A developmentally regulated Na-H exchanger in Dictyostelium discoideum is necessary for cell polarity during chemotaxis

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Increased intracellular H⁺ efflux is speculated to be an evolutionarily conserved mechanism necessary for rapid assembly of cytoskeletal filaments and for morphological polarity during cell motility. In Dictyostelium discoideum, increased intracellular pH through undefined transport mechanisms plays a key role in directed cell movement. We report that a developmentally regulated Na-H exchanger in Dictyostelium discoideum (DdNHE1) localizes to the leading edge of polarized cells and is necessary for intracellular pH homeostasis and for efficient chemotaxis. Starved DdNHE1-null cells (Ddnhe1⁻/⁻) differentiate, and in response to the chemoattractant cAMP they retain directional sensing; however, they cannot attain a polarized morphology, but instead extend mis-localized pseudopodia around the cell and exhibit decreased velocity. Consistent with impaired polarity, in response to chemoattractant, Ddnhe1⁻/⁻ cells lack a leading edge localization of F-actin and have significantly attenuated de novo F-actin polymerization but increased abundance of membrane-associated phosphatidylinositol 3,4,5-trisphosphate (P(3,4,5)P₃). These findings indicate that during chemotaxis DdNHE1 is necessary for establishing the kinetics of actin polymerization and P(3,4,5)P₃ production and for attaining a polarized phenotype.

Introduction

The slime mold Dictyostelium discoideum has been extensively studied as a model for eukaryotic chemotaxis because many of the molecules and principles that underlie chemotaxis are common to both Dictyostelium and mammalian cells (Parent, 2004). Chemotactic competence of Dictyostelium cells toward cAMP can be efficiently and synchronously induced by starvation of vegetative cells. Chemotactically competent cells adopt a classical polarized, elongated morphology while chemotaxing and have a defined front, which is more sensitive to cAMP than the cell rear.

Efficient chemotaxis entails two distinct but functionally linked components: directional sensing and polarity (Devreotes and Janetopoulos, 2003). Directional sensing, defined as the ability of a cell to perceive a signal and set up an internal asymmetry in the direction of the incoming signal, requires an asymmetric production of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (P(3,4,5)P₃) but is independent of actin polymerization. Polarity reinforces internal asymmetries and translates them into relevant morphological changes to execute efficient movement toward chemoattractants. In contrast to directional sensing, localized actin polymerization, regulated predominantly by the localized activation of Rho family low molecular weight GTPases, is a conserved core determinant in cell polarity from yeasts to mammalian cells (Etienne-Manneville and Hall, 2002; Nelson, 2003). In chemotaxing neutrophils, actin polymerization at the leading edge of the cell acts in a positive feedback loop to reinforce localized production of P(3,4,5)P₃ and activation of the Rho family GTPase Rac (Weiner et al., 2002; Srinivasan et al., 2003), and in motile cells, including chemotaxing Dictyostelium cells, de novo actin polymerization is a predominant driving force for membrane protrusions or pseudopodia (Condeelis et al., 1990; Pollard and Borisy, 2003).

A long-speculated but poorly understood mechanism promoting de novo actin polymerization during cell movement is an increase in intracellular pH (pHᵢ). Earlier work on the fertilization of sea urchin eggs (Begg and Rebhun, 1979) and the acrosomal reaction in Echinoderm sperm cells (Tilney et al., 1978) indicates that transient increases in pHᵢ are necessary for actin polymerization and that artificially increasing pHᵢ in the absence of external cues triggers F-actin formation. In Ascaris sperm cells, a pH gradient that is higher at the front of the cell than at the rear is necessary for cell polarity and a leading
edge assembly of cytoskeletal filaments (King et al., 1994; Italiano et al., 1999), even though motile *Ascaris* sperm cells lack actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002) and sperm cells (Wang et al., 2003), *H*. *major* sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein. In mammalian fibroblasts (Denker and Barber, 2002), actin and cytoskeletal filaments consist of bundled fibers of major sperm protein.

Results

*Dd* NHE1 is developmentally expressed and is necessary for pH, homeostasis but not for cell differentiation

A single Na-H exchanger, *Dd* NHE1 (AA052201), has been found by the *Dictyostelium* Sequencing Project. By hydropathy plot analysis, *Dd* NHE1 is predicted to contain twelve transmembrane segments and a hydrophilic COOH-terminal domain, which is consistent with NHE isoforms in other species. Sequence alignment with identified NHEs using ClustalW software (www.ebi.ac.uk/clustalw/) groups *Dd* NHE1 with mammalian NHE8 and *Drosophila melanogaster* NHE1 (Fig. 1A). Mammalian NHE8 groups in a clade distinct from the clade including mammalian NHE1, which is a resident plasma membrane isoform, and from the clade including mammalian NHE6 and NHE7, which are intracellular vesicle isoforms. The mouse orthologue MmNHE8 is a recycling plasma membrane protein and localizes at the apical plasma membrane and at intracellular organelle membranes in kidney proximal tubule cells (Goyal et al., 2003). The localization of *Dd* NHE1 is predicted to be at the plasma membrane with ~74% probability, as determined by the PSORT II localization software (www.psort.org). Immunolabeling of *Dd* NHE1-HA stably expressed in wild-type Ax2 cells indicated a plasma membrane and an intracellular distribution, suggesting it is a recycling plasma membrane isoform, like MmNHE8. In polarized chemotaxing cells, intracellular and plasma membrane *Dd* NHE1-HA localized predominantly at the cell front (Fig. 1B), and cell surface biotinylation was used to confirm that *Dd* NHE1-HA is at the plasma membrane (Fig. 1C). Mammalian NHE1 has a similar localization at the leading edge plasma membrane of lamellipodia in migrating fibroblasts (Denker et al., 2000; Denker and Barber, 2002).

The transition of vegetative *Dictyostelium* cells to chemotactically competent cells is induced by starvation and requires the expression of several genes that allow cells to make, sense, break down, and move toward CAMP. In addition to expressing genes required for chemotaxis, starvation induces genes involved in cell differentiation. The *Dd* NHE1 transcript was detected in chemotactically competent cells, but not in vegetative cells (Fig. 2A), which suggests a role for *Dd* NHE1 in chemotaxis, differentiation, or both processes.
To determine the functional importance of DdNHE1, we generated DdNHE1-null (DdNHE1−/−) cells. Ax2 cells were transformed by linearized DNA constructs designed to disrupt the DdNHE1 gene. Individual clones, each generated by using a different construct to disrupt DdNHE1, were selected and positive clones were identified by the presence of a 1-kb PCR product that is absent from Ax2 cells. Samples from three independently generated DdNHE1−/− clones are shown. (C) Expression of cAR1 and Ga2 is similar in Ax2 and DdNHE1−/− cells, as determined by Northern blot analysis. Expression of the ribosomal gene IG7 was used as a loading control. (D) pH in the absence and presence of cAMP in chemotactically competent Ax2 (closed squares), DdNHE1−/− (open squares), and DdNHE1−/−/DdNHE1 (closed circles) cells was determined in cells loaded with the fluorescent pH-sensitive dye BCECF. Data are expressed as the mean ± SEM of three independent experiments.

Figure 2. DdNHE1 expression is developmentally regulated and is necessary for increased pH in response to cAMP but is not necessary for developmentally regulated expression of cAR1 and Ga2. (A) Expression of the DdNHE1 transcript in Ax2 cells is induced after 6 h of starvation (left). DdNHE1 transcript is lost in a representative clone of DdNHE1−/− cells (right). (B) DdNHE1−/− cells identified by the presence of a 1-kb PCR product that is absent from Ax2 cells. Samples from three independently generated DdNHE1−/− clones are shown. (C) Expression of cAR1 and Ga2 is similar in Ax2 and DdNHE1−/− cells, as determined by Northern blot analysis. Expression of the ribosomal gene IG7 was used as a loading control. (D) pH in the absence and presence of cAMP in chemotactically competent Ax2 (closed squares), DdNHE1−/− (open squares), and DdNHE1−/−/DdNHE1 (closed circles) cells was determined in cells loaded with the fluorescent pH-sensitive dye BCECF. Data are expressed as the mean ± SEM of three independent experiments.

min, the pH had returned to basal levels. In contrast, DdNHE1−/− cells had a lower resting pHi of 6.48 ± 0.06 (n = 7) that increased to only 6.86 ± 0.07 with cAMP. Hence, DdNHE1 is a predominant, although not exclusive, determinant in the cAMP-induced increase in pHi. Stable expression of DdNHE1-HA in DdNHE1−/− cells (DdNHE1+/DdNHE1) restored resting pHi to 6.65 ± 0.07 (n = 3). In response to cAMP, the pHi of 7.29 ± 0.13 in DdNHE1−/−/DdNHE1 cells was higher and sustained for longer than in Ax2 cells, possibly because DdNHE1, driven by the actin-15 promoter, was overexpressed.

DdNHE1−/− cells lack polarity and have impaired chemotaxis

Although DdNHE1 is not necessary for the expression of developmentally regulated genes, chemotaxis in response to cAMP was markedly impaired in all the DdNHE1−/− clones generated. Using time-lapse video microscopy to visualize movement of individual cells toward a point source of cAMP delivered by a micropipette, Ax2 cells rapidly developed an elongated, polarized morphology with a defined front in the di-
rection of the point source of cAMP and pseudopodia that were restricted to the leading edge (Fig. 3, A and B, and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200412145/DC1). When the direction of the point source of cAMP was changed, Ax2 cells executed turns and retained the same leading and trailing edges characteristic of highly polarized cells (Fig. 3 C). In contrast, Ddnhe1−/DdNHE1 cells failed to adopt a polarized morphology in response to cAMP. They retained a rounded shape lacking a clear front and rear and extended pseudopodia around the entire cell (Fig. 3, A and B; and Video 2). In Ddnhe1+/DdNHE1 cells, a polarized morphology and leading edge pseudopodia were restored (Fig. 3, A and B; and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200412145/DC1).

Further analysis of time-lapse recordings indicated that the velocity of chemotaxing Ddnhe1− cells was decreased compared with Ax2 cells and Ddnhe1−/DdNHE1 cells (Fig. 4 A). Tracking of individual cells indicated that at 7 min most of the Ax2 and Ddnhe1−/DdNHE1 cells within the field showed directional movement toward cAMP. Ddnhe1− cells showed some movement, but it was random and not directed toward cAMP (Fig. 4 B, left). At 15 min, all Ax2 and Ddnhe1−/DdNHE1 cells within the field reached the point source of cAMP. Ddnhe1− cells eventually reached the point source but not until 30 min (Fig. 4 B, middle). At 30 min, however, Ddnhe1− cells were still not polarized, retaining mislocated pseudopodia and failing to adopt an elongated morphology. Ddnhe1− cells lacked polarity, regardless of their distance from the chemoattractant source, as indicated by sequential traces of cells at distances of 15 and 45 μm from the micropipette delivering cAMP (Fig. 4 B, right).

**F-actin localization and the kinetics of actin polymerization are impaired in Ddnhe1− cells**

Consistent with impaired polarity, F-actin was mislocalized in Ddnhe1− cells and de novo actin polymerization was markedly attenuated. In polarized Ax2 cells chemotaxing toward a point source of cAMP, F-actin was predominantly at the leading edge and to a slight extent at the sides and the rear (Fig. 5 A). In contrast, F-actin in Ddnhe1− cells extended around the cell cortex and was not localized to the cell pole facing the point source of cAMP (Fig. 5 A). Similar to Ax2 cells, F-actin in Ddnhe1− cells was predominantly localized to areas where pseudopods were extending, although in Ddnhe1− cells pseudopodia were mislocalized and not confined to the direction of chemoattractant.

Because actin polymerization is a predominant driving force for pseudopod extension (Condeelis et al., 1990; Pollard and Borisy, 2003), mislocalized pseudopodia in Ddnhe1− cells suggested an inability to correctly regulate F-actin assembly. This prediction was confirmed by measuring time-dependent actin polymerization in response to cAMP. Total F-actin with a uniform concentration of 2 μM cAMP was determined in chemotactically competent cells in the presence of 5 mM caffeine to inhibit endogenous cAMP production. Ax2 cells had a characteristic biphasic increase in F-actin, as previously described (Condeelis et al., 1988; Chen et al., 2003; Park et al., 2004). The rapid first phase consisted of a 2.2-fold increase in F-actin that peaked between 4 to 8 s and returned to basal level by 16 s. A second, more sustained phase with a smaller amplitude peaked at 120 s after cAMP stimulation (Fig. 5 B). Ddnhe1− cells had similar levels of F-actin in the absence of cAMP compared with Ax2 cells and a biphasic increase in F-actin in response to cAMP. The magnitude of the first phase, however, was reduced by ~60% compared with Ax2 cells (Fig. 5 B). At 16 s, F-actin in Ddnhe1− cells returned to below basal level. The second phase was largely absent with an initial slight increase in F-actin that was not sustained after 30 s. In Ddnhe1−/DdNHE1 cells, the kinetics of actin polymerization was restored and the magnitude of the second phase was slightly increased compared with Ax2 cells (Fig. 5 B). Hence, DdNHE1 does not regulate the abundance of F-actin in starved cells but is necessary for rapid F-actin assembly and for a leading edge localization of F-actin in response to cAMP. These data are consistent with findings in sea urchin.
eggs (Begg and Rebhun, 1979) and in mammalian fibroblasts (unpublished data), which suggest that increases in pH are not necessary for steady-state actin filament assembly but are required for “bursts” of de novo F-actin formation as a dynamic response to extracellular cues.

**Ddnhe1** cells have a sustained increase in membrane-associated PI(3,4,5)P3

cAMP induces a biphasic increase in the abundance of PI(3,4,5)P3 (Chen et al., 2003; Postma et al., 2003), correlating with the biphasic kinetics of actin polymerization. PI(3,4,5)P3 abundance was determined by using a reporter construct of a GFP-tagged pleckstrin homology (PH) domain of the cytosolic regulator of adenylyl cyclase (CRAC; GFP-PH^{CRAC}). The PH domain of CRAC binds PI(3,4,5)P3 and has been used to determine the localization and abundance of PI(3,4,5)P3 in chemotaxing Dictyostelium cells (Parent et al., 1998; Chen et al., 2003; Postma et al., 2003). With a uniform concentration of 2 μM cAMP, Ax2 and Ddnhe1 cells both had biphasic increases in the abundance of membrane-associated (p100 fraction) GFP-PH^{CRAC} (Fig. 6 A); however, there were marked differences. Although the maximal abundance of GFP-PH^{CRAC} in the first phase was similar, in unstimulated Ddnhe1 cells there was significantly more membrane-associated GFP-PH^{CRAC} and hence the fold increase was markedly less (1.2) compared with Ax2 cells (2.2). Additionally, although the kinetics of the second phase was similar, Ddnhe1 cells had markedly more membrane-associated GFP-PH^{CRAC} at all time points.

Figure 5. Localization and production of F-actin in response to cAMP are impaired in chemotactically competent Ddnhe1 cells. (A) In Ax2 cells (top), F-actin was localized predominantly at the leading edge of the cell, and F-actin-rich protrusions were limited to the direction of the chemoattractant source (asterisks). In Ddnhe1 cells (bottom), F-actin was distributed along the cell cortex, and although membrane protrusions (arrowheads) also contained F-actin, protrusions were not limited to the direction of the chemoattractant source but were seen around the cell. (B) Time course of F-actin production in response to a uniform concentration of 2 μM cAMP indicated a characteristic biphasic response in Ax2 cells (blue line), including a rapid (~10 s) first phase of greater magnitude and a slower and smaller second phase. In Ddnhe1 cells (black line), the increase in F-actin in the first phase was reduced by ~60% and the second phase was transient with reduced F-actin formation, compared with Ax2 cells. Ddnhe1/DdNHE1 cells (red line) had a biphasic increase in F-actin, although the magnitude of first and second phases was slightly greater compared with Ax2 cells. Data are expressed as the mean ± SEM of at least three independent cell preparations.

Figure 6. Ddnhe1 cells have increased abundance of membrane-associated PH^{CRAC}-GFP in the absence and presence of cAMP but retain directional sensing. (A) Abundance of PH^{CRAC}-GFP in p100 membrane fractions and in total cell lysates was determined by immunoblotting with anti-GFP antibodies and used as a reporter for PI(3,4,5)P3 production. Ax2 cells and Ddnhe1 cells had a biphasic increase in the abundance of membrane-associated PH^{CRAC}-GFP relative to total PH^{CRAC}-GFP in response to cAMP; however, in Ddnhe1 cells there was more membrane-associated PH^{CRAC}-GFP in the absence of cAMP (time 0) and during the second phase compared with Ax2 cells. Data are expressed as the mean ± SEM of five independent experiments. (A, inset) Ax2 and Ddnhe1 cells have similar expression of PH^{CRAC}-GFP, as indicated by immunoblotting with anti-GFP antibodies of total lysates of Ax2 cells untransformed (lane 1) and transformed with PH^{CRAC}-GFP (lane 2) and Ddnhe1 cells untransformed (lane 3) and transformed with PH^{CRAC}-GFP (lane 4). (B) Asymmetric translocation of cytosolic PH^{CRAC}-GFP to the membrane in latrunculin-treated cells indicates Ax2 and Ddnhe1 sense and respond to a point source of 10 μM cAMP. Intensity of GFP fluorescence at the cortex was expressed by plotting intensity as a function of the percentage of the cell perimeter. Data represent the mean ± SEM of four independent determinations. (C) The cAMP-induced increase in pH of Ax2 cells was attenuated by the presence of LY294002. Data represent the mean ± SEM of four independent determinations.
\textit{Dd}nhe1\textsuperscript{−} cells retain directional sensing

\textit{Dd}nhe1\textsuperscript{−} cells express the developmentally regulated genes cAR1 and Go2 and chemotax toward cAMP, albeit inefficiently, which suggests they retain directional sensing. This finding was confirmed by the ability of \textit{Dd}nhe1\textsuperscript{−} cells to asymmetrically accumulate P\textsubscript{1,3,4,5}P\textsubscript{3} in the direction of a point source of cAMP. Directional sensing, which does not require polarity or rapid actin filament assembly (Devreotes and Janetopulos, 2003), was determined in cells treated with latrunculin A to inhibit actin polymerization. Although latrunculin-treated cells were round and lacked morphological polarity, in Ax2 and \textit{Dd}nhe1\textsuperscript{−} cells GFP-PH\textsuperscript{CRAC} was predominantly localized in an arc along the plasma membrane closest to the pipette delivering cAMP (Fig. 6 B). An analysis of GFP-PH\textsuperscript{CRAC} intensity as a function of cell perimeter revealed nearly superimposable tracings for Ax2 and \textit{Dd}nhe1\textsuperscript{−} cells. Collectively, therefore, our data indicate that \textit{Dd}NHE1 is not necessary for cells to detect an asymmetric extracellular cue but is essential for establishing morphological polarity and efficient chemotaxis toward the cue.

The ability of \textit{Dd}nhe1\textsuperscript{−} cells to acquire asymmetric localization of GFP-PH\textsuperscript{CRAC} suggested \textit{Dd}NHE1 might act downstream of P\textsubscript{1,3,4,5}P\textsubscript{3} production by phosphatidylinositol 3 (PI3)–kinases. This prediction was tested by determining cAMP-induced increases in pH, in the absence and presence of LY294002, which inhibits PI3-kinase activity. In Ax2 cells treated with 30 \textmu M LY294002, the increase in pH, with cAMP was markedly attenuated (Fig. 6 C). In \textit{Dd}nhe1\textsuperscript{−} cells, the pH response to cAMP was similar in the absence and presence of LY294002 (unpublished data). Hence, inhibition of PI3-kinase blocks \textit{Dd}NHE1-dependent, but not \textit{Dd}NHE1-independent, increases pH\textsubscript{r} in response to cAMP.

\section*{Discussion}

In differentiated \textit{Dictyostelium} cells, cAMP induces an increase in pH (Jamieson et al., 1984; Aerts et al., 1987) that is essential for directional movement (Van Duijn and Inouye, 1991). However, the ion transport process regulating pH, homeostasis and the mechanism whereby pH regulates chemotaxis in \textit{Dictyostelium} cells have not been clearly defined. We now report that a \textit{Dictyostelium} Na-H exchanger, \textit{Dd}NHE1, is the predominant regulator of increased pH, in response to cAMP and is necessary for efficient chemotaxis. Although \textit{Dd}nhe1\textsuperscript{−} cells retain directional sensing, they lack morphological polarity, have decreased de novo actin polymerization, and fail to localize F-actin at the leading edge, resulting in impaired chemotaxis.

A rapid increase in actin filament assembly is the major driving force for directed cell motility (Condeelis et al., 1990; Pollard and Borisy, 2003). In response to chemoattractant, \textit{Dictyostelium} cells have a biphasic increase in actin filament assembly (Chen et al., 2003; Park et al., 2004). In \textit{Dd}nhe1\textsuperscript{−} cells, the rapid (<10 s) first phase is markedly attenuated and the prolonged (30 to 180 s) second phase is largely absent. The first phase of actin polymerization may be essential for establishing morphological asymmetry, and \textit{Dd}nhe1\textsuperscript{−} cells never develop polarity, regardless of their distance from the chemoattractant source. Where actin polymerization is sustained determines the direction of cell movement, and \textit{Dd}NHE1, which localizes predominantly at the front of polarized cells, is also necessary for limiting F-actin assembly to the leading edge.

Several models have recently been proposed for how the first and second phases of actin polymerization are controlled (Chen et al., 2003; Mouneimne et al., 2004; Park et al., 2004; Sasaki et al., 2004). All models predict that the first phase is independent of P\textsubscript{1,3,4,5}P\textsubscript{3}, and, consistent with this prediction, in \textit{Dd}nhe1\textsuperscript{−} cells when the first phase of actin polymerization is suppressed the abundance of membrane-associated P\textsubscript{1,3,4,5}P\textsubscript{3} is normal. In \textit{Dictyostelium} cells, the first phase of actin polymerization is in part dependent on Rac1B (Park et al., 2004) and Ras proteins (Sasaki et al., 2004). \textit{Dictyostelium} cells null for \textit{RacB} have an actin polymerization profile similar to that of \textit{Dd}nhe1\textsuperscript{−} cells, including ~60% decrease in the magnitude of the first phase and a markedly suppressed second phase (Park et al., 2004). In \textit{RacB}-null cells, but not in \textit{Dd}nhe1\textsuperscript{−} cells, however, the basal abundance of F-actin is reduced compared with wild-type cells. Another distinction is that \textit{RacB}-null cells exhibit only a small loss of polarity compared with the marked lack of polarity in \textit{Dd}nhe1\textsuperscript{−} cells. \textit{Dictyostelium} Ras proteins also regulate the first phase of actin polymerization and polarity. Although \textit{Dictyostelium} cells null for \textit{RasG} have normal polarity and only slightly reduced F-actin in response to cAMP, expression of a dominant inactive \textit{Dd}RasG-S17N in \textit{RasG}-null mutants to block activation of other Ras proteins results in loss of polarity and ~30% reduction in the initial F-actin peak (Sasaki et al., 2004). The role of \textit{Dd}NHE1 in chemotaxis may be distinct from that of \textit{RacB} or \textit{RasG} because the phenotype of \textit{Dd}nhe1\textsuperscript{−} cells is similar to \textit{RacB}-null cells in F-actin formation, but not in morphological polarity, and similar to \textit{RasG}-null cells expressing \textit{Dd}RasG-S17N in loss of polarity, but not in the regulation of actin polymerization.

In mammalian cells, the first phase of actin polymerization is preceded by an increase in actin free barbed ends generated by the actin-severing protein cofolin (Mouneimne et al., 2004). Because the actin-severing activity of cofolin is pH-sensitive, cofolin is a possible candidate mediating pH-dependent effects of \textit{Dd}NHE1 on the first phase of actin filament assembly. Increased pH is necessary to stimulate cofolin activity and to promote the recruitment of cofolin to the leading edge membrane of migrating cells (Bernstein et al., 2000; Bowman et al., 2000). In addition to cofolin, possible targets of \textit{Dd}NHE1-dependent increases in pH include severin, an actin-severing and -capping protein of the pH-sensitive gelsolin family (Yin et al., 1990; Lamb et al., 1993), and hisactophilin, a pH sensor in \textit{Dictyostelium} that nucleates actin filaments (Hanakam et al., 1996; Stoeckelhuber et al., 1996).

The second phase of actin polymerization is dependent on the abundance and localization of P\textsubscript{1,3,4,5}P\textsubscript{3}, which are regulated by PI3-kinases and the PI3-phosphatase PTEN (Funamoto et al., 2002; Iijima and Devreotes, 2002). In \textit{pik3k1}°/° cells and in cells treated with pharmacological inhibitors of PI3-kinases, the second phase of actin polymerization is suppressed but the first phase is retained (Funamoto et al., 2001;
Chen et al., 2003). Consistent with previous papers that showed mammalian NHE1 may act downstream of PI3-kinases (Ma et al., 1994; Reshkin et al., 2000), the PI3-kinase inhibitor LY294002 completely blocks the DdNHE1-dependent increase in pH$_i$ in response to cAMP. Two predictions may explain why the second phase of actin polymerization is suppressed in DdNHE1$^{-}$ cells despite increased PI$_{3,4,5}$P$_3$. DdNHE1 could act downstream of PI$_{3,4,5}$P$_3$ in a pathway regulating the second phase of actin polymerization, but, in contrast to a positive feedback loop suggested between actin polymerization and PI$_{3,4,5}$P$_3$ (Weiner et al., 2002; Srinivasan et al., 2003; Sasaki et al., 2004), increased pH$_i$ may act in a negative feedback mechanism to limit PI$_{3,4,5}$P$_3$ abundance. Alternatively, DdNHE1 regulates the second phase of actin polymerization independently of PI$_{3,4,5}$P$_3$.

Increased pH$_i$ is predicted to be an evolutionarily conserved mechanism necessary for cytoskeletal filament assembly and for motility (Tilney et al., 1978; Begg and Rebhun, 1979; Italiano et al., 1999). Protons do not directly regulate filament assembly (Tilney et al., 1978; Begg and Rebhun, 1979; Italiano et al., 1999) but likely act on pH-dependent filament-binding proteins, such as cofilin and gelsolin. In Dictyostelium cells, a pH-dependent increase in actin polymerization may be a developmentally regulated mechanism to control polarity because DdNHE1 is expressed in chemotactically competent cells but not in vegetative cells. In neutrophil-like HL60 cells, which are chemotactic only after differentiation, NHE1 is expressed in differentiated, but not in undifferentiated, cells (unpublished data). In fibroblasts (Denker and Barber, 2002) and epithelial cells (Klein et al., 2000), NHE1 also is necessary for directional migration but its expression at the plasma membrane is constitutive.

However, whether DdNHE1 regulates chemotaxis exclusively through increased H$^+$ efflux or also by underdeveloped pH-independent actions remains to be determined. Independently of generating H$^+$ efflux, mammalian NHE1 also anchors actin filaments by binding ERM (ezrin, moesin, and radixin) actin-binding proteins. Actin anchoring by NHE1, but not H$^+$ efflux, is necessary for the bundling of actin stress fibers, localizing NHE1, and tethering actin filaments in specialized membrane domains (Denker and Barber, 2002). Although ERM proteins have not been identified in Dictyostelium, a GST-fusion protein containing the NH$_2$-terminal FERM (protein 4.1 and ERM) domain of moesin binds directly to DdNHE1 (unpublished data), suggesting that binding of a FERM domain–containing protein might maintain DdNHE1 at the cell front. We predict that the localization of NHE1 and DdNHE1 at the front of motile cells retains a leading edge H$^+$ efflux that is necessary for cell polarity and directional movement.

**Materials and methods**

**Cell culture and starvation**

Cells were grown in Knecht medium under standard conditions (Sussman, 1987), and exponentially growing cells were harvested for use in all experiments. Chemotactic competence was induced by starving cells for 6 h in phosphate buffer (PB; 20 mM KH$_2$PO$_4$ and 20 mM K$_2$HPO$_4$, pH 6.8) at RT at a cell density of 1 $\times$ 10$^7$ to 2 $\times$ 10$^7$ cells/ml while shaking at 115 rpm.

**Isolation of DdNHE1 and the generation of DdNhe1$^{-}$ cells**

Total RNA was isolated (Qiagen; UC-100) from wild-type Ax2 cells (provided by C.J. Weijer, University of Dundee, Dundee, Scotland, UK), and RT-PCR was performed on chemotactically competent cells using primers designed to the NH$_2$ terminus and COOH terminus of DdNHE1 (AA052201) (5'/GGATCCCTGAAGAATAAGACAAAAAGTTATATATATTAGTG-3' and 5'/GGGCCTCTATGATGCTGATTGGGTGACACGACATCATCAGAGTCTGATGTCGATTTTGTTATGTTG-3', respectively). The COOH terminus primer also contained an HA-epitope tag (underlined), and both primers contained restriction sites (NH$_2$ terminus, BamHI; COOH terminus, Smal) for cloning. The single PCR product obtained was cloned into the TOPO2.1 cloning vector and subcloned into pBluescript II, a Dictyostelium expression vector containing a neomycin resistance-conferring cassette, which was used in reconstituting DdNHE1-HA into DdNHE1$^{-}$ cells.

To generate DdNHE1$^{-}$ cells, Ax2 cells were electroporated (Howard et al., 1988) with $\sim$0.1 $\mu$g of linearized DNA consisting of a blasticidin resistance cassette (Bst) excised from the Dictyostelium expression vector pUC118 (provided by C.J. Weijer) flanked by regions of DdNHE1. Three different constructs were used for three independent transformations, and individual clones were isolated from each transformation after selection with 10 $\mu$g/ml of Blasticidin. Two of the constructs consisted of the Bst cassette inserted into partial DdNHE1 PCR products of two lengths (1 and 1.5 kb), and the third construct consisted of the Bst cassette inserted into the 1.5kb PCR product after excision of $\sim$200 bp of DdNHE1. Positive clones were identified by the absence of the DdNHE1 transcript from chemotactically competent cells (Fig. 2 A) and by the presence of a PCR product from genomic DNA by using a primer set consisting of one within the Bst cassette and another within the genomic region near DdNHE1 (Fig. 2 B).

**Surface biotinylation**

Starved DdNHE1$^{-}$/DdNHE1-HA cells and Ax2 cells expressing the pH domain of CRAC fused to GFP (pPHAC-GFP; provided by P. Devreotes, Johns Hopkins University, Baltimore, MD) were labeled for 30 min on ice with 0.5 mg/ml of biotin in KK$_2$ buffer (EZLink Sulfo-NHS-LC-Biotin; Pierce Chemical Co.). Cells were washed in ice-cold KK$_2$ buffer three times and lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors, pH 7.4). Samples were cleared with protein G-Sepharose (17–061801; Amersham Biosciences) at 4°C. Supernatant was collected and incubated with anti-HA (1:250; 12CA5; Roche), anti-cAR1 (1:250; provided by C. Parent, National Cancer Institute, Bethesda, MD), or anti-GFP (1:250; J108; Clontech Laboratories, Inc.) antibodies in the presence of protein G–Sepharose at 4°C. Sepharose beads were washed three times in ice-cold RIPA buffer, resuspended in loading buffer, and heated to 100°C for 5 min. Samples were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% milk and probed with HRP/Streptavidin (1:1000; Vector Laboratories).

**Northern blot analysis**

Total RNA was isolated from Ax2 and DdNhe1$^{-}$ cells before starvation and after 3 and 6 h of starvation in PB. 4 $\mu$g of total RNA was separated using gel electrophoresis, transferred to nylon membranes (Hybond-N+; Amersham Biosciences), and probed with 32P-labeled cAR1 or Gq2 DNA probes. The membranes were stripped and reprobed for the ribosomal protein IG7 (provided by P. Schaap, University of Dundee) for loading control. Data are representative of three independent cell preparations.

**pH measurements**

pH$_i$ was determined in cells loaded with the pH-sensitive fluorescent dye 2′,7′-bis(2-carboxyethyl)-5′-(and-6′)-carboxyfluorescein (BCECF; Molecular Probes). During starvation of cells for 6 h, 5 $\mu$M BCECF was added for the final 30 min, 5 mM caffeine was added for the last 20 min, cells were washed once in PB, resuspended in PB containing 5 mM caffeine, and 5 $\times$ 10$^7$ cells were added to wells of a 96-well plate. Cells were allowed to attach for 5 min before removing excess cells and submerging the attached cells in 40 $\mu$L PB, pH 6.8. In plate wells, 40 $\mu$L PB at pH 5.9, 6.8, and 7.0 containing 5 $\mu$M nigericin (Molecular Probes) was added to equalize intracellular and extracellular pH and calibrate fluorescence intensity to pH. After 15 min, the fluorescence intensity was determined by using a SpectraMax Gemini XS Micro Plate fluorometer (Molecular Devices) at excitation wavelengths of 439 nm (H$^+$-insensitive) and 490 nm (H$^+$-sensitive), with an emission wavelength of 530 nm. A plot of the 490/439 nm ratios against the pH values of the...
buffers containing nigericin was used to generate a calibration curve from which pH was determined. To determine pH in response to cAMP, 2 μM was added to cells and the fluorescence intensity was determined at 45-s intervals. After 6 min, the fluorescence intensity at 439 and 490 nm was determined to confirm that fluorescence intensity at 439 remained constant. Determinations were performed in triplicate, and data were expressed as the mean ± SEM of the indicated independent cell preparations. Inhibition of PI3-kinases was achieved by incubating cells with 30 μM Ly294002 during the caffeine treatment.

Chemotaxis assays and cell tracking
For the analysis of individual cell movement, chemotactically competent cells were baselined for 20 min by adding 5 mM caffeine and then plated at a density of ~10^5 cells/cm^2 on nonnutrient 1% phosphate agar plates containing 5 mM caffeine. A micropipette containing 10 μM CAMP was placed next to cells, and images were captured every 30 s using a Spot LCD camera mounted on a microscope (Axiovert S-100; Carl Zeiss Microimaging, Inc.) with a 40× Hoffman modulation objective and operated using Openlab software (Improvision). Cells were tracked using Openlab software, and their coordinates were plotted using Microsoft Excel software.

Phalloidin staining and immunoblotting of DnH1
Cells were submerged in PB, allowed to develop on plastic dishes for 8 h, and washed and fixed for 3 min in PB containing 3.7% glutaraldehyde/0.1% Triton X-100. The fixative was removed, and the cells were washed three times in PB. To visualize F-actin, cells were incubated in PB containing rhodamine phalloidin (Molecular Probes) for 1 h at RT. The cells were washed three times in PB and dried briefly, and a drop of Vectashield was added before a glass coverslip was placed over the cells.

For immunoblotting of DnH1, DnHe / DnH1-α HA-cells were fixed, washed as described in the previous paragraph, followed by blocking in PB containing 5% FBS for 30 min at RT. Cells were labeled for 1 h at RT with anti-HA antibodies (12CA5; Roche) at a dilution of 1:1,500 in PB containing 2% FBS. Cells were washed three times in PB and incubated at RT in PB containing FITC labeled anti–mouse IgG (Jackson ImmunoResearch Laboratories) for 1 h. Cells were washed three times in PB and then left in dry briefly, and a drop of Vectashield was added before a glass coverslip was placed over the cells. Images were captured using an inverted confocal microscope (model LSM500; Carl Zeiss Microimaging, Inc.) and three-dimensional projection images were generated using LSM software.

Actin polymerization assays
Starved cells baselined with 5 mM caffeine at a density of 2 × 10^5 cells/ml were stimulated with a uniform concentration of 2 mM cAMP at a time interval of 5 s) placing a needle containing 10 μM cAMP at a distance of ~20 μm from the cell. Intensity of GFP fluorescence at the perimenter was determined using NIH image software, normalized to maximal fluorescence, and expressed as previously described [Janetopoulos et al., 2004] by plotting intensity as a function of the percent of the perimenter. Data represent the mean ± SEM of four cells from two independent cell preparations.

Online supplemental material
Time-lapse video microscopy (Fig. 3) of 6 h starved cells (Ax2, DnH1), and DnH1 / DnH1-α chemotaxing toward a pipette tip containing 10 μM CAMP is shown in Videos 1, 2, and 3, respectively. Images were captured at 30-s time intervals using a Spot LCD camera mounted on a microscope (Axiovert S-100; Carl Zeiss Microimaging, Inc.) with a 40× Hoffman modulation objective and operated using Openlab software (Improvision). Captured images were compiled into a QuickTime movie using Sorenson Video Compressor software. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200412145/DC1.

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References