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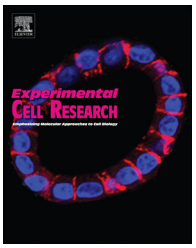

Research Article

Phospho-NHE3 forms membrane patches and interacts with beta-actin to sense and maintain constant direction during cell migration

Srikanth Perike[a,*], *Nurdan Özkucur*[a,b,c,**], *Priyanka Sharma*[a], *Wolfgang Staroske*[d], *Robert Bläsche*[a], *Kathrin Barth*[a], *Richard HW Funk*[a,e]

[a]*Department of Anatomy, Medical Theoretical Center, Medical Faculty Carl Gustav Carus, Technical University of Dresden, Fetscherstrasse 74, 01307 Dresden, Germany*
[b]*Department of Biomedical Engineering, Tufts University, 4 Colby Street, Medford, MA 02155, USA*
[c]*Department of Biology, Tufts University, 200 Boston Ave, Medford, MA 02155, USA*
[d]*Biophysics Group, BIOTEC, Technical University of Dresden, Tatzberg 47/49, 01307 Dresden, Germany*
[e]*CRTD/DFG-Center for Regenerative Therapies Dresden – Cluster of Excellence, Fetscherstrasse 105, 01307 Dresden, Germany*

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ABSTRACT

The Na(+)/H(+) exchanger NHE3 colocalizes with beta-actin at the leading edge of directionally migrating cells. Using human osteosarcoma cells (SaOS-2), rat osteoblasts (calvaria), and human embryonic kidney (HEK) cells, we identified a novel role for NHE3 via beta-actin in anode and cathode directed motility, during electrotaxis. NHE3 knockdown by RNAi revealed that NHE3 expression is required to achieve constant directionality and polarity in migrating cells. Phosphorylated NHE3 (pNHE3) and beta-actin complex formation was impaired by the NHE3 inhibitor S3226 (IC50 0.02 μM). Fluorescence cross-correlation spectroscopy (FCCS) revealed that the molecular interactions between NHE3 and beta-actin in membrane protrusions increased 1.7-fold in the presence of a directional cue and decreased 3.3-fold in the presence of cytochalasin D. Data from flow cytometric analysis showed that membrane potential of cells (V_{mem}) decreases in directionally migrating, NHE3-deficient osteoblasts and osteosarcoma cells whereas only V_{mem} of wild type osteoblasts is affected during directional migration. These findings suggest that pNHE3 has a mechanical function via beta-actin that is dependent on its physiological activity and V_{mem}. Furthermore, phosphatidylinositol 3,4,5-trisphosphate (PIP3) levels increase while PIP2 remains stable when cells have persistent directionality. Both PI3 kinase (PI3K) and Akt expression levels change proportionally to NHE3 levels. Interestingly, however, the content of pNHE3 level does not change when PI3K/Akt is inhibited. Therefore, we conclude that NHE3 can act as a direction sensor for cells and that NHE3 phosphorylation in persistent directional cell migration does not involve PI3K/Akt during electrotaxis.

*Corresponding author. Fax: +49 351 458 6303.
**Corresponding author at: Department of Biomedical Engineering, and Department of Biology, Tufts University, 200 Boston Ave, Suite 2600, Medford, MA, 02155, USA. Fax: +1 617 627 0909.
E-mail addresses: srikanthperike@gmail.com (S. Perike), nurdan.oezkucur@tufts.edu (N. Özkucur).

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Background

In the process of cell migration the persistent movement into one direction is a fundamental process in fertilization, morphogenesis of embryos, tissue repair, wound healing and regeneration [1–5]. Single or collective cell migration in vitro can perceive and maintain constant direction in response to electric fields (electrotaxis), chemical gradients (chemotaxis), extracellular matrix gradients (haptotaxis) or mechanical cues (mechanotaxis). Thus, persistent directional cell migration is defined as a multifactorial biological process at the cellular and tissue levels that requires simultaneous interactions with a number of cues in vivo. The cues that underlie persistent directional migration may differ from those that drive random migration [6]. Understanding this process can provide deep insights into how the misperception of directional cues by migrating cells can cause autoimmune diseases, neuronal disorders, tumor cell invasion, poor wound healing or birth defects [7–11].

Persistent directional cell migration involves complex biological interactions, implying mechanical, physical and physiological rules that need to be periodically reapplied at the leading edge until the cell has arrived at the correct site. The reorganization of actin and microtubules, mediated by Rho GTPases, Cdc42 and PIP2/3 as well as by AMPK through CLIP-170, are integral events in building cell polarity and geometry and in forming membrane protrusions as the cells move forward [12–17]. Moreover, it has been shown that biochemical and biomechanical mechanisms alone cannot efficiently shape the lamellipodium [18]; robust cell polarity cannot be maintained without the influence of physical factors emerging from the microenvironment [19–22]. Notably, directional cell migration relies on both physiological [23,24] and bioelectrical [25–27] factors, which may even override signaling from well-known chemotactic cues during wound healing [28]. A few studies have focused specifically on the molecules or complexes that are responsible for sensing a constant direction in migrating cells. Quinones et al. showed that in metastasis, the absence of the inverse Bin/Amphiphysin/Rvs domain can increase EGF-induced directional cell migration by reducing receptor mediated endocytosis in mouse embryonic fibroblasts (MEF) in vitro, and this domain also plays a role in sensing and responding to border cell migration in isolated Drosophila egg chambers [29,30]. The internalization and control of signaling by the chemokine receptor CXCR4 is essential for fine-tuning SDF-1-guided zebrafish primordial germ cell migration in vivo [31]. The drosophila homologue of the serine/threonine kinase PAR-1 has been shown to promote the directionality of transient cell protrusions that are required for sensing direction during chemotaxis in border cells [32]. A very recent study reported that Daydreamer (DydA), a Ras effector and GSK-3 substrate, is important for directional sensing and cell motility [33]. It is important to note that we preferred to use the term 'persistent directional cue' instead of physiological strengths of applied electric fields (EF). However it remains unknown how cells attain, and subsequently maintain, constant polarity and directivity during persistent directional migration. Therefore, a signaling molecule localized at the leading edge may translate asymmetric membrane receptor signaling to generate polarity.

In mammals, ten Na(+)/H(+) exchanger (NHE) isoforms are present and are located primarily in the plasma membrane and in intracellular organelles [34,35]. In addition to their roles in ion translocation, NHEs act as scaffolds that facilitate the interaction of many ancillary proteins, including CHP (calcineurin homologous protein, p22), ezrin, NHERFI/II and calmodulin-dependent kinase [34]. Recent studies demonstrated their roles in renal epithelial cell motility [36], the directional cell migration of human melanoma cells [37], MEFs [38] and *Dictyostelium discoideum* [39] as well as in planar epithelial cell polarity [40–42]. In particular, NHE1 serves to regulate intracellular pH and volume, while NHE3 is involved in Na^+ reabsorption and proton secretion [43]. Due to the electrically silent nature of NHEs [44], we suggest our data represents the effects induced by the presence of directional cue per se, not those governed directly by EF such as electrophoretic movements of proteins along the cell axis. Therefore, NHE3 might be relevant not only to electrotaxis, but to all directional migration processes under a constant cue instead.

A link between NHEs and cell polarity has been shown in different cell types [36,39,41,45]. In our previous studies, we reported that patchy accumulations of physiologically active pNHE3 (H^+ bubbles) outward proton fluxes localize specifically to the leading edge of migrating cells, together with a pseudopodia marker protein β actin [46]. In addition, we also showed that pNHE3 is involved in the cellular directivity of directionally migrating osteogenic cells [46]. The conclusions from our previous studies were largely based on the pharmacological inhibition of NHE3. However, the molecules and mechanisms that are involved in the activation of NHE3 membrane patches and their physio-mechanical role affecting cell migration have not been studied in detail.

In the present study, we investigated the spatiotemporal mobility and localization of NHE3 interaction with beta-actin in HEK293T cells, and correlated their dynamic movements with respect to cellular directivity. In addition to it, we employed advanced fluorescence cross-correlation spectroscopy coupled with confocal microscopy to show that the NHE3-beta-actin interaction is enhanced in the pseudopodia when persistent directional cue is present (physiological electric fields). Using gene knockdown methods, we produced NHE3-deficient rat osteoblastic (calvaria) and human osteosarcoma (SaOS-2) cells and found that their cell polarity and directional migration is associated with NHE3 activity in the presence of persistent directional cues. Furthermore, we show here that by modulating NHE3 activity the interaction between NHE3 and beta-actin can be disrupted in normal cells but not in tumor cells which reflects the differences in cell migration. There is evidence for involvement of beta-actin in directional cell migration during embryo development, reported by Bunnell et. al. [47]. Regarding directional migration, we show that changes in both the expression and content of NHE3 can be correlated with the changes in cell membrane potential. In our efforts to understand how pNHE3 is involved in the perception of direction in migrating cells, we found that PI3K and Akt expression levels change proportionally to NHE3 expression. PI3K is recently reported as regulator of cell polarity in neutrophils [48,49] and randomly migrating fibroblasts reorient polarity through PI3K-dependent branching and pivoting of protrusions [50]. PI3K/Akt is also known for stimulating NHE3 [51,52]. However, our data shows that when PI3K/Akt was inhibited, the phospho-NHE3 content did not change, suggesting additional regulatory mechanisms for perception of direction during electrotaxis.