distributed along the membrane at  $pH_e$  7.5. At the same time the access for the integrin  $\beta 1$  mAb to its extracellular epitope is impaired as  $pH_e$  decreases. This is consistent with the idea that a higher extracellular proton concentration increases the integrin/collagen binding force thereby sterically blocking the mAb's access to its binding site. Fifth, the expression of integrin  $\beta 1$  was not affected by  $pH_e$  (Fig. 8). Palecek *et al.* (1997) demonstrated that varying the quantity of integrins or its ligands



**Figure 7. Cells exposed to low  $pH_e$  values ( $pH_e$  6.8) pull on the matrix when they retract their lamellipodia**

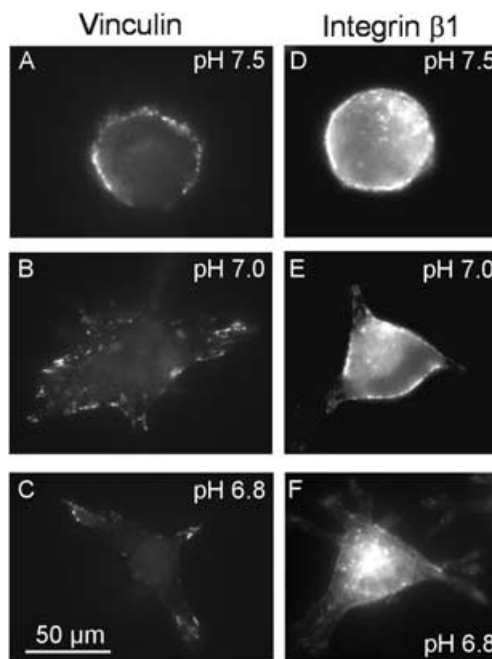
This is shown by the displacement of microbeads incorporated in the collagen matrix. *A*, bead 1 moved  $1.1 \mu m$  within 30 min, beads 2 and 3 were displaced by  $1.3$  and  $1.9 \mu m$ , respectively. At high  $pH_e$  ( $pH_e$  7.5) cells did not exert traction on the matrix. The labelled bead moved  $0.2 \mu m$ . Scale bar =  $10 \mu m$ . *B*, statistics of bead displacement at  $pH_e$  6.8 ( $n = 12$  beads from four different trials, three beads per cell) and at  $pH_e$  7.5 ( $n = 15$  beads from five different trials, three beads per cell).



**Figure 8. In MV3 cells the expression of integrin  $\beta 1$  does not depend on  $pH_e$**

*A*, Western blot of human integrin  $\beta 1$ . Equal amounts of protein ( $2 \mu g$ ) were loaded. *B*, the 110 kDa (precursor protein, open bars) as well as the 130 kDa bands (mature protein, filled bars) did not differ depending on  $pH_e$  (mean  $\pm$  s.e.m.;  $n = 5$  blots).

has a strong impact on cell-substratum adhesiveness in CHO cells and thereby affects migration. We cannot completely rule out a  $pH_e$ -dependent incorporation of integrin  $\beta 1$  into the plasma membrane of MV3 cells. However, the  $pH_e$ -independent integrin  $\beta 1$ -expression implies that besides the quantity of cell matrix interactions their binding strength, probably affected by  $pH_e$ , may also



**Figure 9. Comparison of the distributions of vinculin (A-C) and integrin  $\beta 1$  (D-F) in MV3 cells at different  $pH_e$**

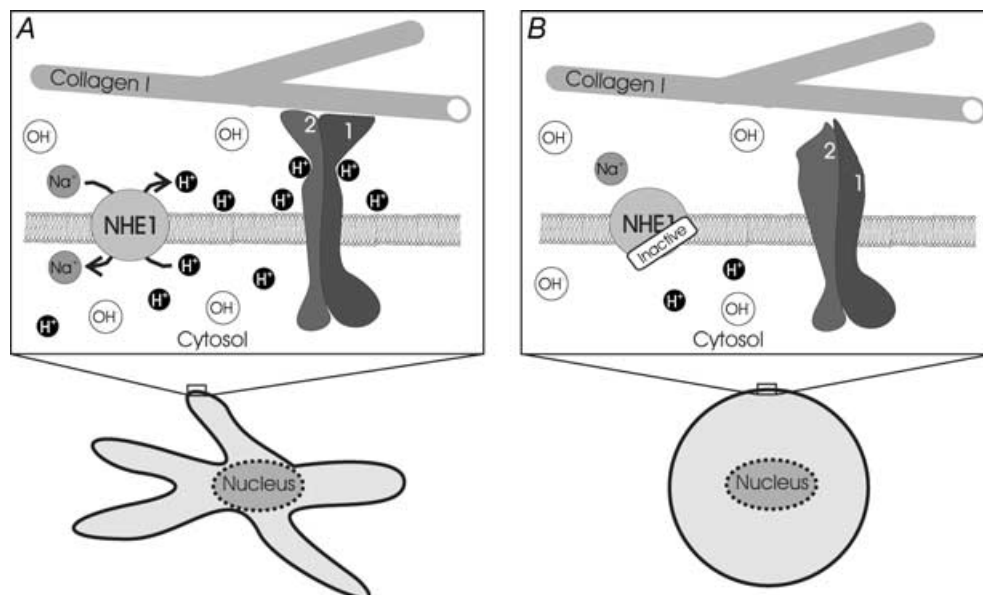
Cells were seeded on collagen at different  $pH_e$  values and then fixed 60 min after seeding.  $pH_e$  varied between  $pH_e$  6.8 and  $pH_e$  7.5. Scale bar:  $50 \mu m$ .

modify adhesion and migration. Sixth, applying atomic force microscopy Lehenkari & Horton (1999) found the pH optimum for the binding force of the fibronectin ligand-sequence GRGDSP to integrin  $\alpha\nu\beta3$  to be pH 6.5. This is consistent with the present finding that at  $\text{pH}_e$  6.4 the binding loosens and the cells are of spherical shape. The NHE1 is very active at  $\text{pH}_e$  6.4, which may decrease the local  $\text{pH}_e$  at the outer face of the plasma membrane to even lower values. When NHE is inhibited by EIPA at  $\text{pH}_e$  6.4 no more protons are exported from the cytosol to the outer face of the plasma membrane and the local  $\text{pH}_e$  may be less acidic than it would be in the absence of EIPA. Consequently, cells are less spherical in the presence of EIPA.

Interestingly, the NHE and the integrin receptor molecules are often colocalized at the focal adhesion sites of the leading edges of lamellipodia (Grinstein *et al.* 1993; Plopper *et al.* 1995). Thus, the NHE could create a proton-enriched microenvironment in the immediate vicinity of the focal adhesion complexes, i.e. close to integrin molecules. In fact, protons can be laterally transferred along the surface of biological membranes. They move along lipid/water interfaces rather than diffusing away (Prats *et al.* 1986; Tessié *et al.* 1990). The current state of literature combined with the present observations led us to hypothesize that the local pH at focal adhesion sites may influence the strength of cell adhesion, i.e. of the integrin/collagen bond, and thereby affect cell

migration (Fig. 10). A surplus of protons or a lively NHE activity would lead to a tight adhesion whereas alkalosis, a lack of protons or an inhibited NHE activity would prevent adhesion. Future experiments will have to verify this hypothesis and to show the existence of such a pH microenvironment at the outer surface of the plasma membrane.

Integrin binding to the extracellular matrix triggers a multitude of intracellular signals that are in part transmitted by the focal adhesion kinase (FAK) and the integrin linked kinase (ILK; Wu & Dedhar, 2001). They bind to integrin  $\beta$  tails (Liu *et al.* 2000). The phosphorylation state of FAK and its kinase activity are regulated by the binding of cells to the extracellular matrix (Schlaepfer & Hunter, 1998; Hauck *et al.* 2001; Hauck *et al.* 2002). On the assumption that extracellular protons stabilize the integrin–collagen bond, it is to be expected that at  $\text{pH}_e$  7.5 (weak adhesion) no or less FAK is phosphorylated than at  $\text{pH}_e$  6.6 (strong adhesion). Alternatively, other integrin pathways such as the RACK1–Src interaction (Cox *et al.* 2003) may be involved. Future studies will show if and how those signal transduction pathways starting from the integrin–collagen interaction are affected by the proton concentration at the cell surface. The binding of collagen to integrin  $\alpha2\beta1$  is stimulated by  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  and inhibited by  $\text{Ca}^{2+}$  (Humphries, 2002). We are now trying to imitate the effects  $\text{pH}_e$  has on cell adhesion, migration and morphology by (i) varying the  $\text{Mg}^{2+}/\text{Ca}^{2+}$



**Figure 10. Hypothetical model of the integrin–collagen interaction**

Integrins and NHE1 are colocalized. Protons stabilize the collagen–integrin ( $\alpha2\beta1$  dimer) bond. A, acidosis, a surplus of protons or a lively NHE activity, leads to tight adhesion which generates a dendritic cell shape. B, alkalosis, a lack of protons or an inhibited NHE activity, prevents adhesion and cells become spherical.

ratio in the experimental solution and (ii) adding  $Mn^{2+}$ .

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**Supplemental material**

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and contains supplemental material consisting of five videos: MV3 cells embedded in collagen at  $\text{pH}_e$  7.35 (video 1), 6.75

(video 2), and 8.1 (video 3) or in the presence of either  $10 \mu\text{mol l}^{-1}$  EIPA (video 4,) or  $10 \mu\text{mol l}^{-1}$  HOE642 (video 5) at  $\text{pH}_e$  7.18 in each case were recorded for 5 h. Frames collected every 10 min are displayed at a rate of 4 frames  $\text{s}^{-1}$ .

This material can also be found as part of the full-text HTML version available from <http://www.blackwell-synergy.com>