

# Electric Field-Induced Polarization of Charged Cell Surface Proteins Does Not Determine the Direction of Galvanotaxis

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Galvanotaxis, that is, migration induced by DC electric fields, is thought to play a significant role in development and wound healing, however, the mechanisms by which extrinsic electric fields orchestrate intrinsic motility responses are unknown. Using mammalian cell lines (3T3, HeLa, and CHO cells), we tested one prevailing hypothesis, namely, that electric fields polarize charged cell surface molecules, and that these polarized molecules drive directional motility. Negatively charged sialic acids, which contribute the bulk of cell surface charge, redistribute preferentially to the surface facing the direction of motility, as measured by labeling with fluorescent wheat germ agglutinin. We treated cells with neuraminidase to remove sialic acids; as expected, this decreased total cell surface charge. We also changed cell surface charge independent of sialic acid moieties, by conjugating cationic avidin to the surface of live cells. Neuraminidase inhibited the electric field-induced directional polarization of membrane ruffling and  $\alpha 4$  integrin, while avidin treatment actually reversed the directional polarization of sialic acids. Neuraminidase treatment inhibited directionality but did not alter speed of motility. Surprisingly, avidin treatment did not significantly alter either directionality or speed of motility. Thus, our results demonstrate that electric field-induced polarization of charged species indeed occurs. However, polarization of the bulk of charged cell surface proteins is neither necessary nor sufficient to cause motility, thus contradicting the second part of our hypothesis. Because neuraminidase inhibited directional motility, we also conclude that sialic acids are required constituents of some cell surface molecule(s) through which electric fields mount a polarized transmembrane response. *Cell Motil. Cytoskeleton* 64: 833–846, 2007. © 2007 Wiley-Liss, Inc.

**Key words:** DC electric fields; live cell biotinylation; directional migration; sialic acid; neuraminidase

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## INTRODUCTION

Cellular galvanotaxis, or electric field (EF)-directed motility, has been observed in vitro in many cell types [Robinson, 1985; Mycielska and Djamgoz, 2004; McCaig et al., 2005], and endogenous EFs are thought to be physiological regulators of motility during development [Altizer et al., 2001; Levin et al., 2002] and wound healing [Farboud et al., 2000; Song et al., 2002; Zhao et al., 2006; Huttenlocher and Horwitz, 2007]. However, much remains unknown about the molecular mechanism(s) by which EFs produce cellular responses.

EFs have been hypothesized to exert a direct influence on the activity of ion transporters on either the cathode- or anode-facing side of the plasma membrane [Cooper and Keller, 1984; Mycielska and Djamgoz, 2004]. For example, calcium transport is typically implicated, since several cell types, including keratinocytes [Fang et al., 1998], and C3H/10T1/2 fibroblasts [Onuma and Hui, 1988], require calcium for galvanotaxis. In contrast, calcium transport appears to be irrelevant to galvanotaxis of 3T3 fibroblasts [Brown and Loew, 1994] and galvanotropism of *Xenopus* neurons [Palmer et al., 2000], since both occur in the absence of external calcium. Sodium ions may also be involved, as EF migration has been shown to require activity of sodium channels in MAT-LyLu rat prostate cancer cells [Djamgoz et al., 2001], and the sodium-hydrogen transporter, NHE1, in WT-PSN fibroblasts [Zhao et al., 2006].

In addition to polarized effects on ion transport, another prevailing hypothesis is that EFs cause the redistribution of charged cell-surface molecules. Many molecules on the cell surface are negatively charged due to the presence of sialic acids (Sials), which account for the bulk of the charge on the cell surface [Cook, 1968; Schauer, 1982; Angata and Varki, 2002]. Charged molecules may redistribute via electrophoresis through the plasma membrane [Jaffe, 1977; Orida and Poo, 1978; McLaughlin and Poo, 1981]. Alternatively, redistribution of charged species may happen because the EF induces an electroosmotic flow of positive counter-ions above the cell surface, towards the cathode-facing side, and this drags, and thus polarizes, negatively charged cell surface components [McLaughlin and Poo, 1981; McCaig et al., 2005]. EF-induced redistribution of charged cell surface molecules has been proposed as the mechanism by which EFs exert galvanotropic effects on neurons [Patel and Poo, 1982], although the role of EF-induced redistribution in galvanotaxis has not previously been tested.

A number of cell surface molecules have been shown to become polarized in response to EFs; for example, the EGF receptor [Giugni et al., 1987; Fang et al., 1999] and the acetylcholine receptor [Orida and Poo, 1978; Stollberg and Fraser, 1988]. A number of investi-

gators have reported EF-induced asymmetric cell-surface labeling with concanavalin A (ConA) [McLaughlin and Poo, 1981; Stollberg and Fraser, 1988; Brown and Loew, 1994]. In addition, pretreatments with lectins such as ConA or wheat germ agglutinin (WGA) have been shown to inhibit EF motility [Patel and Poo, 1982; Brown and Loew, 1994; Stewart et al., 1996]; although this inhibition has been attributed to prevention of EF-induced redistribution of charged cell surface proteins, it is possible that other effects may be involved instead, such as sterically blocking or cross-linking of surface molecules [Stewart et al., 1996].

Previous experiments have implicated polarized cell surface species in galvanotropism. For example, treatment with neuraminidase, an enzyme that removes Sials [Rosenberg and Schengrund, 1976], has been used in classic studies by Poo and coworkers [McLaughlin and Poo, 1981; McCloskey et al., 1984] who demonstrated that removal of Sials inhibited protein reorientation on the surface of EF-exposed cells. Further, neuraminidase treatment was shown to reverse the polarization of acetylcholine receptor in EF-exposed *Xenopus* muscle cells [Stollberg and Fraser, 1990]. However, in one study, neuraminidase did not inhibit the cathodal turning of *Xenopus* neurites [McCaig, 1989]. We are unaware of any previous studies examining the effects of neuraminidase on galvanotaxis. Thus, in this paper, we test the EF motility of cells in which we have altered cell surface charge. Our experiments extend previous findings by using two different treatments to alter surface charge, namely, neuraminidase treatment and avidin conjugation.

## MATERIALS AND METHODS

### Cell Culture and Migration Assay

NIH 3T3 cells, HeLa cells, and CHO cells stably expressing GFP- $\alpha$ 4 integrin (CHO GFP- $\alpha$ 4), a gift of Dr. Joy Yang, Johns Hopkins University [Pinco et al., 2002], were maintained in the following media: 3T3 in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) with 10% calf serum (HyClone, Logan, UT) and 25 mM HEPES (pH 7.3), HeLa in DMEM with 10% calf serum, and CHO GFP- $\alpha$ 4 in Ham's F-12 media (Invitrogen) plus 10% fetal bovine serum (HyClone) and 400  $\mu$ g/ml Geneticin (Invitrogen). EF motility was measured in the growth media used for each cell type.

For EF motility, cells were plated overnight in Flexi-Perm silicon chambers (Sigma, St. Louis, MO) on HCl-washed glass slides, using  $2.0 \times 10^4$  to  $6.5 \times 10^4$  cells per 18-mm diameter chamber. EF motility [using equipment described in Chao et al., 2000] was performed at 6 V/cm for 1 h. CHO cells (only) were starved in

serum-free medium for 3 h before application of EF, as described [Pu and Zhao, 2005]. Migration speed (total distance traveled per hour) and directional velocity (speed towards the cathode) were quantified according to Finkelstein et al. [2004]. Directionality, calculated as, (directional velocity) divided by (speed), was used to quantify the proportion of cell movement that was cathodally-directed.

### Avidin Conjugation to the Cell Surface

HeLa cells grown in Flexi-Perm chambers were first biotinylated by 20-min incubation with 200  $\mu$ l per chamber of a freshly prepared solution of 1 mM Sulfo-NHS-Biotin (Sigma) in phosphate-buffered saline (PBS) containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , pH 7.4. Next, 10  $\mu$ l of a 10 mg/ml solution of avidin or succinylated avidin (Sigma) was applied for 20 min, prior to EF exposure.

Based on the following approximations, we calculated that the avidin we applied was in sufficient excess so that, if  $\geq 1\%$  of the solution avidin bound to the cell surface, the net positive charge of added avidin would outweigh the net negative charge of Sials on the surface of treated cells: To estimate the number of Sials per cell, we used the value of 3.2 nmol Sials per  $10^7$  cells, reported for guinea pig neutrophils [Schauer, 1982]. Each galvanotaxis chamber contained  $6.5 \times 10^4$  cells, thus there were  $\sim 2 \times 10^{-11}$  moles of Sials per chamber. One hundred micrograms avidin was applied per chamber, therefore  $\sim 1.5 \times 10^{-9}$  moles of avidin tetramer. From this calculation, each chamber contained  $\sim 100$  times as many molecules of avidin as of Sial; we also note that one avidin molecule carries a larger net charge than one Sial.

### Neuraminidase and Cytochalasin D Treatment

Neuraminidase from *Clostridium perfringens* (Sigma, type V) was resuspended at 10 U/ml in treatment buffer, consisting of PBS, pH 6.5, and 0.05 % BSA; aliquots were stored at  $-70^\circ\text{C}$ . Live cells were treated with neuraminidase diluted in growth media (media with serum). Coverslips or slides were placed cell-side down on drops of neuraminidase solution (control cells were mock-treated by placing on drops of treatment buffer alone) for 30 min in a humidified chamber at  $37^\circ\text{C}$ . Cytochalasin D treatment was 2  $\mu\text{M}$  for 1 h before application of EF; the drug was also included in the media inside the EF motility chamber.

### Zeta Potential

Total surface charge (zeta potential) was determined using confluent monolayers of cells; 3T3 cells were used in these experiments because 3T3 monolayers are sufficiently uniform to avoid excessive noise in the measurements. The zeta potential was calculated from streaming potential measurements performed with a modified parallel-

plate flow chamber under well-developed, laminar flow conditions [Van Wagenen et al., 1976; Hung et al., 1996]. Glass microscope slides and  $24 \times 50 \text{ mm}^2$  coverslips were pre-washed with HCl, placed in 100-mm square dishes, and  $2 \times 10^6$  cells and culture medium were added and grown until confluent. For each run, one cell-covered slide and one cell-covered coverslip were placed on opposite sides of the chamber, with the cells facing inward. With this setup, the cells covered more than 97% of the chamber surface, while the rest of the surface was covered by the edge of the silicon gasket that separated the slide from the coverslip. A syringe pump connected to the chamber was used to apply oscillating fluid flow (using PBS) at 0.133 Hz. Flow rates of 5–15 ml/min were applied, corresponding to  $\sim 200$ –700 Pa, calculated from the chamber geometry. A volt meter connected to the chamber measured changes in electrical potential ( $V$ ); from plots of  $\Delta V$  against  $\Delta P$ , zeta-potential was calculated from the equation

$$\zeta = (\eta\sigma\Delta E_{\text{str}})/(\epsilon\Delta P)$$

in which  $\eta$  is the viscosity (0.001 N s/m<sup>2</sup>),  $\sigma$  is the conductivity (1.7 S/m), and  $\epsilon$  is the dielectric constant ( $6.67 \times 10^{-10}$  Coulomb<sup>2</sup>/(N m<sup>2</sup>)) [Van Wagenen and Andrade, 1980]. The average slope from the best fit line of the  $\Delta E_{\text{str}}$  vs.  $\Delta P$  data was used in performing zeta potential calculations, in light of the unpredictable asymmetry potentials associated with these measurements [Van Wagenen and Andrade, 1980].

### Fluorescent Staining and Western Blotting

To stain cells with Wheat Germ Agglutinin (WGA), coverslips were fixed (20 min in ice-cold 10% formalin in PBS), rinsed in TRIS-buffered saline (TBS), and then stained in 60  $\mu$ l of 2- $\mu\text{g}/\text{ml}$  Rhodamine-WGA (Invitrogen) in TBS for 20 min. For avidin staining, slides fixed in 10% formalin were blocked with drops of 3% milk in TBS for 2 h, then treated for 1 h with anti-avidin (Sigma; 1:500 dilution in TBS), followed by 1 h with anti-rabbit IgG conjugated to rhodamine (ICN/Cappell, Solon, OH; 1:100 dilution in TBS). After staining, slides and coverslips were rinsed in TBS, mounted in Fluormount G (Southern Biotech, Birmingham, AL) and images were captured by fluorescent microscopy [Finkelstein et al., 2004]. For western blotting, extracts were prepared by scraping up cells in SDS-PAGE sample buffer. We performed electrophoresis and transfer to nitrocellulose membranes, then blocked with in TBS with 3% milk, 0.1% Tween-20. Avidin-conjugated proteins were detected using anti-avidin (1:1000), anti-Rabbit IgG-peroxidase (ICN/Cappell; 1:10,000), and West Pico ECL reagents (Pierce, Rockford, IL).

### Quantification of Staining and Polarization

The level of cell-edge labeling in WGA-stained or avidin-stained cells (e.g., Figs. 1b and 2b) was quantified using the Linescan function in MetaMorph software (Molecular Devices, Downingtown, PA) to quantify pixel intensity along two horizontal lines drawn across the image of each cell. Lines were drawn above and below, not through, the nucleus (which was often brightly stained in neuraminidase-treated cells, presumably because some permeabilization occurred during fixation, allowing WGA to label nuclear envelope O-linked carbohydrates). For each line, the average intensity of the 10 pixels that represented each cell edge was divided by the average intensity along the entire line. This ratio was used to estimate the degree of edge-specific staining.

To quantify polarization of WGA or  $\alpha 4$  integrin staining of cell edges (e.g., Figs. 3b and 4d), the free-draw tool in MetaMorph was used to draw regions around the cathode-facing and anode-facing halves of the cell membrane (regions drawn included only the cell edge, minimizing inclusion of the interior). The average pixel intensity for the cathode facing ( $I_C$ ) and anode facing ( $I_A$ ) regions were corrected for background intensity and used to calculate an asymmetry index for each cell, according to McLaughlin and Poo [1981]

$$\text{asymmetry index} = (I_C - I_A) / (\text{average of } I_C \text{ and } I_A).$$

Polarization of cell edge ruffling (e.g., Fig. 4b) was measured from phase-contrast images of cells taken after 1 h of EF motility. Cells were scored as polarized towards the cathode only if they showed clear evidence of a ruffling membrane, and if the center of the phase-dark area of the membrane ruffling was included within the cathode-facing quadrant of the cell.

### Cell Viability

Viability was assessed by trypan blue exclusion. Adherent cells were washed with PBS, incubated in dilute trypan blue solution ( $\sim 0.1\%$  in media) for  $\sim 10$  min, washed, and visualized. At least 100 cells in at least 5 microscope fields were counted for each condition.

### Statistical Analysis

Comparisons between treatment groups were made by one-way ANOVA with Dunnett's post test, using GraphPad Prism (GraphPad Software, San Diego, CA), except where otherwise noted (e.g.,  $\chi^2$ , Fig. 4b). Results were considered significant at  $P < 0.05$ .

## RESULTS

### Sials Contribute to the Net Negative Charge of the Cell Surface

When we placed non-adherent cells in the EF, they initially moved towards the anode by passive electrophoresis. However, after they attached to the glass, they began active crawling toward the cathode (see Supplementary Movie 1). This result suggests that, although the EF induces cathode-directed motility, the cell surface has a net negative charge, which allows passive electrophoresis of non-adherent cells. To study possible roles of negatively charged cell surface molecules in EF motility, we employed two protocols to alter surface charge. We first used a well-established method: We pretreated cells with neuraminidase to remove negatively charged Sials from the surface of living cells. In a second, novel approach, we used biotin-avidin conjugation to append excess positively charged molecules to the cell surface, outweighing the usual negative charge of the cell surface.

### Neuraminidase Treatment Removes Sials and Decreases Cell Surface Charge

We treated cells with the Sial-specific glycosidase, neuraminidase, assaying digestion by staining treated cells with the lectin WGA (Fig. 1a). Although WGA binds to Sials, it also exhibits high affinity binding to other O-linked carbohydrates, e.g., *N*-acetyl glucosamine [Monsigny et al., 1980]. However, the prevalence of Sials on the cell surface should allow us to attribute most WGA labeling on unpermeabilized cells to Sials. Indeed, in 3T3 and HeLa cells, a 30-min treatment with  $\geq 1$  U/ml neuraminidase (in fact, 0.5 U/ml in 3T3 cells) abolished most cell surface WGA staining, demonstrating the efficacy of digestion conditions, and verifying that most O-linked carbohydrates on the cell surface are Sials, as they are removed by neuraminidase. Quantification showed that the reduction in cell surface Sials was significant (Fig. 1b). We also found that Sials are rapidly replaced at the cell surface; WGA labeling recovered nearly to the intensity found in untreated cells within 1 h after neuraminidase wash-out (Fig. 1b, light gray columns).

To assess the cytotoxicity of neuraminidase treatment, we used a trypan blue exclusion test. Neuraminidase treatment was not toxic; at concentrations up to 1 U/ml in 3T3 cells, viability was  $>95\%$ , and viability of treated and mock-treated control cells did not differ significantly (data not shown). We also tested the motility of neuraminidase-digested cells in the absence of an EF, using a wound-healing assay. Neuraminidase-treated cells still migrated, however, the rapid recovery of cell surface Sials precluded a quantitative comparison of the

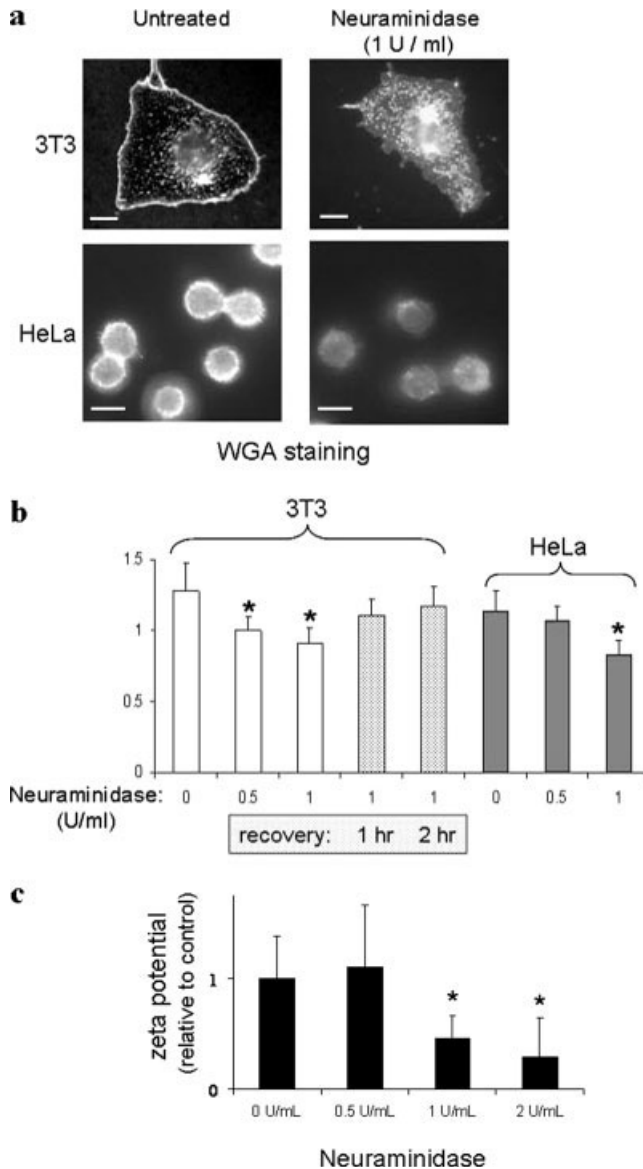


Fig. 1. Neuraminidase removes charged cell surface Sials. (a) Labeling with rhodamine-labeled WGA of cells fixed immediately after 30-min treatments with 1 U/ml neuraminidase or with buffer alone (untreated). Scale bars = 10  $\mu$ m. (b) Intensity of cell edge WGA staining was quantified from linescans across cells after 30-min treatment with the indicated quantities of neuraminidase, as described in Materials and Methods. The two light gray columns represent the recovery of WGA staining of cell edges in 3T3 cells that were first treated with 1 U/ml neuraminidase for 30 min, then allowed to recover in culture media without neuraminidase for 1 or 2 h, as indicated. At least 15 cells were measured per condition. (c) Zeta potential of control and neuraminidase treated 3T3 cells as indicated (30-min neuraminidase treatments; control cells were mock-treated, with buffer alone). Chart shows the absolute value of the zeta potential, relative to the value for untreated cells. Averages from four experiments are shown. In (b) and (c), asterisks (\*) indicate significant differences from control cells ( $P < 0.05$ ).

motility of neuraminidase-treated and control cells (data not shown).

To test whether loss of WGA staining corresponded to a loss of cell surface charge, we measured the zeta potential of 3T3 cells with and without neuraminidase treatment. Zeta potential for a solid–liquid interface (such as the interface between a cell and the medium covering it) is defined as the difference in electrical potential between a surface and the liquid flowing over that surface [Hunter, 2001]. Thus, we hypothesized that changes in cell surface charge brought about by neuraminidase treatment of cell monolayers should be reflected by changes in zeta potential. Figure 1c shows that the absolute value of the zeta potential, relative to the value for untreated cells, was significantly reduced by treatment with 1 U/ml neuraminidase. This reduction in negative cell surface charge corroborated WGA staining results and further demonstrated that neuraminidase treatment was efficacious in removing abundant, charged Sials from the cell surface.

#### Avidin Conjugation Can Alter Surface Charge in Living Cells

As another means of altering cell surface charge, we employed a novel method to add excess positive charges, instead of removing negative charges: We derivatized the surface of live cells with biotin and then conjugated the highly cationic protein, avidin ( $pI \sim 10$ ), to the surface. As a control for any avidin effects unrelated to charge, we also conjugated biotinylated cells with succinylated avidin ( $pI = 7.0$ ), in place of avidin.

Since derivatization and conjugation of live, motile cells had not been previously reported, we first examined the efficiency of our protocol for conjugating avidin to cell surface proteins. Immunofluorescent staining confirmed that avidin was bound to the cell surface after treatment with biotin plus avidin (Fig. 2a). Next, we measured the brightness of anti-avidin staining of cell edges in cells fixed at 20–80 min following avidin addition (Fig. 2b). These time intervals corresponded to the 60-min period during which we subjected cells to the EF, since we initially placed cells in the EF 20 min after avidin addition. Figure 2b shows that we detected no decrease in avidin staining of cell edges during this time period, indicating that cell surface avidin remained attached to the cell surface throughout standard EF motility experiments.

To further demonstrate the efficiency of avidin conjugation, we performed western blotting with an antibody to avidin (Fig. 2c). In cells treated with avidin without prior biotinylation (Fig. 2c, lane 2), the prominent band near the bottom of the western blot presumably represents unconjugated avidin monomer, which in the absence of biotin would be expected to non-specifically

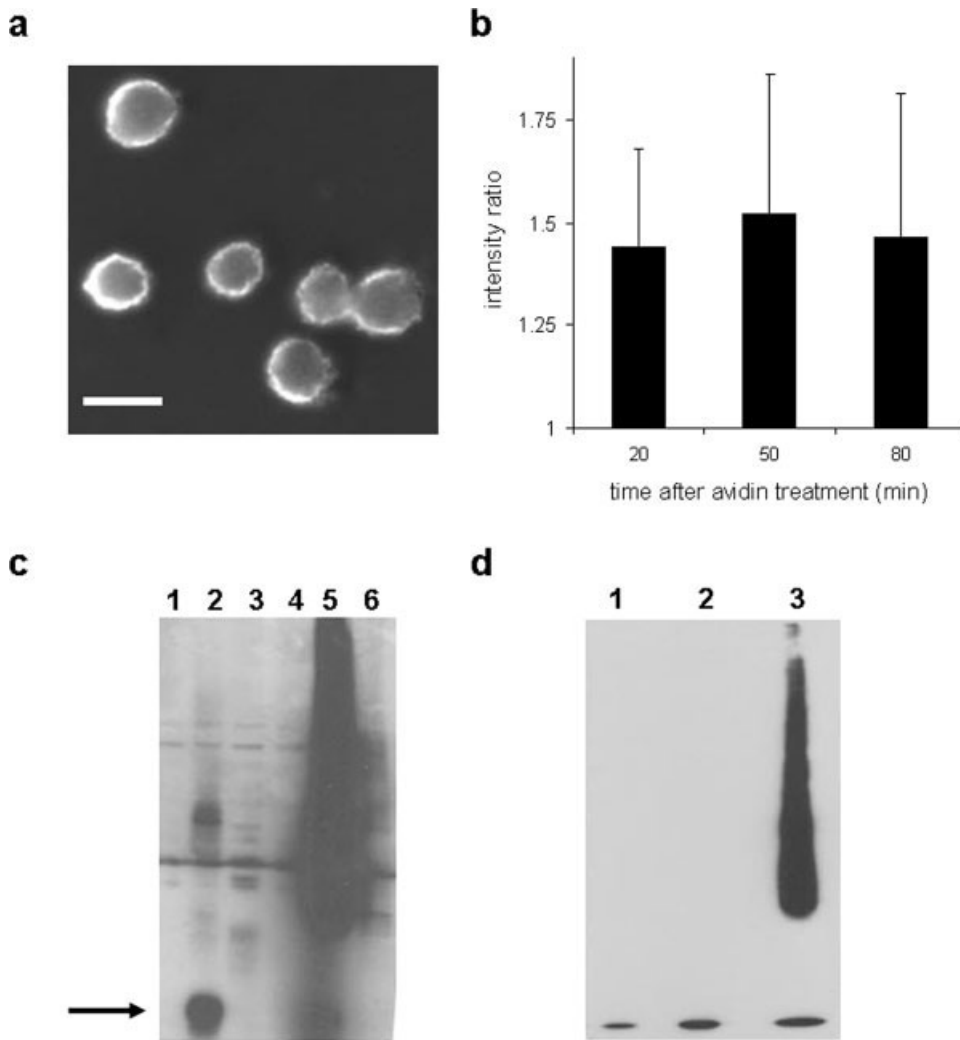


Fig. 2. Avidin alters cell surface charge in HeLa cells. (a) Representative anti-avidin immunofluorescence image of HeLa cells fixed 20 min. after biotin-avidin treatment. Scale bar = 10  $\mu$ m. (b) Edge-specific staining quantified by line-scans (see Materials and Methods); cells were fixed at the indicated times after avidin treatment. None of the time points differed significantly from one another. (c) Anti-avidin western blot. Lanes show 1, untreated; 2, avidin only; 3, succinylated avidin only; 4, biotin only; 5, biotin plus avidin; 6, biotin plus succinylated avidin. Arrow ( $\rightarrow$ ) indicates position of monomeric avidin, whereas other anti-avidin staining represents avidin covalently coupled to cell proteins. (d) Anti-avidin Western blot. Lanes show 1, avidin standard (30 ng); 2, avidin standard (100 ng); 3, lysate from cells treated with biotin plus avidin.

adsorb to the negative cell surface due to its cationic charge. In contrast, cells treated with biotin plus avidin (Fig. 2c, lane 5) showed a continuum of bands of varying  $M_r$ , and only a minor band at the position of avidin monomer, suggesting that, as expected, most avidin was covalently coupled to cell surface proteins of various molecular masses. Cells treated with succinylated avidin are also shown in Fig. 2c, lanes 3 and 6; here, the immunolabeled bands appear much weaker, most likely because succinylated avidin reacted only weakly with available anti-avidin antibodies, as verified by slot blots (data not shown).

In order to quantify the amount of avidin conjugated after treatment with biotin plus avidin, we performed additional western blotting, in which small amounts of lysates from cells treated with biotin plus avidin were electrophoresed along with standard amounts of pure avidin (Fig. 2d). From densitometric quantification of the avidin labeling on these blots, we calculated

that the cell lysate contained 20  $\mu$ g avidin. Since this lysate was derived from two treatment chambers, each incubated with 100  $\mu$ g avidin, it appears that  $\sim$ 10% of added avidin became conjugated to cells. As described in the Materials and Methods, we calculated that conjugation of only 1% of added avidin would suffice to counteract the charge of all Sials on the cell surface. Thus, we believe that avidin treatment was at least as effective as neuraminidase in decreasing the net negative charge on the cell surface.

We used the standard trypan blue exclusion test to assess viability of treated cells. Biotin and/or avidin treatments were not toxic; the percentage of trypan blue-excluding cells was  $\sim$ 100%, indistinguishable between biotin/avidin-treated and untreated control cells. Based on the results of these experiments, we conclude that conjugation with biotin plus avidin is a useful, effective, and gentle method for conjugating avidin to live cells and changing cell surface charge.

### EF Exposure Polarizes Cell Surface Molecules Based Upon Charge

We used rhodamine-labeled WGA to examine the distribution of charged cell surface Sials in EF-exposed cells. Cell edge WGA staining was significantly biased towards the cathode in HeLa cells, following a 1-h EF exposure (Fig. 3a, top panel). Next, we asked if Sials were redistributed directly by the EF, or as an indirect consequence of the cell's motility response. Accordingly, we treated cells with cytochalasin D to inhibit motility, and placed them in the EF. As shown in Fig. 3a, bottom panel, we found that cell surface Sials still polarized in the EF, even in cells that mounted no motility response.

Next, we tested whether we could alter the passive reorientation of Sials in EF-exposed cells by changing the net charge of the cell surface. Compared to EF-exposed HeLa cell controls, in which WGA-labeling showed Sials polarized towards the cathode (Fig. 3b, top panels), EF-exposed cells that had been pre-treated with biotin plus avidin exhibited strong polarization of Sials towards the anode (Fig. 3b, middle panels). Pre-treatment of cells with biotin plus succinylated avidin appeared to abrogate their EF-induced polarization; that is, the cells showed essentially no net polarization of Sials (Fig. 3b, lower panels). As shown in the histograms (Fig. 3c), quantification of the asymmetry index [McLaughlin and Poo, 1981; also see Materials and Methods] of WGA labeling confirmed these visual impressions: The cathodal polarization of Sials seen in untreated, EF-exposed cells (Fig. 3c, upper panel), was reversed in cells conjugated with biotin plus avidin (Fig. 3c, middle panel), and was randomized with biotin plus succinylated avidin (i.e., Fig. 3c, lower panel shows an average asymmetry index  $\sim +0.1$ , indicating insignificant net polarization). The biotin-plus-avidin and biotin-plus-succinylated conjugations showed asymmetry indices statistically different from each other and from that measured in untreated cells (Fig. 3c).

### Neuraminidase Inhibits Leading Edge Polarization Towards the Cathode

To test for effects of neuraminidase on morphological polarization, we obviously could not use WGA labeling, since neuraminidase efficiently removes Sials, the primary WGA-binding species. Instead, we examined the polarization of membrane ruffling and of  $\alpha 4$  integrin in EF-treated cells, with and without prior neuraminidase treatment. Unlike Sials, we presume that redistribution of these markers towards the leading edge is mediated by the cell's motility response, i.e., not mediated directly by the interaction of the EF with the cell surface.

CHO cells transfected with  $\alpha 4$  integrin (CHO GFP- $\alpha 4$  cells) were particularly amenable to this experiment because CHO cells show a robust motility response upon EF exposure [Pu and Zhao, 2005], and because CHO GFP- $\alpha 4$  cells exhibit faster wound-healing motility than untransfected CHO cells [Pinco et al., 2002], suggesting that they may be highly motile in response to EFs as well. Indeed, CHO GFP- $\alpha 4$  cells showed faster and more consistent EF motility than naïve CHO K1 cells (data not shown). Furthermore,  $\alpha 4$  integrin is localized to the leading edge in migrating cells [Pinco et al., 2002] and thus constitutes a good marker for leading edge polarization in EF-motility. In fact, we found that many cells showed a distinct, ruffled membrane at one edge, which allowed us to score the polarization of the leading edge relative to the applied EF.

Representative micrographs document the EF-induced polarization of membrane ruffling (Fig. 4a) and GFP- $\alpha 4$  integrin fluorescence (Fig. 4c), and their inhibition by neuraminidase pre-treatment. Quantification showed that polarization of membrane ruffling (Fig. 4b) in EF-exposed cells was consistently biased towards the cathode, and this bias was reduced or eliminated by neuraminidase pre-treatment. In fact, neuraminidase reduced polarization of membrane ruffling to control (no EF) levels in both 3T3 and in CHO GFP- $\alpha 4$  cells (Fig. 4b). We did not attempt to quantify membrane ruffling in HeLa cells, whose more spherical shape renders any morphological polarization difficult to detect. Next, we calculated the asymmetry index of  $\alpha 4$  integrin in CHO GFP- $\alpha 4$  cells; after a 30 min EF exposure,  $\alpha 4$  integrin was significantly polarized towards the cathode in untreated, but not in neuraminidase-treated, cells (Fig. 4d). Thus, neuraminidase pre-treatment effectively prevented redistribution towards the cathode of membrane markers involved in the motile response.

### Neuraminidase, But Not Avidin, Inhibits Directionality of EF Motility

Next, we used neuraminidase and biotin-avidin treatments to change cell surface charge, as a direct test of the hypothesis that interaction of the EF with charged cell surface species is a determinant of motility direction. We limited the interval during which we measured EF motility responses to 1 h, because after neuraminidase digestion, cells appear to regenerate their Sials significantly during a 2-h interval (Fig. 1b). We tried including neuraminidase in the motility chamber, to permit us to monitor the EF-motility of neuraminidase-digested cells over longer intervals. However, with or without neuraminidase present in the motility chamber, cell surface Sials recovered with similar rapidity to the level characteristic of untreated cells. Presumably, neuraminidase activity in the chamber is lost due to electrophoresis of this

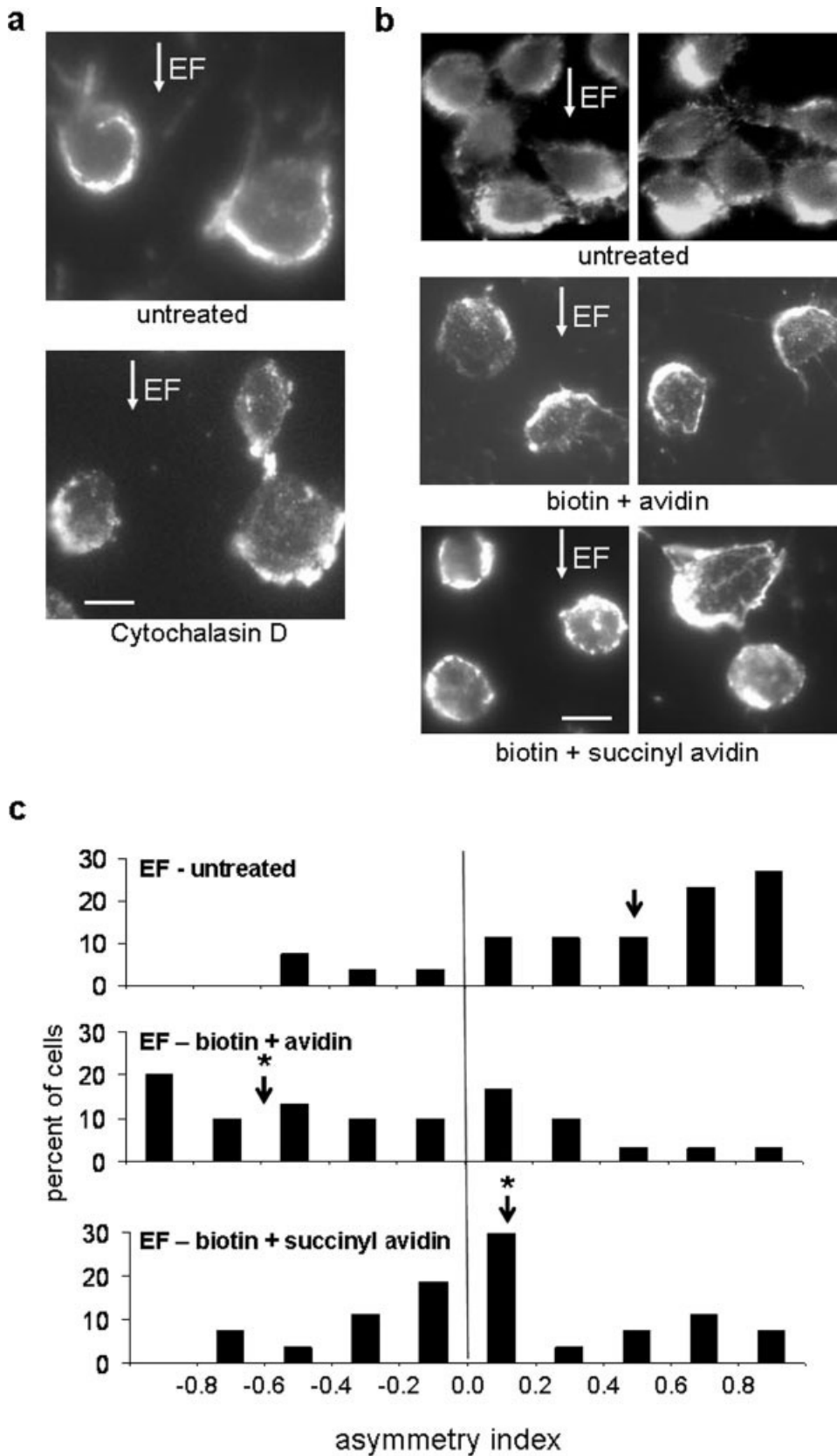


Fig. 3. Polarization of Sial after EF exposure is reversed by biotin + avidin. (a) WGA labeling is polarized towards the cathode in HeLa cells that were untreated (top panel), or treated with cytochalasin D (bottom panel). Scale bar = 10  $\mu$ m. (b) Polarization of WGA labeling is altered by cell surface derivatization. Micrographs of HeLa cells (untreated control, treated with biotin + avidin, or treated with biotin + succinyl-avidin) exposed to EF for 1 h, fixed, stained with WGA-rhodamine, and visualized by fluorescence. Two representative images are shown for each treatment. As indicated by the arrows ( $\rightarrow$  EF), the cathode faces down in all images. Scale bar = 10  $\mu$ m. (c) Histograms showing the asymmetry index of WGA polarization (see Materials and Methods) after 1 h EF exposures as in (b). Note that a positive or a negative asymmetry index denotes WGA staining polarized towards the cathode or anode, respectively. Arrows indicate the mean value of each asymmetry index. Asterisks (\*) indicate significant differences, compared to controls ( $P < 0.05$ ). At least 25 cells were measured per condition.

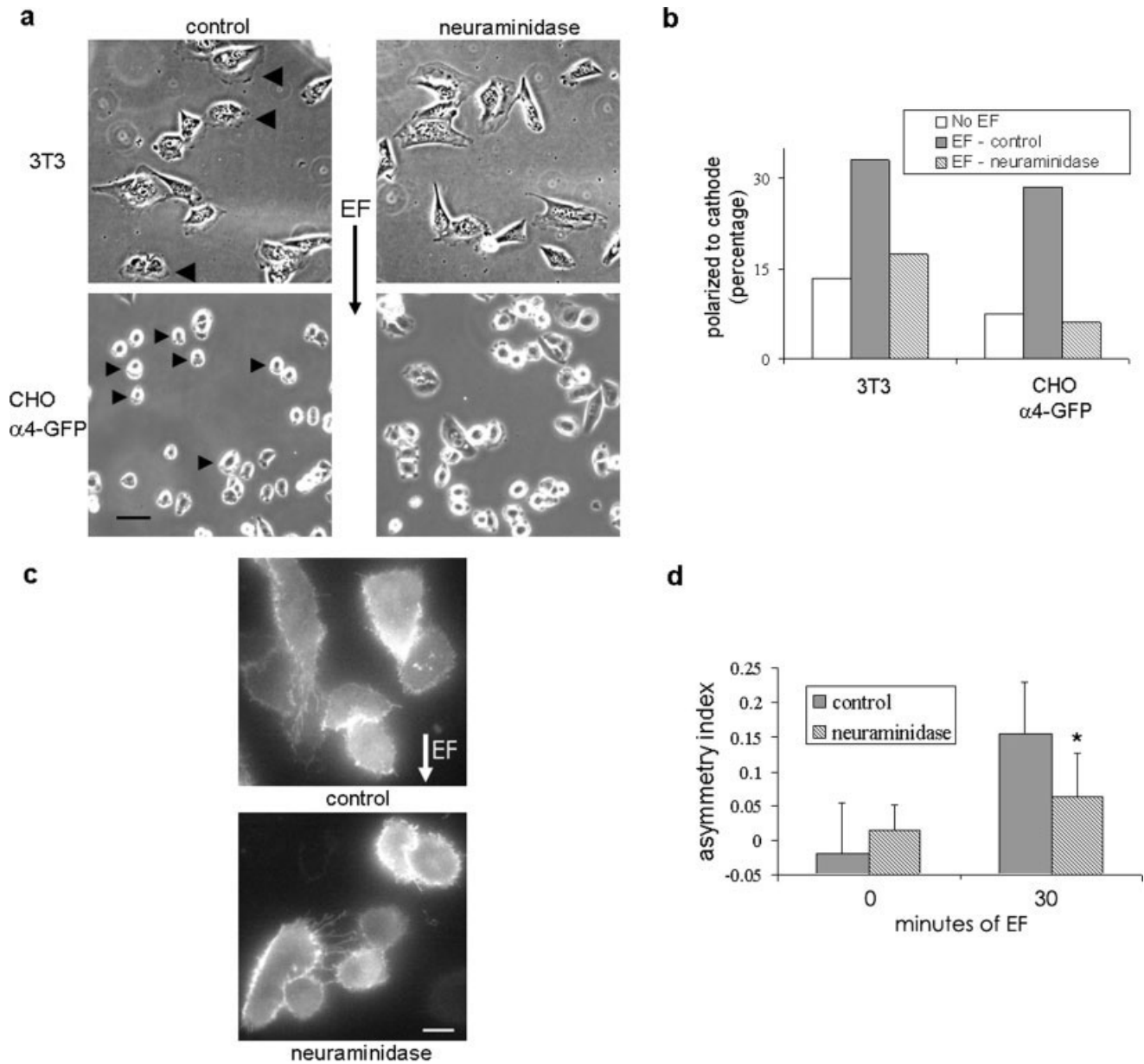


Fig. 4. Cell polarization towards cathode is prevented by neuraminidase. (a) Phase contrast images of 3T3 and CHO GFP- $\alpha 4$  cells after 60-min EF in the absence or presence of neuraminidase pre-treatment. Arrowheads indicate examples of cells that possessed membrane ruffling that was scored as polarized to the cathode (see Materials and Methods). Scale bar = 50  $\mu\text{m}$ . (b) Graph shows the percentage of cells scored as having membrane ruffling polarized towards the cathode under each condition. At least 60 cells were measured per condition.

Differences were significant for both cell types, as measured by the  $\chi^2$  test. (c) Fluorescent images of CHO GFP- $\alpha 4$  cells, following 30-min EF exposure. Scale bar = 10  $\mu\text{m}$ . (d) Quantification of integrin  $\alpha 4$  polarization in CHO GFP- $\alpha 4$  cells after 0 and 30 min EF exposure; with and without neuraminidase pre-treatment. The cathode is down in all images (arrow). Asterisk indicates that asymmetry value was significantly altered by neuraminidase pre-treatment ( $P < 0.05$ ). 12 cells were measured for each condition.

acidic enzyme to the cathode [pI = 5.1; Groome and Belyavin, 1975]. Limiting EF motility experiments on neuraminidase-treated cells to a 1-h duration was an unavoidable challenge; however, statistical analysis of data from many short experiments should allow us to overcome this problem.

To test the role of surface charge on EF motility, we measured speed and directionality of EF motility after each surface perturbation protocol. Figure 5 shows some examples of the observed cell migration, and illustrates the method we used to record the direction and distance of cell movement. In order to make cell

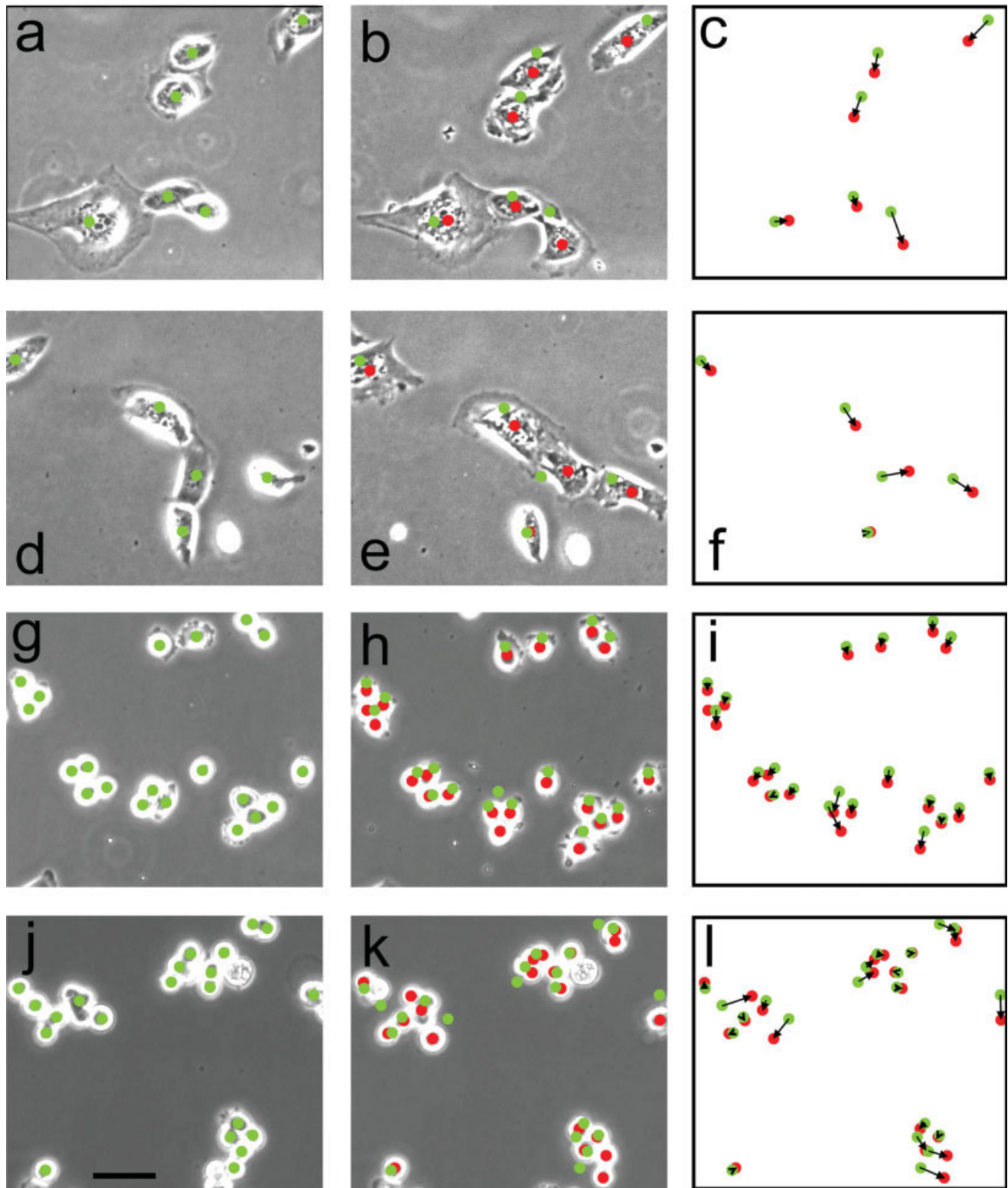


Fig. 5. Examples of electric field migration showing how motility was measured. (a–c) 3T3 cells, control conditions. (d–f) 3T3 cells treated with neuraminidase. (g–i) HeLa cells, control conditions. (j–l) HeLa cells treated with neuraminidase. (a,d,g,j) Cells at the beginning of the electric field exposure (time 0). Cell centroids marked by green dots. (b,e,h,k) The same cells as the previous panel, after 1 h electric

field, with centroids marked by red dots. The green dots from the previous panel (time 0 position) are overlaid to show cell movement. (c,f,i,l): The same view as the previous panel, with arrows showing the movement of individual cells, and the background removed for clarity. Cathode is down in all images. Scale bar in panel j = 50  $\mu\text{m}$ .

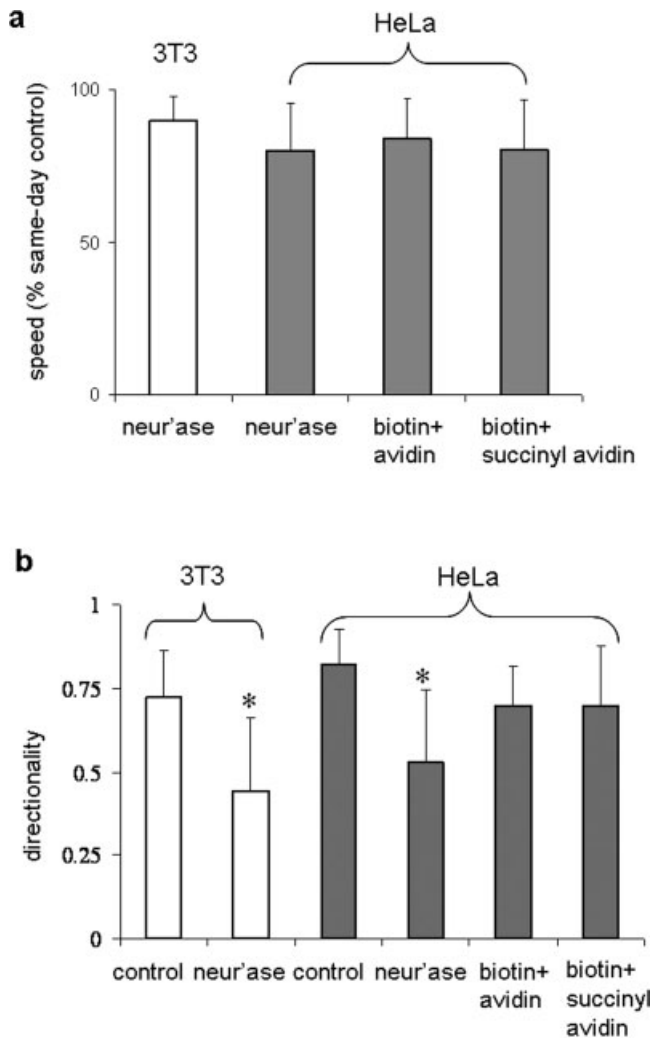


Fig. 6. Neuraminidase, but not biotin + avidin, inhibits directional EF motility. (a) Total speed of 1-h EF motility of 3T3 and HeLa cells, expressed as percentage of the speed of untreated control cells (treated with buffer alone). (b) Directionality of migration of 3T3 and HeLa cells during 1-h of EF motility. Positive directionality indicates migration towards the cathode. Asterisks in panel b indicate significant difference from control for the same cell type ( $P < 0.05$ ); no statistically significant differences were found in panel a. All EF-motility experiments were performed for 1 h; data presented are from four separate experiments per condition, and more than 100 cells were measured per condition.

movement more obvious, only a small portion of each microscope image is shown in Fig. 5. The entire images from which these panels are taken can be seen in Supplementary Fig. 1. To control for day-to-day variation in cell behavior, we expressed the speed data (Fig. 6a) as the percentage of the control cells assayed the same day. For reference, average motility speed, in  $\mu\text{m}/\text{h} \pm$  standard deviation, for control 3T3 cells was  $14.4 \pm 3.4$ , and for control HeLa cells was  $7.4 \pm 2.7$ . As shown in Fig. 6a, neither neuraminidase nor biotin-avidin treatments signif-

icantly affected speed of motility. In contrast, neuraminidase significantly inhibited directionality of motility in both cell types (Fig. 6b). The fact that cells were able to move at normal speed, but were impaired in their ability to respond directionally to the EF indicates that neuraminidase specifically inhibited cells' capacity to determine direction, rather than simply impairing the mechanics of their motility. In HeLa cells treated with biotin-plus-avidin (Fig. 6b, two right bars), directionality appeared to be decreased, but this was not significant. Besides, treatment with biotin-plus-avidin showed the same, insignificant decrement in directionality as biotin-plus-succinylated avidin, again arguing that altering cell surface charge was insufficient to effect a change in motility direction. Thus, we concluded that, unlike neuraminidase treatment, changing cell surface charge via avidin conjugation did not alter directionality of motility.

## DISCUSSION

In order to assess the effect(s) of surface charge on EF motility, we used two treatments to decrease the net negative charge of the cell surface: neuraminidase to remove negative charges, and avidin to add positive charges. Although neuraminidase has been used in classical studies of surface charge and galvanotropism [but not galvanotaxis; McLaughlin and Poo, 1981; Patel and Poo, 1982], we know of no previous studies in which the behavior of live cells was studied following conjugation with avidin. The avidin protocol was not overtly toxic, as measured by trypan blue exclusion, and confirmed by cells' ability to migrate normally after biotin-avidin treatment. Based on western blotting with avidin antibody, it appears that sufficient avidin was conjugated to alter the surface charge as desired. Our results suggest that this coupling protocol is a useful tool for manipulating cells and/or investigating surface proteins, especially given the availability of avidin analogs with a range of  $pI$ 's [Kang et al., 1995] that make it possible to investigate charge-specific effects on cell behaviors.

In the current study, electrokinetic measurements (zeta potential) were performed to provide a quantitative means for describing cell surface alterations with neuraminidase enzymatic treatment. Direct comparison of our values to zeta potential values previously reported for glass or for living cells is difficult as a variety of different electrolyte components, ionic strengths, and buffers have been described, in addition to different cell types. In addition, most previous values derived from experiments using a different technique for measuring surface charge, microelectrophoresis performed on cells in suspension. With this caveat, our values are similar to those reported from previous studies. For the flow channel without cultured cells (i.e., glass surfaces alone), the

measured zeta potential was  $-53$  mV for the control (medium and glass alone) which compares well with the literature [approximately  $-25$  mV, Van Wagenen et al., 1976]. This value was unchanged when neuraminidase was included in the glass-alone control. In our studies, the zeta potential for NIH-3T3 fibroblasts was  $-68$  mV, which considering the caveat above, is very close to reported values. For example, the zeta potential of living mouse BALB/c 3T12 cells (mouse tumorigenic fibroblasts) was  $-29$  mV [Van Wagenen et al., 1976] from cells cultured in monolayer in a glass capillary tube measurement system. We found that neuraminidase treatment of cell monolayers decreased the negative surface charge by  $\sim 38\%$ . To focus attention on the relative effect of neuraminidase on the cell surface charge, data were normalized to control as presented in Fig. 1c. The decrease in cell surface charge is consistent with effects of neuraminidase reported from experiments using a different technique for measuring surface charge, microelectrophoresis. For example, Greig et al. [1976] found a 26% decrease in cell surface charge for neuraminidase treated mouse lymphoma P388-F36 cells. In protozoa, Matta et al. [1992] observed a  $\sim 33\%$  decrease of the negative surface charge with neuraminidase treatment.

Our results suggest that polarization of cell surface molecules in the EF is not causally linked to directional motility. Specifically, treatment with biotin plus avidin reversed the direction of WGA polarization, from cathodal to anodal, yet this neither reversed, nor even significantly altered, motility direction. This result is inconsistent with the hypothesis that cathodal polarization of bulk, cell surface proteins is either necessary or sufficient to generate cathodally-directed motility. A reversal in WGA polarization, following conjugation with avidin, is consistent with the finding of reversed ConA staining in EF-exposed cells that had been pre-incubated with positively charged lipids [McLaughlin and Poo, 1981]. Thus, the first part of our working hypothesis is true, and the second part, false. That is, polarization of charged surface molecules, as measured by WGA, is induced by the EF, but is not functionally coupled to EF induction of directional motility.

Previous studies have suggested that reorganization of cell surface molecules is required for galvanotaxis in 3T3 cells [Brown and Loew, 1994] and for galvanotropism in frog neurites [Patel and Poo, 1982]. These researchers based their conclusions on (1) the correlation of motility with polarization of surface markers, and (2) the inhibition of motility by ConA, which also inhibits redistribution of surface ConA ligands. There are several plausible explanations for the seeming contradiction between our findings and these earlier results. First, it should be noted that our WGA polarization and avidin conjugation experiments were performed only in HeLa

cells, and it is possible that our findings are cell type-specific. A more likely possibility, though, is that the difference lies in the technique for altering redistribution of cell surface molecules. All results are consistent with the notion that, although bulk redistribution of cell surface molecules is not needed for EF motility, redistribution of a specific critical species is needed, and external ConA prevents the redistribution of this critical surface molecule. Alternatively, since previous reports described correlative, rather than functional studies, the polarization of cell surface molecules may be an epiphenomenon, not an essential facet, of EF motility. Other concerns in interpreting the effects of external ConA abound; e.g., in addition to inhibiting protein redistribution, ConA might block the function of critical surface proteins by cross-linking or sterically masking them [Stewart et al., 1996].

Of course, several previous studies that did not utilize lectin binding have described cell surface polarization in EF-exposed cells. The list of molecular markers that become polarized or redistributed includes EGF receptor [Fang et al., 1998], acetylcholine receptor [Stollberg and Fraser, 1988], phosphoinositide-3 kinase [Pu and Zhao, 2005], Rho [Rajnicek et al., 2006], Src kinase [Zhao et al., 2006], and  $\alpha$ -4 integrin (Fig. 4). Since these proteins are already known to be involved in cellular motility responses in non-EF motility models such as chemotaxis or wound-healing, it makes sense that they would also play a role in EF motility. However, it is not known whether each of these redistributes as a direct response to the EF, as a target of another primary signal, or as part of the downstream motility response that ensues.

For practical reasons, as discussed in the Results section, we used only 1 h of EF treatment for motility measurements. As a consequence, our results address the mechanism of the early stages of EF motility, which may not be identical to the mechanism of persistent motility. It has been suggested that cells have both short-term and long-term responses to EFs, and that translocation of proteins may be important only for the long-term response [Mycielska and Djamgoz, 2004], which is consistent with our conclusion that movement of charged cell surface molecules is not involved in the early stage of EF motility. Another potential limitation of our findings is that we used a relatively high electric field. It has been suggested that endogenous electric fields at wound edges can typically be 1–2 V/cm [Nishimura et al., 1996; Wang et al., 2003]. In our previous work [Finkelstein et al., 2004] we found that 3T3 cells responded similarly to fields of 1–6 V/cm, but with a larger response at the larger voltage. For that reason, we feel that the 6 V/cm voltage used in this paper provides a good model for the mechanism of the physiological response.

Another finding of our study is that cells lost directional EF motility after neuraminidase treatment.

Since our results with avidin suggest that negative cell surface charge is not needed for EF motility, it is likely that the requirement for Sials in EF motility is not due simply to their negative charge. Plasma membrane Sials have numerous biological functions [Jeanloz and Codrington, 1976] that may contribute to EF motility; e.g., loss of Sials can influence the activity of calcium channels [Marengo et al., 1998], potassium channels [Cook, 1968], and sodium channels [Bennett et al., 1997]. In addition, Sials can bind  $\text{Ca}^{2+}$  ions [Schauer, 1982] and influence membrane fluidity [Schauer, 1982]. The ion exchanger NHE1, which has been implicated in EF motility [Zhao et al., 2006], also has neuraminidase-sensitive glycosylation [Counillon et al., 1994], so it is possible that this protein contains Sials that are critical for EF motility. However, when we tested the EF motility of 3T3 cells treated with EIPA (an NHE1 blocker), we found that in our system, directional EF motility was not inhibited (data not shown). This suggests that NHE1 function is not critical for EF motility in all cell types, and that neuraminidase effects on our HeLa and 3T3 cells cannot be attributed to loss of NHE1 function.

It should be noted that neuraminidase removes Sials from glycolipids, as well as from glycoproteins. Our initial bias was that the effect of neuraminidase on EF motility was due to the loss of Sials from critical glycoproteins, based on numerous studies that demonstrated polarization of specific proteins in EF. However, gangliosides, which are glycolipids that contain Sials [Huwiler et al., 2000], could comprise a critical neuraminidase target, whose polarization in the EF is important for the motility response. Cell membrane gangliosides are important regulators of growth factor signaling [Liu et al., 1980; Meuillet et al., 1999] and calcium homeostasis [Ledeen and Wu, 2002]; thus, it is plausible that the effect of neuraminidase on EF motility might involve the loss of Sials from gangliosides. Gangliosides, unlike proteins, are not subject to biotin-avidin conjugation, since they lack primary amines to react with the NHS-biotin. A different approach, such as a hydrazine-based biotinylation, would be required to specifically derivatize glycolipids in order to investigate the role of gangliosides. Although WGA staining suggests that all Sial-containing species, including gangliosides, lose their cathodal polarization in avidin-treated cells, it is at least a formal possibility that a low level of gangliosides still became polarized in the EF, but that they form too small of a fraction of total cell surface WGA staining to be detectable.

Our results suggest that initiation of EF motility involves specific changes in cell membrane function (as opposed to overall surface charge or bulk reorientation), which are likely to involve changes in ion channel func-

tion. Our finding that Sials are critical for EF motility suggests that cell surface molecule(s) critical to the induction of EF motility contain Sials; this may facilitate the identification of specific macromolecules involved in the primary cellular response to EFs.

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