

Cell Migration Does Not Produce Membrane Flow

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Abstract. We have previously reported that rearward migration of surface particles on slowly moving cells is not driven by membrane flow (Sheetz, M. P., S. Turney, H. Qian, and E. L. Elson. 1989. *Nature (Lond.)*. 340:284–288) and recent photobleaching measurements have ruled out any rapid rearward lipid flow (Lee, J., M. Gustafsson, D. E. Magnussen, and K. Jacobson. 1990. *Science (Wash. DC.)* 247:1229–1233). It was not possible, however, to conclude from those studies that a slower or tank-tread membrane lipid flow does not occur. Therefore, we have used the technology of single particle tracking to examine the movements of diffusing particles on rapidly locomoting fish keratocytes where the membrane current is likely

to be greatest. The keratocytes had a smooth lamellipodial surface on which bound Con A-coated gold particles were observed either to track toward the nuclear region (velocity of $0.35 \pm 0.15 \mu\text{m/s}$) or to diffuse randomly (apparent diffusion coefficient of $[3.5 \pm 2.0] \times 10^{-10} \text{ cm}^2/\text{s}$). We detected no systematic drift relative to the cell edge of particles undergoing random diffusion even after the cell had moved many micrometers. The average net particle displacement was $0.01 \pm 2.7\%$ of the cell displacement. These results strongly suggest that neither the motions of membrane proteins driven by the cytoskeleton nor other possible factors produce a bulk flow of membrane lipid.

THE forward migration of cells has often been linked to the rearward movement of particles on their surfaces (Abercrombie et al., 1970; Bray, 1970; Dembo and Harris, 1981; Bretscher, 1984). The rearward migration of material has consistently been observed in a wide variety of cells under different conditions. The capping of antigen-antibody complexes and the clearance of particles bound to the surface of lamellipodia are driven by this rearward motion. In addition, filamentous actin, the dominant structural component of the lamellipodium, has been found to move rearward. Recently, the rearward migration of the actin cytoskeleton has been correlated with the rearward movement of surface particles (Forscher and Smith, 1988; Fisher et al., 1988). The general movement of material toward the nucleus from the leading edge of the lamellipodium suggests that there might be a general flow of the membrane in that direction. Furthermore, the observation that newly synthesized viral glycoproteins are inserted in the plasma membrane toward the front of the cell has prompted the suggestion that there is a counterbalancing flow of membrane from the front to the rear of the cell (Bretscher, 1984). A recent study, however, has demonstrated that the systematic rearward transport of cell surface proteins on slowly moving macrophages is not caused by membrane lipid flow (Sheetz et al., 1989). Direct measurements have shown that randomly diffusing glycoproteins are not flowing toward the rear of the cell even though adjacent particles are actively

transported rearward. Further, photobleaching studies have not observed rearward movement of membrane lipids (Lee et al., 1990). Thus, no bulk membrane flow toward the nucleus at the rate of surface particle or cytoskeleton movement in lamellipodia has been found.

Although the measurements of Sheetz et al. (1989) demonstrate that membrane lipid flow does not drive rearward transport of membrane glycoproteins, they do not establish that there are no lipid flows. The slow velocity of cell movement and the rapid diffusion rate of phospholipids (Lee et al., 1990) place limitations on the sensitivity of the previous measurements of lipid flow. Therefore, we have attempted to detect membrane lipid flow by measuring the trajectories of particle motions on the surfaces of rapidly locomoting fish epidermal keratocytes (Euteneuer and Schliwa, 1984; Cooper and Schliwa, 1986). On these cells the rate of membrane flow, if coupled to cell locomotion, should be relatively rapid and could superimpose a detectable systematic drift on randomly diffusing surface particles. The fish epidermal keratocytes have additional advantages for these measurements. They have a uniform crescent shape with a broad featureless lamella extending more than halfway around the cell body on which the surface particles are readily observed (see Fig. 5). On typical tissue culture substrata they preserve this shape as they crawl forward with a smooth, almost gliding motion at velocities up to $30 \mu\text{m}/\text{min}$. Despite this unusual style of locomotion on conventional substrata, many keratocytes adopt the morphology and pulsatile locomotory behavior of fibroblasts on substrata coated with adhesive glycoproteins such as fibronectin (Kucik, D. F., unpublished observations). Hence,

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it is likely that the cellular processes which move fibroblasts forward also drive keratocyte movement. The fish keratocyte is, therefore, a good system in which to probe the important features of membrane glycoprotein movements during the process of cell migration.

There are several different mechanisms by which lipid flow could be driven and each would have different observable effects on the trajectories of diffusing particles. For example, rearward flow could result from the selective insertion of membrane in forward regions of the cell (Fig. 1 *a*). If the region of insertion near the leading edge is spatially separated from a primary region of internalization at the rear of the cell, there will be a net rearward flow of lipid from the former to the latter (Bretscher, 1984). Another possibility is that the rearward moving cytoskeleton could be tightly associated with the upper regions of the membrane and would drag that part of the membrane rearward as well. (To compensate for this, other regions of membrane would have to be transported from more rearward regions of the cell to the forward edge, possibly from the surface adjacent to the substratum.) In both cases a particle on the dorsal surface of the cell should be carried rearward from the leading edge toward the nucleus. Alternatively, if the lamellipodium were to act as a tank track and the lower surface were to interact with the substrate, the upper surface would flow forward at a velocity twice the speed of cell locomotion (Fig. 1 *b*) (although partial coupling could occur with a lower velocity of movement). Finally, the membrane might passively move forward with the lamellipodium (Fig. 1 *c*). Then a particle attached to the membrane but not influenced by active cytoskeletal

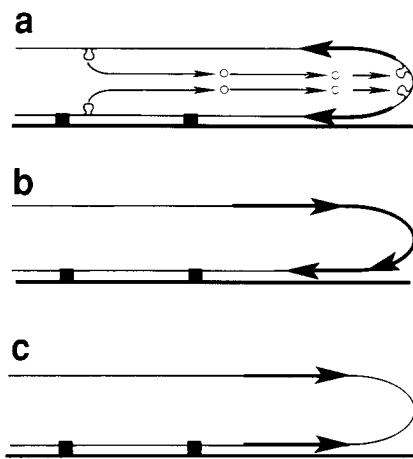


Figure 1. Three possible models of membrane motion on the dorsal surface of the lamellipodium as the cell locomotes. (*a*) The retrograde membrane flow model. Membrane is supplied to the leading edge by exocytosis, and recycled by endocytosis elsewhere. The membrane thus moves rearward with respect to the leading edge on both the dorsal and the ventral surface of the cell as it locomotes. The rearward motion of the membrane on the ventral surface provides the force to propel the cell forward. (*b*) The "tank track" model. The membrane flows forward on the dorsal surface of the cell and rearward on the ventral surface with respect to the leading edge. This model predicts that the dorsal membrane will move forward at twice the speed of locomotion. (*c*) The passive membrane model. The membrane moves forward with the cell passively, on both the dorsal and ventral surfaces.

forces would simply move along with the cell. Tracking diffusing particles on the surface of rapidly moving keratocytes allows us to measure the velocity of surface movement to an accuracy within 3% of the overall cell migration velocity.

Materials and Methods

Fish epidermal keratocytes from goldfish (*Carassius auratus*) were grown on glass coverslips from whole-scale explants (Kolega, 1986) and cultured in fish Ringer supplemented with Gibco Amphibian Medium (Cooper and Schliwa, 1986). Latex beads (Polysciences, Inc., Warrington, PA) and gold beads (Janssen Pharmaceuticals, Piscataway, NJ) were coated as described earlier (Sheetz et al., 1989) with Con A, wheat germ agglutinin (Sigma Chemical Co., St. Louis, MO), fibronectin (obtained from John A. McDonald, Washington University), or laminin (obtained from Robert P. Mecham, Washington University). These were added to the stage medium. As the particles settled onto the dorsal surfaces of the cells, their motion was observed by video-enhanced differential interference contrast microscopy on a Zeiss IM-35 inverted microscope. Images obtained with a Dage 70 camera were processed with a Hughes model 794 video digitizer and recorded on videotape. Particle positions were determined for each video frame (30 s^{-1}) by the method of Gelles et al. (1988). Edge positions were determined by a similar method, but with lower precision (50 nm). Diffusion coefficients and rates of systematic transport were determined as described by Sheetz et al. (1989).

Results

Membrane Surface of Keratocytes

Although the surface of the keratocyte lamella appears smooth in the light microscope, it is important to determine the detailed structure of the surface at the electron microscopic level. Examination of crescent-shaped cells in the scanning electron microscope has revealed that there are few irregularities in the membrane surface; therefore, it is valid to consider the surface to be a smooth plane. Diffusing particles on this broad, flat, rapidly moving lamella will indicate motion of the membrane. The uniformity of the shape of the cells facilitates comparisons of measurements from different regions of a single cell and among cells.

For the cells observed, the average velocity of forward motion was $0.3 \pm 0.15 \mu\text{m/s}$. Forward movement was continuous with relatively little fluctuation in the velocity or direction over the course of the measurements. Some subtle variations in the lamellar thickness were evident as regions of higher or lower intensity in the video-enhanced differential interference contrast images. The pattern of these variations in intensity moved toward the nuclear region with a velocity of $0.35 \pm 0.15 \mu\text{m/s}$ (in the cell frame of reference) and they became undetectable as they approached the thicker regions of the cell near the nucleus.

Single Particle Movements

When Con A-coated gold particles (40 nm) were added to the medium, they bound to the surface and their motion was restricted to the two-dimensional plane of the membrane. Although the majority of the particles diffused randomly over the surface, rare particles, usually aggregates (as determined by their significantly greater contrast), became restricted in diffusion and tracked rearward to the nuclear region. There they began again to diffuse randomly. The fact that the particles which tracked rearward were predominantly aggregates suggested that the probability of systematic

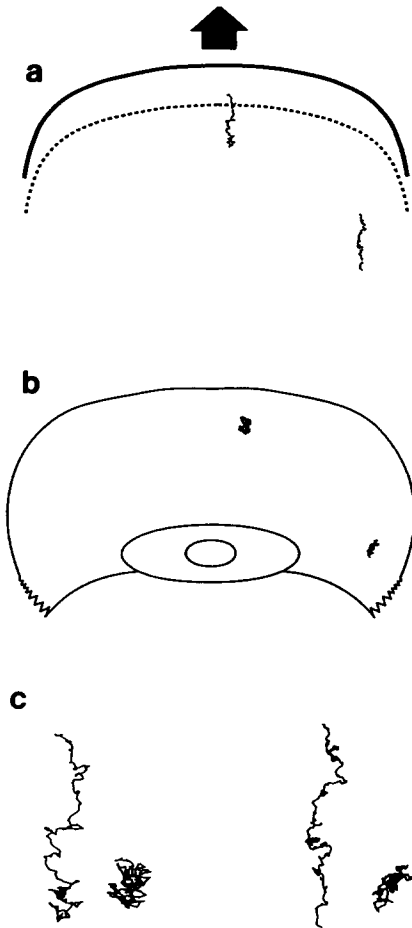


Figure 2. Examples of diffusing wheat germ agglutinin-coated 40-nm gold particles in different regions of the lamella. (a) Trajectories of two particles plotted in the frame of reference of the substratum. The original position of the leading edge is shown as a dashed line; the final position by the solid line. Both particles move forward with the cell. (b) Trajectories of the same two particles in the frame of reference of the cell are plotted to show their relative locations on the lamella. (c) Enlarged view of the trajectories of the two particles in both frames of reference. Both particles are in random motion in the frame of reference of the cell. One can compute the probability that a particle diffusing with diffusion coefficient D will traverse at least a certain distance (x) in a given direction simply by executing a random walk (Berg, 1983). The equation $P(x)dx = (1/4\pi Dt) \exp(-x^2/4Dt) dx$ gives the probability that a particle initially at $x = 0$ will diffuse to a location between x and $x + dx$ in time t . The probability that $x > x_0$ is $\int_{x_0}^{\infty} P(x) dx$, the error function $\frac{\sqrt{\pi}}{2} \operatorname{erfc}(x_0)$. We applied this analysis to the particle trajectories in this figure plus 15 others with t from 9.2 to 48 s (mean = 30 s). When plotted in the frame of reference of the substrate, the trajectories are very unlikely to have resulted from random motion ($p < 0.01$ in each case). When plotted in the frame of reference of the cell, however, they are consistent with purely random motion ($p > 0.5$ in each case). This is compatible only with passive membrane movement.

rearward transport depended on particle size. But even larger latex spheres (70–300 nm) would often diffuse randomly.

To test whether or not other surface glycoproteins would behave similarly to the Con A receptors, we added gold particles coated with other ligands including wheat germ agglu-

tinin, laminin, and fibronectin. We saw no qualitative differences in the behavior of the particles coated with any of these proteins and those coated with Con A, although there may be some quantitative differences in the fractions of particles that diffused randomly versus those that were systematically transported.

Location of Particles on the Dorsal Surface

There are several reasons for concluding that all the tracks studied were those of particles on the dorsal surface of the cell. Beads of this size would not be expected to diffuse in the cytoplasm within the lamella. Although smaller fluorescently labeled ficolls diffuse throughout the cytoplasm, those >24 nm in diameter were excluded from the lamellipodium (Luby-Phelps and Taylor, 1988). The smallest particles used in our study were 40 nm, and some were as large as 300 nm (Fig. 2). Hence, any of these particles within the lamellar cytoplasm should have failed to diffuse. Yet most particles of all sizes did diffuse, including a significant fraction of the particles which also underwent systematic transport toward the rear of the cell. In particles occasionally detached from the surface and diffused away into the medium. Therefore we conclude that the particles which we have observed associated with the lamella were on its surface, not within its cytoplasm. Particles trapped beneath the cell sometimes moved as the cell crawled over them, but their motion was nonrandom and had a diffusive component with a diffusion coefficient 100 times smaller than that of particles randomly diffusing on the dorsal surface. Also, beads on the dorsal surface were in a different focal plane than particles adhering to the glass substrate. Finally, beads on the cell surface were easily distinguished from those in solution by their much slower rate of diffusion and their tendency to remain in focus.

Diffusion Behavior

Small colloidal gold particles, although below the limit of resolution of the light microscope, can be observed and tracked with video-enhanced differential interference contrast microscopy to yield a precise indication of the movements of attached membrane proteins, including those of randomly diffusing proteins (Sheetz et al., 1989; Kucik et al., 1989). Analysis of the motions of diffusing particles on all regions of the lamella showed that their rate of diffusion ($D = [3.5 \pm 2.0] \times 10^{-10}$ cm²/s, the mean \pm SD for 28 particles) is in the same range as membrane protein diffusion coefficients previously measured by fluorescence photobleaching recovery (Jacobson et al., 1987; Kucik et al., 1989). In these rapidly moving cells, however, this rate of diffusion was slow enough compared to the velocity of cell locomotion that the cell could move a considerable distance over the substratum before a particle could diffuse very far over the cell surface. For example, a particle in random motion with a diffusion coefficient of 10^{-10} cm²/s would take 225 s to diffuse a root-mean-square distance of 3.0 μ m over the cell surface; the cell, however, can locomote 3.0 μ m in only 10 s.

Estimate of Particle Flow Rate

Diffusing particles maintained their approximate position relative to the cell's leading edge while the cell moved forward, regardless of their location on the lamellipodium. Fig.

2 shows the trajectories of two particles on the surface of a rapidly moving cell. One of the particles is at the front of the lamellipodium, and the other is near the rear lateral edge. Both particles maintain their positions relative to the leading edge as the cell and its surface particles move forward as a unit. When plotted relative to the leading edge, the motion of the particles is random (Fig. 2 *b*), indicating that in this frame of reference, the particles are simply diffusing. When plotted with respect to the stationary reference frame, the particle trajectory reflects the motion of the cell edge (Fig. 2 *a*). Of the three models, this is compatible only with the model in Fig. 1 *c*.

The lack of systematic drift of the diffusing particles in the frame of reference of the cell was particularly striking in light of the dramatic centripetal cytoplasmic waves visible within the lamellipodium. We observed centripetal transport of some surface-bound particles in concert with these waves on all parts of the lamellipodium, particularly with large (0.3 μm) latex beads and with large aggregates of gold particles, but only rarely with individual gold particles. This centripe-

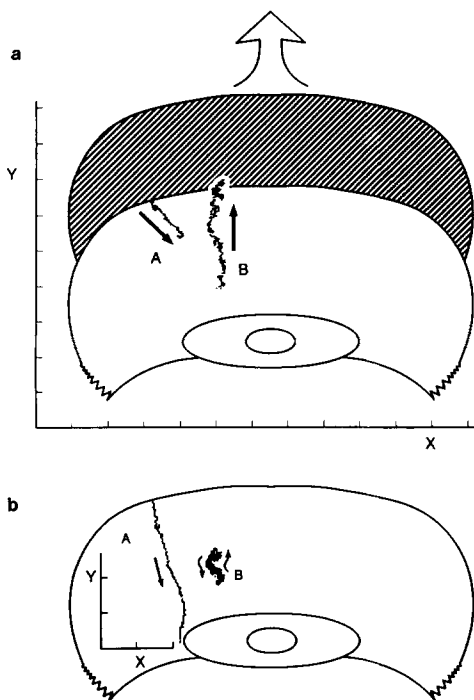


Figure 3. Comparison of a particle undergoing rearward transport with a particle in random motion in the same area of the lamella. (*a*) The trajectories of two 0.3- μm Con A-coated latex beads are plotted in the frame of reference of the substratum. The same behavior was seen with both gold and latex beads coated with Con A, wheat germ agglutinin, laminin, and fibronectin. The shaded area shows the extent of forward movement of the cell during the period of observation. Bead *B* is clearly moving forward with the advancing leading edge, while Bead *A* is moving rearward at an angle. Note the large component of random motion in the trajectory of Bead *B*. Analysis of the trajectories (Sheetz et al., 1989; Kucik et al., 1989) yields an off-axis diffusion coefficient of 2×10^{-11} cm^2/s for bead *A* and 1×10^{-10} cm^2/s for bead *B*. (*b*) The same particle trajectories plotted in the frame of reference of the cell. It is clear that in this frame of reference bead *A* is undergoing retrograde transport; analysis (see Fig. 2 caption) of the trajectory of bead *B* shows that its motion is random.

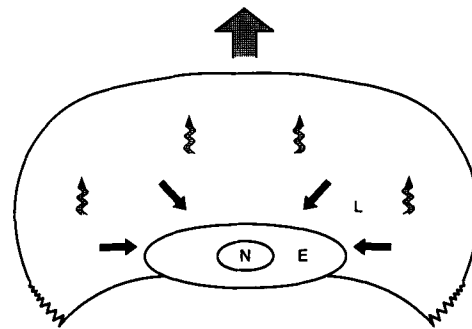


Figure 4. The two different types of particle motion observed on locomoting cells. The short, thick arrows indicate retrograde transport of particles. As shown, the transport is actually centripetal, as is the motion of cytoplasmic waves (Forscher and Smith, 1988). The stippled arrows indicate motion in the frame of reference of the substratum. The large arrow at the leading edge indicates forward motion of the cell, and the wavy stippled arrows indicate the direction of motion of diffusing particles with respect to the substratum as the cell crawls forward. This motion of diffusing particles with respect to the substrate is directly forward in all cases, regardless of the location of the particle on the lamella. When plotted in the frame of reference of the cell, the motion is isotropic and random. E, endoplasm; L, lamella; N, nucleus.

tal transport was easily distinguished from diffusion by the steady rearward migration and relative lack of Brownian motion as compared to diffusing particles in the same region of the lamella (Fig. 3; also Sheetz et al., 1989). The diffusing particles were not influenced by the centripetal motion of the underlying cytoplasmic waves even in the lateral regions of the cell, where the motion of the waves is at right angles to the direction of cell migration (Fig. 4).

To obtain a quantitative estimate of the net flow velocity of the diffusing particles we determined the net displacement of 26 particles relative to the leading edge during a period of time (10–54 s). The positions of 10 particles before and after 54 s are shown in Fig. 5. The overall displacements were random relative to the leading edge. In a different cell, digital analysis of the displacements of 16 particles in the cell frame of reference yielded velocity vectors which are displayed in Fig. 6 relative to the cell motion vector. The average velocity of the cell movement was considerably greater than the apparent velocities of systematic motion of the individual particles (Fig. 6 *a*). The average diffusing particle velocity relative to the cell edge (0.042 ± 5.0 nm/s) was not statistically different from 0.

Discussion

The movement of diffusing particles in the membranes of migrating cells does provide a reasonable measure of the rate of bulk membrane flow. In the keratocyte the high rates both of cellular locomotion and of systematic centripetal transport of particles make it an especially favorable system for detection of membrane flow. The high rate of centripetal transport ensures that randomly diffusing particles are readily distinguishable from particles undergoing systematic transport. The high velocity of cell movement provides an optimal situation for detection of membrane flow coupled to cell locomotion. Using single particle tracking it should be possible

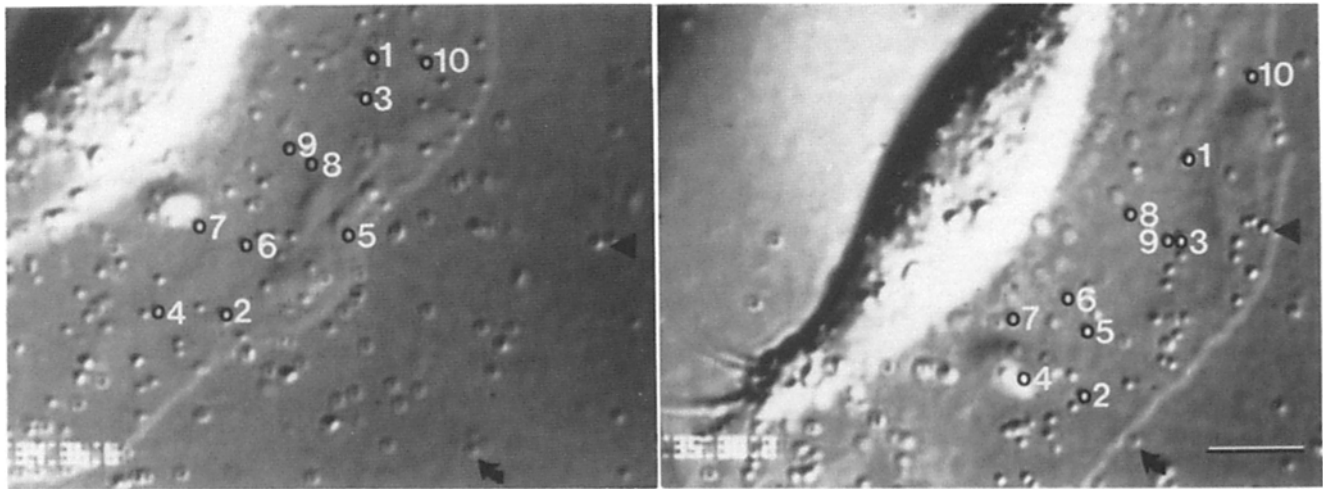


Figure 5. Video micrographs showing the positions of 10 diffusing particles at $t = 0$ s and $t = 54$ s which illustrates the random nature of their movements relative to the leading edge. Particles 2 and 10 both reached the leading edge during this period. During this period the cell covered a distance of $12 \mu\text{m}$ (the arrows mark the same points on the glass). Bar, $5 \mu\text{m}$.

to measure a velocity of systematic transport of particles in the range of 4–10 nm/s. This is 1–2% of the cell migration rate. Furthermore, it is likely that the beads which we have observed are coupled to several membrane glycoproteins. The effect of a lipid flow should therefore be greater in these beads than on a single glycoprotein (Bretscher, 1984). This further enhances the sensitivity of our approach for detecting membrane flow. Hence, if membrane flow were coupled to cell locomotion, it should have been detected in our measurements.

One should note, however, that the diffusion coefficient of the diffusing beads is a factor of 10–20 lower than expected for glycoproteins diffusing in a pure lipid bilayer (Jacobson et al., 1987). This lower diffusion coefficient could result from interaction with a stationary matrix. This supposed stationary matrix would, however, have to be free from substantial interaction with the centripetally moving waves which seem to be correlated with systematic transport of surface

particles. Furthermore, recent observations that the diffusion coefficients of beads of different sizes of keratocyte lamellae depend weakly on bead size also argue against a model based on the retardation of diffusion of membrane particles by multiple interactions with a stationary cytoskeletal matrix (Kucik, D. F., E. L. Elson, and M. P. Sheetz, manuscript in preparation). Finally, we could suppose that the forces which retarded membrane protein diffusion by 10–20-fold acted to retard in similar proportion the systematic transport of membrane proteins driven by a lipid flow. Then, if the velocity of the lipid flow were comparable to that of the cell locomotion, we would expect a systematic transport of the diffusing proteins at a rate of 5–10% that of the cells. This velocity of systematic transport would have been detectable in our measurements. Thus, there is no reason to believe that a membrane flow of this magnitude to the rear was undetected by this work.

Generality of Keratocyte Membrane Properties

Under some conditions keratocyte locomotion involves lamellipodial movement very similar to that seen in fibroblasts and other types of adherent cells. Because of the relative enlargement of the lamella in keratocytes, we presume that it is the major source of motive force for this cell. Hence, the keratocyte should be ideally suited to elucidating the role of the lamellipodium in cell migration. These cells commonly show rearward motion both of surface particles and of cytoplasmic structures, presumably based on an actin matrix. The fact that we observe no membrane flow in the keratocyte membrane supports other findings in macrophages and polymorphonuclear leukocytes (Sheetz et al., 1989; Lee et al., 1990).

Directed Rearward Migration

There is ample evidence that a major component of the cytoskeleton in the lamellipodium of locomoting cells is moving rearward (Heath, 1983; Wang, 1985). In neuronal growth cones the actin-based cytoskeleton appears continually to assemble at the leading edge and disassemble at the

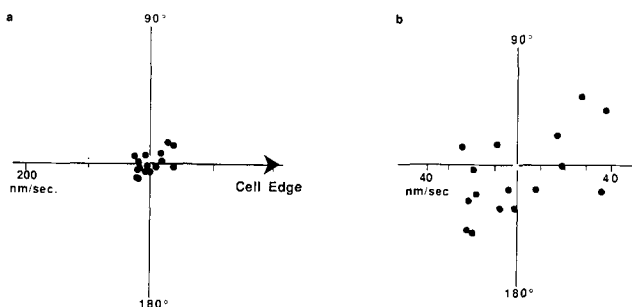


Figure 6. Two-dimensional vectorial plots of the velocities of movement of 16 diffusing particles on migrating keratocytes (●) relative to the velocity vector of the leading edge (arrow in *a*). An expanded scale for the vectors is shown in *b* which indicates a random distribution of the velocity vectors (the average in the direction of cell movement was $+0.024 \pm 5.4$ nm/s). Particles chosen were located on the leading lamella at least $4 \mu\text{m}$ from the leading edge and the endoplasmic region.

endoplasmic region (Forscher and Smith, 1988). If a significant fraction of the membrane glycoproteins were attached to this moving cytoskeleton, their movement might be expected to induce a membrane lipid flow (which must be matched by a forward flow in a neighboring region). Our observations show no evidence of membrane flows, which suggests that the attachments between the rearward-moving cytoskeleton and membrane glycoproteins are not extensive.

Absence of Detectable Membrane Flow

The evidence for membrane flow has always been circumstantial and has been based on findings which could be interpreted without invoking membrane flow (Bretscher, 1984; Abercrombie et al., 1970). Although some studies show that newly synthesized membrane proteins appear at the forward regions of locomoting cells (Bergmann et al., 1983), other studies have indicated that the major site for exocytosis was at the forward boundary of the vesicle-rich endoplasm with the actin-rich lamella (Hopkins, C., personal communication). Here the endoplasm is defined as the relatively thick, organelle-containing cell body, as opposed to the thin, organelle-free lamella. Likewise, the major site of endocytosis appears to be at the forward edge of the endoplasm (and oriented toward the Golgi apparatus, as suggested by Bergmann et al., 1983). If the sites of both endocytosis and exocytosis are closely spaced, then there should be little net long range membrane flow. There is no persuasive evidence that the extent of membrane insertion is great enough and that the locations of the sites of exocytosis and endocytosis are sufficiently far separated to generate a lipid flow detectable by our methods. Of course, slower or more localized flows of lipid between sites of exocytosis and endocytosis are very likely to occur in the plasma membrane, but it seems unlikely that these flows which are below our limit of detection are significantly involved in cell locomotion.

Our results are thus consistent with a purely cytoskeletal mechanism of cell locomotion, in which the actin in the lamellipodium transduces the force by which the cell is pulled forward. The membrane plays a passive role, simply moving forward with the cell.

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