

# Membrane Expansion Increases Endocytosis Rate during Mitosis

Drazen Raucher and Michael P. Sheetz

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

**Abstract.** Mitosis in mammalian cells is accompanied by a dramatic inhibition of endocytosis. We have found that the addition of amphiphilic compounds to metaphase cells increases the endocytosis rate even to interphase levels. Detergents and solvents all increased endocytosis rate, and the extent of increase was in direct proportion to the concentration added. Although the compounds could produce a variety of different effects, we have found a strong correlation with a physical alteration in the membrane tension as measured by the laser tweezers. Plasma membrane tethers formed by latex beads pull back on the beads with a force that was related to the in-plane bilayer tension and membrane-cytoskeletal adhesion. We found that as cells enter mitosis, the membrane tension rises as the endocytosis rate decreases; and as cells exited mitosis, the endocytosis rate increased as the membrane tension decreased. The addition of amphiphilic compounds decreased membrane tension and increased the

endocytosis rate. With the detergent, deoxycholate, the endocytosis rate was restored to interphase levels when the membrane tension was restored to interphase levels. Although biochemical factors are clearly involved in the alterations in mitosis, we suggest that endocytosis is blocked primarily by the increase in apparent plasma membrane tension. Higher tensions inhibit both the binding of the endocytic complex to the membrane and mechanical deformation of the membrane during invagination. We suggest that membrane tension is an important regulator of the endocytosis rate and alteration of tension is sufficient to modify endocytosis rates during mitosis. Further, we postulate that the rise in membrane tension causes cell rounding and the inhibition of motility, characteristic of mitosis.

**Key words:** mitosis • endocytosis • membrane tension • laser optical tweezers • cell cycle

**C**ELLULAR endocytosis is critical for many aspects of cellular function including nutrition, signaling, transepithelial transport, and control of the membrane surface area (Matter and Mellman, 1994; Riezman et al., 1997). The transition from interphase to mitosis in eukaryotic cells is accompanied by dramatic inhibition of endocytosis as well as profound changes in cellular architecture. A wide range of dynamic membrane functions are inhibited including protein secretion (Featherstone et al., 1985; Warren et al., 1983), pinocytosis (Berlin et al., 1978; Berlin and Oliver, 1980), and receptor-mediated endocytosis (Warren et al., 1983; Pypaert et al., 1987). Many of the changes in mitosis have been correlated with post-translational modifications of critical proteins. Reorganization of the cytoskeleton into a mitotic spindle and many of the related events clearly involve cyclin-dependent kinases and biochemical events downstream (reviewed by Pines, 1994). Previous studies strongly suggest that phos-

phorylation is involved in inhibiting invagination (Pypaert et al., 1991), and the involvement of Cdc2 kinase in the inhibition of fusion of endocytic vesicles in vitro (Tuomikoski et al., 1989) and of invagination (Pypaert et al., 1991) is very well documented. How the endocytic machinery is controlled is currently not understood but to be able to reverse the inhibition in mitosis might indicate how the inhibition occurred initially.

Fluid-phase endocytosis has been studied most extensively in the case of clathrin-dependent endocytosis, although there are clearly situations where clathrin-independent and actin-dependent endocytosis are prominent. In the case of clathrin, the baskets deform the membrane; and once the membrane is invaginated, a protein such as dynactin may be responsible for the fission event. Certain membrane proteins are endocytosed preferentially; but the budding process can occur in vitro through dynactin interactions with lipid (Sweitzer and Hinshaw, 1998; Takei et al., 1998). This implies that the cytoskeletal proteins should be displaced for the endocytosis process to occur (Hinshaw and Schmid, 1995). The overall process of endocytosis is mechanochemical in that the chemical energy in clathrin and dynactin complexes is transduced into membrane bending and fission.

Address correspondence to Dr. Michael P. Sheetz, Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. Tel.: (919) 684-8091. Fax: (919) 684-8592. E-mail: m.sheetz@cellbio.duke.edu

A well-studied case where the endocytosis rate is altered dramatically is after stimulated secretion, which results in a dramatic increase in the membrane surface area. The endocytosis rate increases dramatically in neuronal synapses after transmitter release and in rat basophilic leukemia (RBL) cells after antigen-induced secretion. In the case where measurements have been made, the increase in endocytosis rate correlates with a decrease in tension in the membrane as measured by the force generated on tethers (Dai et al., 1997). Membrane tension has been suggested as a regulator of endocytosis rate for both plant (Kell and Glaser, 1993) and animal cells (Sheetz and Dai, 1996). To inhibit endocytosis, either the endocytic machinery could be biochemically inhibited or the physical resistance to endocytic vesicle formation could increase. If the membrane tension was increased in mitosis, higher membrane tension could also induce rounding of the cells and inhibit motility, which is seen in mitosis.

Recent developments have made it simpler to measure the tension of biological membranes from the force that is exerted on membrane tethers. Tethers can be formed by pulling on membrane-attached beads with laser tweezers and the displacement of the beads in the laser tweezers gives a rapid readout of the force on the beads. Tether force is typically modulated in plasma membranes by alterations in the in-plane tension in the membrane bilayer and membrane-cytoskeleton adhesion. In-plane tension is dependent upon the balance of endocytosis and exocytosis as well as osmotic pressure across the membrane. Membrane-cytoskeleton adhesion contributes to tether force because the cytoskeleton does not enter the tethers and the separation of the membrane from the cytoskeleton creates a membrane osmotic pressure (Sheetz and Dai, 1996).

Phospholipid bilayers can be expanded by the addition of amphiphilic compounds (Jain et al., 1975; Zhelev, 1998). They intercalate between the phospholipid molecules at the air-water interface. The change in bilayer area is in rough proportion to the concentration of the compound added. Proteins may affect the degree of expansion, but the compounds clearly cause similar expansion of biological membranes as was shown by the protection of red cells from hypotonic lysis by the binding of amphiphilic compounds to the red cell membrane. Such agents when asymmetrically expanding one half of the bilayer can cause cell shape change by creating an imbalance in the membrane bilayer couple (Sheetz and Singer, 1974).

If the decrease in endocytosis during mitosis is the result of an increase in membrane tension and not an inhibition of the endocytic apparatus, then agents that physically expand the membrane and decrease tension should increase endocytosis in mitotic cells. The findings presented here indicate that not only biochemical factors in cell cytosol but also physical force in the membrane plays an important role in controlling membrane traffic and perhaps in a variety of cellular activities.

## Materials and Methods

### Cell Culture

HeLa cells were grown in monolayers at 37°C in an atmosphere of 5%

CO<sub>2</sub> and 95% air in the presence of 0.1% penicillin and streptomycin in DME (Sigma Chemical Co.) supplemented with 10% FBS (Sigma Chemical Co.) and 10 mM L-glutamine (GIBCO BRL). To induce the mitotic block at the metaphase/anaphase boundary, cells were incubated for 20 h with 8 nM taxol or 2 nM vinblastine (Jordan et al., 1993; Wendell et al., 1993).

### Microscopy and Membrane Tension Determination by Optical Tweezers

The cells were viewed by a video enhanced differential interference contrast (DIC)<sup>1</sup> microscope equipped with laser optical tweezers (Choquet et al., 1996). Carboxylated, 1- $\mu$ m-diam latex beads (Polyscience) were coated with rat IgG or ConA (Sigma Chemical Co.) by use of carbodiimide linkage (Kuo and Sheetz, 1993). Beads were attached to the cell membrane at central part of the cell (in later phases of mitosis beads were attached at the cell poles) and pulled away from the cell surface to form a membrane tether. The tether force is calculated from the displacement of the bead from the center of the laser tweezers during tether formation and the calibration of the trap (Dai and Sheetz, 1995b). Membrane tension is related to the square of the tether force as deduced from the lipid bilayer systems (Vaughn et al., 1992; Hochmuth et al., 1996).

### Fluorescence Labeling and Quantification of Endocytosis

Unsynchronized cell populations on coverslips were incubated 5–10 min with 1  $\mu$ M FM1-43 (Molecular Probes) or 5 mg/ml fluorescein-dextran (average molecular weight 4,000; Sigma Chemical Co.) in HBSS (Sigma Chemical Co.), gently rinsed, fixed 5 min in 2% formaldehyde, and stained 5 min with 1.5  $\mu$ M DAPI to reveal mitotic phase. Fields of interest containing mitotic and interphase cells were identified by the DAPI-stained image and cells were recorded for several seconds. The filters were then changed to allow visualization and subsequent recording of the FM1-43 or fluorescein-dextran-labeled endocytic vesicles. After subtraction of background fluorescence, all spots which had intensity values  $\geq 60\%$  of that of the brightest spot were scored. Endocytosis was also quantified by measuring fluorescence intensities of endocytic vesicles. As described in Maxfield and Dunn (1990) the brightness of each endosome was quantified as the sum of all pixel intensity values  $\geq 60\%$  of that of the brightest pixel in each spot. If labeled vesicles were not randomly distributed throughout the cell, it is possible that the selection of the focal plane would introduce bias into our results. To check whether or not the number of labeled vesicles is dependent upon the focal plane selected, we scored vesicles for 50 interphase and 20 metaphase cells in two different focal planes. There was no significant difference in the number of labeled vesicles between the chromosomal focal plane (as determined by DAPI) and another focal plane 1–2  $\mu$ m apart. Furthermore, similar results were obtained with fluorescein labeled dextran, suggesting that quantification of endocytosis was not only independent of focal plane, but also independent of the choice of the dye. Endocytosis rate in taxol and vinblastine arrested mitotic cells was determined using a FACScan<sup>®</sup> cytometer (Becton Dickinson). Fluorescence intensity of 10,000 cells was measured immediately after addition of fluorescent dye FM1-43 and after 5 min of incubation. The data are reported as the ratio of the mean fluorescence intensity of cells incubated 5 min with fluorescent dye FM1-43 and control cells.

## Results

### Effect of Amphiphilic Compounds on Endocytosis in Mitosis

In mitosis, a dramatic decrease in endocytosis rate has been reported (Berlin et al., 1978). We repeated those observations (Fig. 1) using either fluorescent dextran or FM1-43 uptake as a measure of endocytosis rate by measuring the number of vesicles per cell and the total fluorescence intensity. With fluorescent dextran, the endocytosis

1. Abbreviations used in this paper: DIC, differential interference contrast; RBL, rat basophilic leukemia.

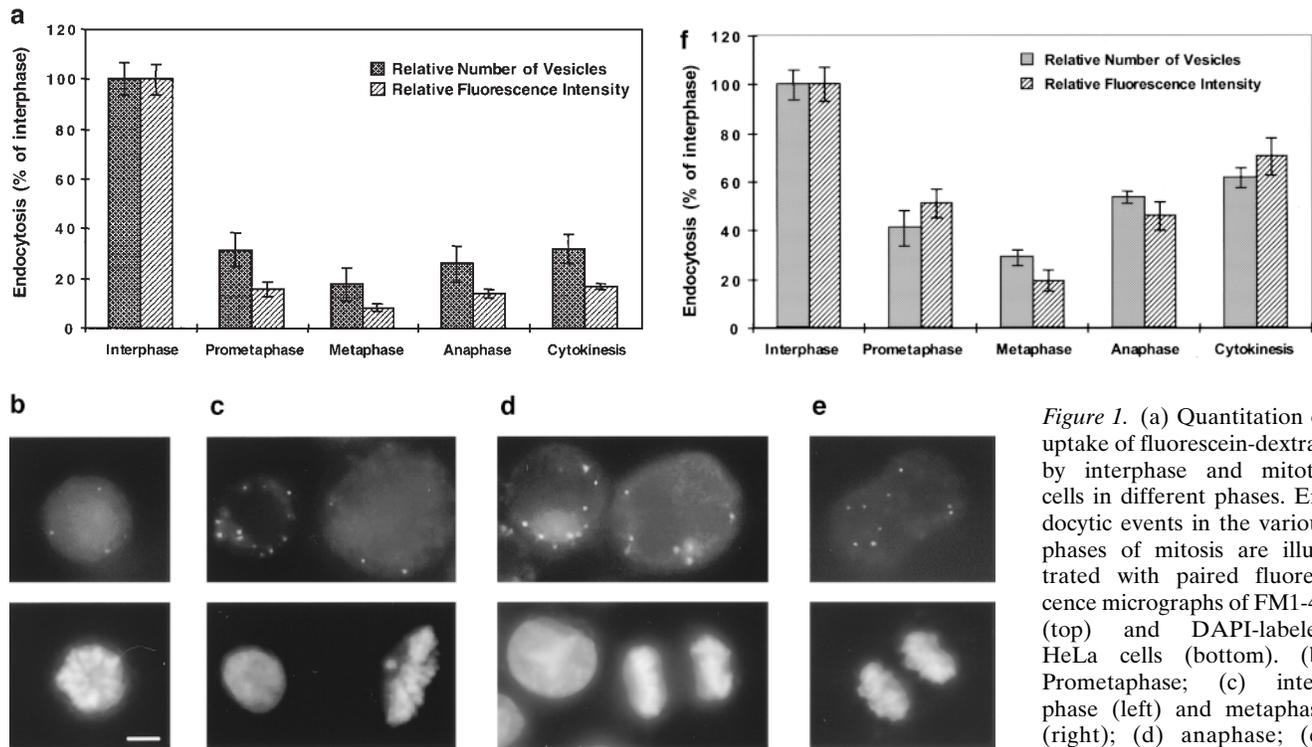


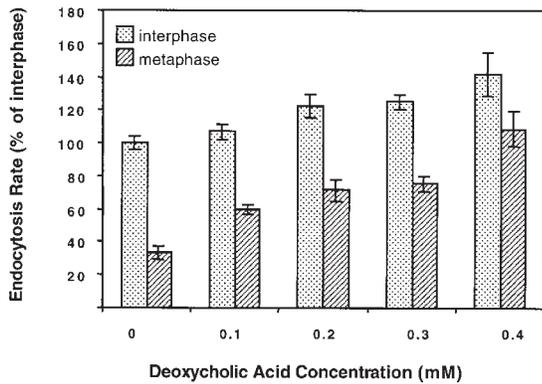
Figure 1. (a) Quantitation of uptake of fluorescein-dextran by interphase and mitotic cells in different phases. Endocytic events in the various phases of mitosis are illustrated with paired fluorescence micrographs of FM1-43 (top) and DAPI-labeled HeLa cells (bottom). (b) Prometaphase; (c) interphase (left) and metaphase (right); (d) anaphase; (e) telophase; (f) quantitation of

uptake of FM1-43 labeled endocytic vesicles by interphase and mitotic cells in different phases. The data given are average from seven different experiments and the number of labeled endocytic vesicles was counted in 10–60 cells at each mitotic phase. Bars indicate SEM.

rate normally decreased to  $\sim 10\%$  of interphase level in metaphase. FM1-43 is a water-soluble dye that is membrane impermeable, but it is readily incorporated into endocytic vesicles and is retained after cell fixation (Betz and Bewick, 1992; Terasaki, 1995). Fig. 1, b–e shows FM1-43 labeling of HeLa cells in various stages of mitosis in parallel with the DAPI staining patterns that enable rapid identification of mitotic phases in HeLa cells (Fig. 1, b–e, bottom). While interphase cells were brightly stained with FM1-43-labeled endocytic vesicles spread throughout the cytoplasm (low numerical aperture objectives were used to detect even out of focus vesicles), many fewer labeled vesicles were found in mitotic cells (Fig. 1, b–e, top). The number of endocytic events in each image frame was counted for each mitotic phase and the results are summarized in Fig. 1 e. In prometaphase, uptake of endocytic vesicles decreased to 40% of interphase value. Metaphase cells had the lowest endocytosis rate (20% of interphase). In progressing to anaphase and cytokinesis, endocytosis increased, corresponding to 60% of the interphase value. It should be noted that the value for endocytosis rate in cytokinesis is most likely higher than shown in Fig. 1, a or f. This is because during the incubation period before observation the cells probably moved from the short anaphase period into cytokinesis. We have also quantified endocytosis by measuring the sum of the fluorescence intensities of the FM1-43-labeled endocytic vesicles. Results obtained by this method were similar to results obtained by counting the number of fluorescent spots. A similar decrease in FM1-43 uptake, as compared with interphase cells, was ev-

ident from prometaphase through cytokinesis reaching the lowest value in metaphase (Fig. 1 f). Since quantification of endocytosis by counting the number of fluorescent vesicles is simple and reproducible and the data are consistent with that obtained by measuring the sum of the fluorescence intensities, all endocytosis measurements are presented in this form. The smaller decrease in FM1-43 uptake to 20% of interphase compared with 10% for dextran could be result of a decrease in average vesicle size, since the ratio of vesicle membrane area to volume increases in smaller vesicles.

There are many practical reasons to want to increase fluid-phase uptake in mitotic cells, including the understanding of which factors may be primarily responsible for the decrease in endocytosis rate. A clear case where the rate of endocytosis is increased is after stimulated secretion. Secretory membrane that is added to the plasma membrane is rapidly recovered possibly because of membrane expansion. Amphiphilic compounds can also cause a significant increase in the area of plasma membranes. They could mimic the effect of secretion in expansion of the plasma membrane. When we added the detergent deoxycholate, we found that there was an increase in the membrane endocytosis rate of cells in metaphase (Fig. 2). As shown in Fig. 2, with increasing concentrations of deoxycholate, there was a proportional increase in the rate of endocytosis, indicating that the effect was proportional to detergent concentration. Endocytosis rates in mitotic cells reached interphase levels when 0.4 mM of deoxycholic acid was added which is still below the critical micelle con-



**Figure 2.** Uptake of FM1-43 in interphase and metaphase cells with increasing concentrations of deoxycholic acid. Nonsynchronized HeLa cells plated on a glass coverslips, labeled with FM1-43, treated 10 min with deoxycholic acid, fixed and stained with DAPI, as described in Materials and Methods. Uptake of FM1-43 is expressed as a percentage of the value of interphase cells.

centration for deoxycholate. Thus, deoxycholic acid can cause a dramatic increase in the endocytosis rate even to interphase levels.

Deoxycholic acid also causes an increase in the endocytosis rate of interphase cells. Similar effects were observed before for a variety of amphiphilic compounds (Table I). Although the degree of increase in the endocytosis rate with deoxycholate concentration was greater in mitotic cells, the rate of endocytosis in mitotic cells never equaled the interphase endocytosis rate in the same medium. Thus, it seems that a general change in the cell membranes following deoxycholate addition results in an increase in endocytosis rates.

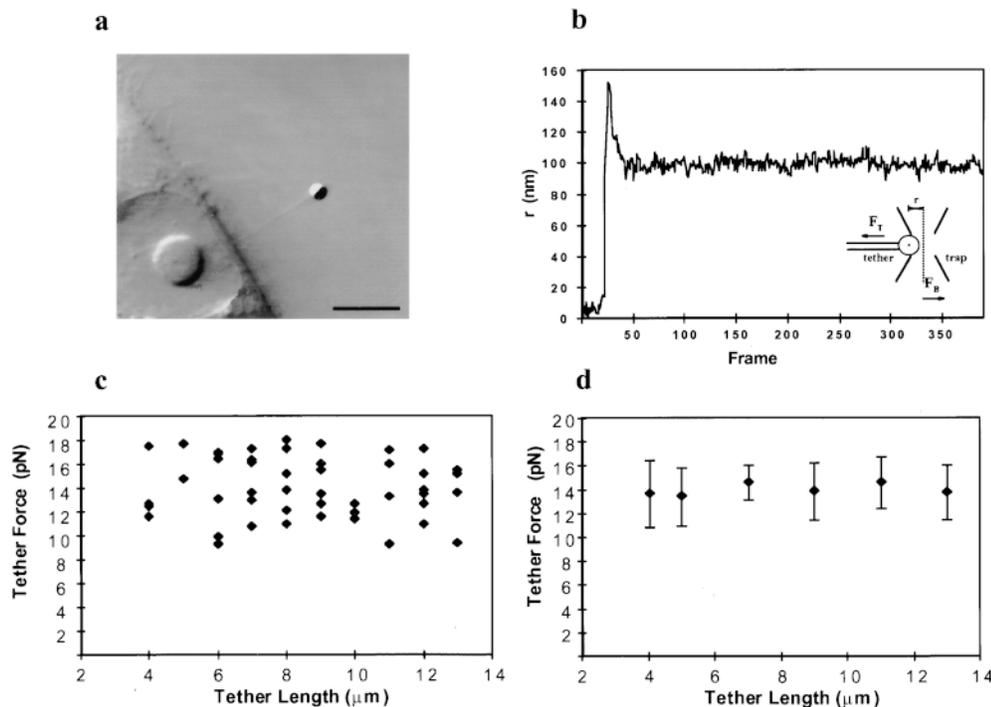
**Table I.** Effect of Membrane Expanding Reagents on Endocytosis and Tether Force in Interphase and Metaphase

	Interphase		Metaphase	
	Tether force	Endocytosis	Tether force	Endocytosis
Control	100 ± 6	100 ± 4	292 ± 27	29 ± 3
Deoxycholic acid	74 ± 8	107 ± 4	211 ± 25	40 ± 3
Ethanol	61 ± 10	133 ± 9	134 ± 16	65 ± 7
DMSO	76 ± 9	122 ± 7	97 ± 10	67 ± 4

Incubation of HeLa cells with deoxycholic acid (0.1 mM), ethanol (1%), DMSO (3%) for 10 min reduced the tether force in interphase and metaphase cells, and at the same time the uptake of endocytic vesicles was increased. To compare different experiments, tether force and endocytosis rate are expressed as a percentage of the value of interphase cells.

### Membrane Tension Increase in Mitosis

In previous studies, we have found that the increase in endocytosis rate after secretion was correlated with a decrease in membrane tension indicating that a decrease in membrane tension in mitotic cells could increase tension. To measure apparent membrane tension, a polystyrene bead coated with rat IgG or ConA was attached to the cell plasma membrane and pulled away from the cell surface with laser tweezers to form a membrane tether as previously described (Dai and Sheetz, 1995b; Fig. 3 a). The force pulling the bead back to the cell (tether force) causes displacement of the bead from the center of the tweezers (Fig. 3 b, inset), and the displacement ( $r$ ) is directly proportional to the tether force (Kuo and Sheetz, 1993). In the actual force measurements, there is a peak during initial tether extension because of viscous flow into the tether (Fig. 3 b). When extension ceases, force decreases to the plateau level representing the static tether force. In HeLa cells there was no detectable change in static tether force for tethers pulled at different lengths (Fig. 3 c). Similarly,



**Figure 3.** Measurement of tether force. (a) Membrane tether formed with laser tweezers on a HeLa cell in interphase. Bar, 5  $\mu$ m. (b) Position of the bead in the trap ( $r$ ) with time during the tether formation. Inset, schematic diagram illustrating the opposition of the force of the trap ( $F_B$ ) and tether force ( $F_T$ ) pulling the bead back towards the cell. That tether force is not dependent on tether length is confirmed in c, the data given are single static tether force measurements plotted against corresponding tether length. (d) A single tether was pulled to different lengths and the corresponding static tether force was measured.

when the same bead was used to form membrane tethers varying in length from 3 to 14  $\mu\text{m}$ , static tether force did not change (Fig. 3 d) indicating that static tether force is independent of tether length. In general, static tether force is dependent on bending stiffness, in-plane tension, and membrane-cytoskeleton interaction (Waugh et al., 1992; Dai and Sheetz, 1995b; Hochmuth et al., 1996). However, our results (data not shown) and results from the previous studies show that bending stiffness is constant. Since bending stiffness is constant, the static tether force is related to the square root of membrane tension (Waugh et al., 1992; Hochmuth et al., 1996). In the membrane tension term as it is defined here (Sheetz and Dai, 1996), there are contributions from membrane-cytoskeleton adhesion and in-plane tension that can not be separated cleanly. Both of those terms are relevant to the process of endocytosis because the membrane must be curved and separated from the cytoskeleton.

As cells entered into mitosis, there was a nearly twofold increase in the static tether force in prometaphase (Fig. 4 a). In metaphase, the tether force peaked at a level threefold greater than interphase cells. With progression through anaphase, the tether force dropped to twice that of interphase. Finally, as cells reached cytokinesis, tether force returned to the interphase value.

From tether force (Fig. 4 a) and fluorescence (Fig. 1) results, it is evident that the increase in membrane tension from interphase through metaphase is accompanied by a decrease in endocytosis. Similarly, after the peak in metaphase, the decrease in tension is followed by an increase in endocytosis rate, suggesting that membrane tension may modulate endocytosis rate during mitosis.

### Tether Force and Endocytosis Rate Are Inversely Related in 3T3 Cells

To examine whether the increase in membrane tension

and decrease in endocytosis in mitotic cells is a unique property of HeLa cells, we measured static tether force and endocytosis rates in NIH-3T3 mouse fibroblasts. Fig. 4 b shows a threefold increase in tether force in metaphase cells with respect to interphase cells that is accompanied by twofold decrease in FM1-43 uptake (Fig. 4 c), suggesting that elevated tension and decreased endocytosis in mitotic cells is a general phenomenon.

### Mitotic Block Results in Tension Increase

To check whether the rise in tension is a result of a transitory modification or if it persists over longer periods when the cells are locked at the prometaphase/metaphase boundary, we examined membrane tension in mitotic HeLa cells arrested by vinblastine or taxol. Since, membrane tension is sensitive to drugs that affect microtubule polymerization (data not shown; Dai and Sheetz, 1995a), it is essential to inhibit mitosis and cell proliferation without causing changes in microtubule polymer mass. Addition of very low concentrations of vinblastine or taxol to the growth media of HeLa cell cultures induces significant cell cycle arrest, without causing net depolymerization or polymerization of microtubules (Wendell, 1993). As shown in Fig. 5 a, the anti-tumor drug taxol inhibited HeLa cell proliferation by inducing a mitotic block at the metaphase/anaphase boundary (Jordan et al., 1993). Similarly in vinblastine-treated cells, chromosomes had not undergone anaphase segregation, and the cell cycle was blocked in a stage that resembled prometaphase or metaphase (Fig. 5 b). Interestingly, static tether force almost doubled in taxol-arrested cells and increased 40% in vinblastine-treated cells, with respect to interphase cells (Fig. 5 c). The uptake of FM1-43 in taxol and vinblastine arrested mitotic cells was determined using a FACScan<sup>®</sup> cytometer. The increase in tether force was accompanied by a 60% decrease in uptake of FM1-43 in taxol-arrested cells and a

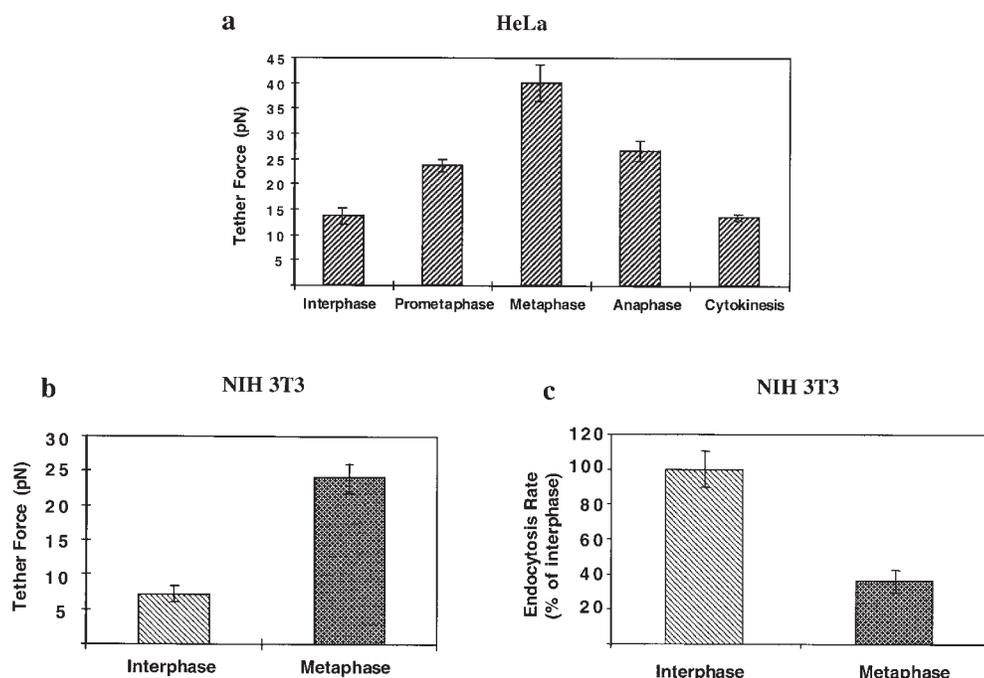
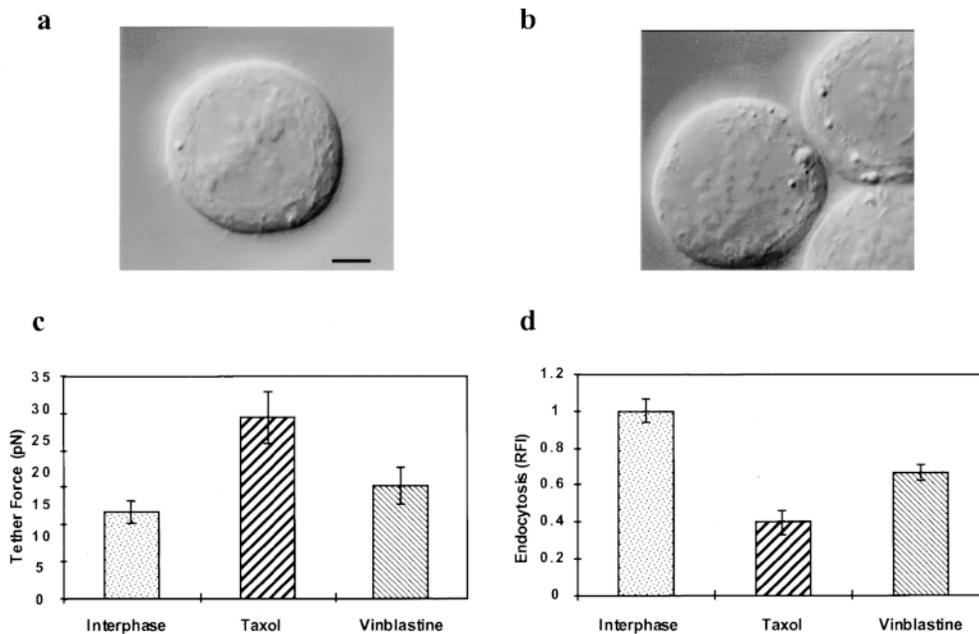


Figure 4. Tether force in interphase and mitotic HeLa cells. (a) Average tether force in different mitotic stages. While previous studies of membrane properties were not capable of resolving each phase, the laser optical tweezers enabled us to directly measure tether force in each cell phase and to follow the same cell through mitosis. (b) Tether force in interphase and metaphase of NIH-3T3 mouse fibroblast cells. Error bars represents SEM for 10–25 measurements. (c) Uptake of FM1-43 labeled vesicles by interphase and metaphase cells.



**Figure 5.** Tether force in taxol or vinblastine arrested cells. (a) Photomicrograph of HeLa cells incubated for one cell cycle with vinblastine (cell cycle was blocked in a stage which resembled prometaphase or metaphase since chromosomes had not undergone anaphase segregation). (b) Photomicrograph of HeLa cells blocked by taxol at the metaphase-anaphase boundary. Bar, 5  $\mu$ m. (c) 30% increase in tether force in vinblastine arrested cells and twofold increase in tether force in taxol arrested cells with respect to tether force of untreated interphase cells. (d) Endocytosis was determined by FACS<sup>®</sup> analysis. The data are expressed as the relative fluorescence index (RFI), relative to control cells, and represents the mean of triplicate determination  $\pm$  SD.

35% decrease in vinblastine-treated cells (Fig. 5 d). These results suggest that in cells locked in mitosis the endocytosis rate is decreased and membrane tension is increased without alteration in microtubule polymer mass.

#### ***Amphiphilic Compounds Decrease Membrane Tension***

If the primary cause of the decrease in endocytosis rate in metaphase cells is the increase in membrane tension, then agents that reduce membrane tension should cause an increase in endocytosis rate. In separate studies we have found that detergents (Dai, J., and M.P. Sheetz, unpublished data) and the solvents ethanol and DMSO (Dai and Sheetz, 1995b) all cause a decrease in membrane tension, which correlates with the expansion of the membrane caused by such agents. Likewise in HeLa cells, deoxycholic acid, ethanol, and DMSO cause a decrease in tether force in both interphase and metaphase cells (Table I). In parallel, there was an increase in endocytosis rate with all treatments (Table I). Thus, mitotic cells can increase the rate of endocytosis if membrane tension is decreased by membrane-expanding detergents and solvents.

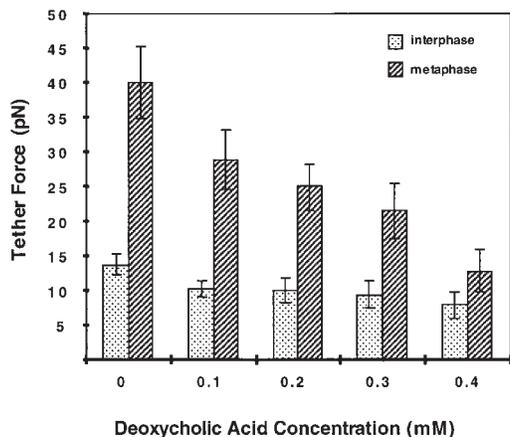
#### ***Dependence of Membrane Tension on Deoxycholate Concentration***

If there is an inverse dependence of endocytosis rate on membrane tension, then the endocytosis rate of metaphase cells should continuously increase to interphase levels as the membrane tension is decreased to interphase levels. To test this, we measured tether force and endocytosis rate immediately after the addition of increasing amounts of deoxycholic acid (Fig. 6). Increasing deoxycholic acid concentrations caused a linear decrease in

tether force in interphase and metaphase cells (Fig. 6). In 0.4 mM deoxycholic acid, the tether force in interphase cells and metaphase cells decreased by 45 and 65%, respectively compared with control cells. The metaphase cells were more sensitive to detergent concentration than interphase cells. At 0.4 mM deoxycholic acid, tether force in metaphase cells corresponded to tether force in untreated interphase cells. Uptake of endocytic vesicles in interphase and metaphase cells increases in parallel with the increase in concentration of deoxycholic acid (Fig. 2). Moreover, treatment of the cells with 0.4 mM deoxycholic acid increased endocytosis rate of metaphase cells to the value that matches the rate of untreated interphase cells. The return to interphase endocytosis rates was confirmed by measuring endocytosis of fluorescein dextran. Upon addition of 0.4 mM deoxycholic acid, metaphase cells increased their uptake of fluorescein dextran to  $93 \pm 7\%$  (STD,  $n = 11$ ) of untreated interphase cells. Results obtained by both methods and fluorescent dyes indicated that when membrane tension in metaphase cells is adjusted to the interphase level with detergent addition, the endocytosis rate reaches the interphase level. This treatment did not obviously alter the mitotic process, since cells in mitosis completed cytokinesis in the presence of 0.4 mM deoxycholic acid.

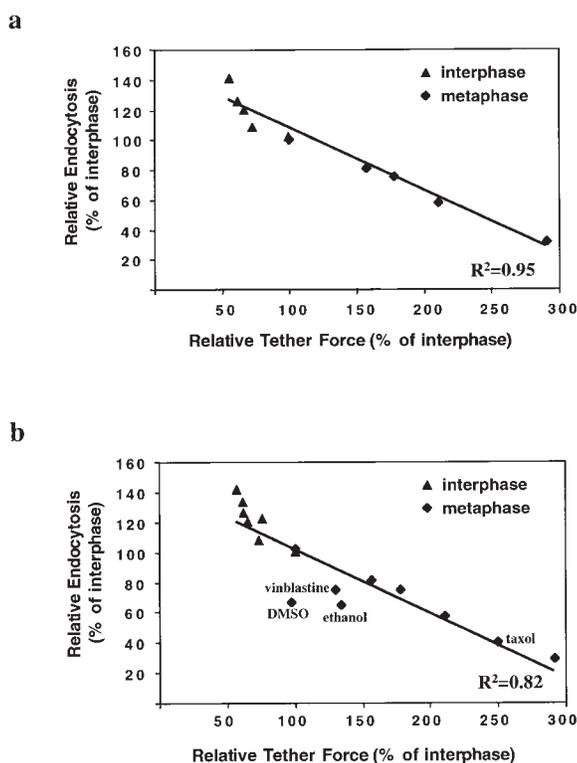
#### ***Correlation of Relative FM1-43 Uptake and Tether Force in Mitotic Cells***

To further clarify the relationship between tether force and endocytosis we have plotted relative tether force and relative endocytosis rate in for different concentrations of deoxycholate, DMSO, ethanol, and cells arrested with



**Figure 6.** Titration of membrane tension with increasing concentrations of deoxycholic acid. (a) Nonsynchronized HeLa cells plated on a glass coverslips were incubated for 10 min in various concentrations of deoxycholic acid and the tether force measured as described in Fig. 3. Error bars represents SEM for 10–25 measurements.

taxol or vinblastine. With different concentrations of deoxycholate there is a linear correlation between membrane endocytosis rate and tether force for both mitotic and interphase cells (Fig. 7 a;  $R^2 = 0.95$ ). When we plot



**Figure 7.** Relative tether force and relative endocytosis rate in interphase and metaphase cells for: (a) different concentrations of deoxycholate; (b) different concentrations of deoxycholate, DMSO, ethanol, and cells arrested with taxol or vinblastine. Tether force and endocytosis rate are expressed as a percentage of the value of interphase cells. Line represents linear least square fit of the data points.

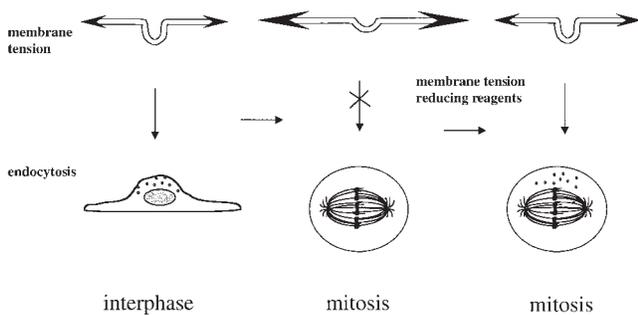
the values for the other conditions on the same graph (Fig. 7 b), there is more scatter but the linear correlation is still very good ( $R^2 = 0.82$ ). The major deviation from the linear trend is DMSO. DMSO is known to affect actin polymerization (Sanger et al., 1980), which may explain higher inhibition of endocytosis rate in that case. We conclude that there is a roughly linear correlation between membrane tension and endocytosis rate in both metaphase and interphase cells. Different agents are expected to have different secondary effects on cell endocytosis which may cause greater scatter in the plot and indicates that other factors than membrane tension can significantly affect endocytosis rate.

## Discussion

Tether force measurements and quantitative fluorescence measurements of single cells have demonstrated an inverse relationship between tether force and endocytosis in mitotic and interphase cells. Whether we measure the rate of fluid or membrane uptake using fluorescein dextran or FM1-43 respectively, we find a dramatic decrease in endocytosis rate in mitosis as was previously observed (Berlin and Oliver, 1980). Endocytosis rate in the metaphase could be increased dramatically with the addition of deoxycholate, ethanol, and DMSO, which had the common effect of expanding the membrane bilayer. In line with the expansion of the membrane by these amphiphilic compounds, there was an apparent decrease in membrane tension as measured by tether force. Therefore, we postulate that the physical parameter of apparent membrane tension has a major effect on cell endocytosis rate.

All of the membrane-expanding agents caused a decrease in tension and an increase in endocytosis rate. For all of the treatments, a linear fit of membrane tension with endocytosis rate had a correlation coefficient of 0.82. This is despite the fact that DMSO has unusual effects on the cytoskeleton (Sanger et al., 1980) and ethanol has a wide variety of metabolic effects. Although it is extremely unlikely that a better correlation will be found between the amphiphilic compounds effects on endocytosis rate and their effects on a specific biochemical mechanism, a causal relationship is only inferred at this time. The deoxycholate effects on interphase and metaphase cells show a different slope in an endocytosis rate versus tether force plot, which could be explained as a difference in the endocytic machinery as a function of the cell cycle (Pypaert et al., 1987). The biochemical changes in mitosis may cause major changes in the endocytic pathway but the strong correlation between endocytosis rate and tether force indicates that tether force reflects an important factor controlling endocytosis rate.

In previous analyses, the relationship between tether force and apparent membrane tension was established. The two components of membrane tension, bilayer in-plane tension, and membrane-cytoskeleton adhesion are linked for animal cells because the adhesive forces mold the shape of the membrane to the cytoskeleton (see Sheetz and Dai, 1996 for further clarification). We consequently suggest a very simple model for regulation of endocytosis during mitosis (see Fig. 8). Relatively low membrane tension in interphase cells allows rapid invagination of ves-



**Figure 8.** Model for regulation of endocytosis during mitosis. The formation of endocytic vesicles requires substantial force to bend the membrane and to overcome the membrane tension. The magnitude of membrane tension is denoted by the thickness of the arrow and endocytosis is represented by the small vesicles inside the cell. Low membrane tension in interphase allows bending of the membrane, formation of endocytic vesicles and endocytosis proceed. During the transition from interphase to mitosis membrane tension increases dramatically preventing the invagination of endocytic vesicles and resulting in inhibition of endocytosis. However, as shown in this study, if the membrane tension in mitotic cells is decreased to interphase levels by addition of tension reducing reagents, endocytosis is restored to interphase levels.

icles. During the transition from interphase to mitosis, membrane tension increases dramatically preventing the invagination of endocytic vesicles. However, endocytosis in mitotic cells may be restored by reducing the membrane tension with membrane expanding reagents, indicating that membrane tension is an important factor in regulating cellular endocytosis rate.

This model is consistent with Fawcett's morphological observation of coated pits in dividing erythroblasts (Fawcett, 1965). He found that flatter coated pits appeared more frequently in mitotic cells, suggesting that curvature of the membrane by the coated pit was inhibited. Pypaert et al. (1987) have shown that all categories of coated pits, from shallow to deeply invaginated were present in both interphase and mitotic A431 cells. However, the flatter coated pits occupied over a twofold greater area of the plasma membrane in mitotic cells than in interphase whereas the curved pits occupied a similar area in both cases. Accumulation, i.e., higher density, of flatter coated pits in mitotic cells may well be explained by inhibition of the invagination process in a step which converts the shallow coated pits into deeply invaginated ones. High membrane tension would inhibit the curvature of membrane by the clathrin coats. We feel that the tether force provides a very good measure of the resistance that a clathrin-dependent or other endocytic process must overcome to form an endocytic vesicle. The endocytic machinery must displace the cytoskeleton as well as bend the membrane against the force generated by the in-plane tension. Both of those terms contribute to the membrane tension as measured by the static tether force.

What is the molecular mechanism of membrane tension regulation during the cell cycle? The changes in membrane tension might well be explained as direct effects of alterations in the compositional or structural properties of

the membrane and underlying cytoskeletal network or as an indirect effect of a decrease in exocytosis rate. In the first case, membrane tension would be modulated by changes in plasma membrane phospholipid and protein composition that accompany mitosis; perhaps in a manner analogous to the decrease in tension that occurs within 10 s of stimulation of secretion (Dai et al., 1997). Indeed, transferrin receptors and active  $\text{Na}^+/\text{K}^+$  ATPase pump sites are internalized during prophase and metaphase and subsequently recycled to the cell surface (Rabito and Tchao, 1980; Sager et al., 1984; Warren et al., 1984); and the resynthesis of plasma membrane sphingomyelin is greatly decreased in cells undergoing mitosis (Kallen et al., 1994). One of the parameters that has the most obvious role in determining membrane tension is the membrane-cytoskeleton interaction (Sheetz and Dai, 1996). This raises the possibility that membrane tension in mitotic cells may be modulated by changes in the cytoskeletal lattice that underlies the plasma membrane. Sanger reported different staining patterns of chick fibroblasts labeled with fluorescent heavy meromyosin indicating dramatic redistribution of actin during cell division (Sanger, 1975). Whether these or other changes in membrane composition and structure during mitosis could alter membrane tension is not known. Alternatively, the drop in secretion during mitosis will cause an increase in membrane tension until the endocytosis rate matches the secretion rate. Since the endocytosis rate will rapidly match the exocytosis rate (membranes do not stretch) and the endocytosis rate is nearly linearly related to the inverse of the membrane tension, a decrease in exocytosis rate will rapidly cause a rise in membrane tension. The rise in membrane tension will cause the membrane to stretch by 0.1%. Because an area of membrane equal to the plasma membrane will be endocytosed in  $\sim 100$  min, the decrease in exocytosis rate will result in a rise in membrane tension in 6 s (the same time scale was found for the decrease in membrane tension after stimulation of secretion of RBL cells [Dai et al., 1997]). Further studies to directly measure the membrane-cytoskeleton adhesion are needed to differentiate between these possibilities although both effects could contribute to the rise in tension.

Cells normally transit through mitosis in 20–30 min, but taxol or vinblastine arrested cells are locked in mitosis for an average of 10 h in these experiments. They still maintain an elevated tension after that period irrespective of whether a microtubule stabilizing or destabilizing drug was used. The rise in tension is therefore not a result of a transitory modification but persists when the cells are locked in metaphase. Increased tension over such long periods will dramatically decrease the uptake of fluid phase nutrients. Thus, some of the problems with synchronizing cells by arresting them in mitosis could be the result of the decreased endocytosis rate.

Regardless of the actual mechanism that is used by cells to regulate tension in mitosis, this study clearly shows that endocytosis rate correlates inversely with membrane tension. Moreover, the rise in membrane tension during mitosis is not an unique property of HeLa cells but is readily observed in NIH-3T3 fibroblasts (Fig. 4). Thus, we speculate that the physical change of increasing membrane tension and correlated inhibition of endocytosis are impor-

tant elements in the control of cellular activities during mitosis.

Physical parameters such as membrane tension can exert control over many important cellular biochemical activities because tension is an intensive variable that is felt throughout the plasma membrane. A similar physical parameter is the trans-membrane potential. However, membrane tension acts as a force to smooth the membrane and produces a resistance to mechanical deformation. When any protein undergoes a mechanical change during a biochemical process, the application of mechanical force to the protein can alter that process (reviewed in Khan and Sheetz, 1997) and many proteins are mechanically linked to the plasma membrane. In the case of endocytosis, one hypothesis is that the cell uses tension-dependent endocytosis to maintain the proper amount of membrane on the cell surface as the cell changes morphology and secretes membrane vesicles. If the endocytosis machinery is always active but the activity is regulated by tension in the membrane, then a drop in tension is readily corrected by an increase in the endocytosis rate. An increase in tension would normally be corrected by decreasing endocytosis while maintaining or increasing secretion. In mitotic cells, the biochemical alterations of the cell result in a decreased secretion rate that could cause a higher tension. Because the endocytosis rate is lower in high tension, the endocytosis rate would then match the lower secretion rate. This simple physical explanation is consistent with the fact that the changes in endocytosis (and presumably exocytosis) rate and tether force are approximately proportional. Alternative hypotheses for the regulation of the proper cell surface area are necessarily complicated because they require enzymatic linkage between exocytosis and endocytosis as well as cell morphology. Tension-dependent control of membrane area provides a way of properly adjusting to complex morphological changes and also of altering a host of other cellular activities in concert.

In this study, we have focused on regulation of endocytosis by membrane tension in mitosis; however, the alteration of other cell parameters by increased tension in mitosis such as cell shape and motility may be also important for the mitotic process. Our preliminary results show that tension is inversely correlated with the rate of actin-based lamellipodial and filopodial extension in cells (Raucher, D., J. Dai, and P. Sheetz, unpublished results). Decreased cell motility and spreading would lead to the cell rounding seen in mitosis. A reasonable postulate is that the entry into mitosis decreases secretion which causes membrane tension to rise and then increased membrane tension would inhibit motility and round the cell. Thus, the decrease in secretion would alter the physical state of the cell membrane during mitosis inducing a number of the observed changes that are characteristic of mitosis through action on proteins mechanically linked to the plasma membrane.

We have found that stimulation of endocytosis in mitotic cells does not block mitosis. These results suggest a new cellular mechanism for the regulation of endocytosis rate in mitotic cells and may provide new methods to stimulate endocytic uptake in mitotic cells. That may be important in anti-tumor drug delivery in cancer cells. Namely, one of the major characteristics of cancer cells is rapid

growth and frequent cell division accompanied by inhibition of endocytosis. Since alteration of tension is sufficient to modify endocytosis rate, this raises the possibility that factors which reduce membrane tension may increase susceptibility of dividing cells to anti-mitotic, anti-tumor drugs by increasing their rate of endocytosis (Raucher, D., and M.P. Sheetz, preliminary results). The control of endocytosis by membrane tension indicates that physical forces in membranes may play significant roles in cellular biochemical reactions. The inelasticity of the plasma membrane assures that tension forces generated locally in the membrane can globally affect tension-dependent cellular enzymes.

We thank Dr. R.D. Berlin for helpful comments in the early phase of this project and Dr. D. Felsenfeld and L. Lindesmith for critical reading of the manuscript.

We thank the National Institutes of Health for financial support.

Received for publication 14 October 1998 and in revised form 4 January 1999.

### References

- Berlin, R.D., and J.M. Oliver. 1980. Quantitation of pinocytosis and kinetic characterization of the mitotic cycle with a new fluorescence technique. *J. Cell Biol.* 85:660–671.
- Berlin, R.D., J.M. Oliver, and R.J. Walter. 1978. Surface function during mitosis 1: phagocytosis, pinocytosis and mobility of surface-bound ConA. *Cell.* 15:327–341.
- Betz, W.J., and G.S. Bewick. 1992. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science.* 255:200–203.
- Choquet, D., D.P. Felsenfeld, and M.P. Sheetz. 1996. Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell.* 88:39–48.
- Dai, J., and M.P. Sheetz. 1995a. Regulation of endocytosis, exocytosis and shape by membrane tension. In *Protein Kinesin: Dynamics of Protein Trafficking and Stability*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 567 pp.
- Dai, J., and M.P. Sheetz. 1995b. Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers. *Biophys. J.* 68:988–996.
- Dai, J., H.P. Ting-Beall, and M.P. Sheetz. 1997. Secretion-coupled endocytosis correlates with membrane tension changes in RBL 2H3 cells. *J. Gen. Physiol.* 110:1–10.
- Fawcett, D.W. 1965. Surface specializations of absorbing cells. *J. Histochem. Cytochem.* 13:75–91.
- Featherstone, C., G. Griffiths, and G. Warren. 1985. Newly synthesized G protein of vesicular stomatitis virus is not transported to the Golgi complex in mitotic cells. *J. Cell Biol.* 101:2036–2046.
- Hinshaw, J., and S.L. Schmid. 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature.* 374:190–192.
- Hochmuth, R.M., J. Shao, J. Dai, and M.P. Sheetz. 1996. Deformation and flow of membrane into tethers extracted from neuronal growth cones. *Biophys. J.* 70:358–369.
- Jain, M.K., N.Y. Wu, and L.V. Wray. 1975. Drug-induced phase change in bilayer as possible mode of action of membrane expanding drugs. *Nature.* 255: 494–496.
- Jordan, M.A., R.J. Toso, D. Thrower, and L. Wilson. 1993. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. USA.* 90:9552–9556.
- Kallen, K.J., D. Allan, J. Whatmore, and P. Quinn. 1994. Synthesis of surface sphingomyelin in the plasma membrane recycling pathway of BHK cells. *Biochim. Biophys. Acta.* 1191:52–58.
- Kell, A., and R.F. Glaser. 1993. On the mechanical and dynamic properties of plant cell membranes: their role in growth, direct gene transfer and protoplast function. *J. Theor. Biol.* 160:41–62.
- Khan, S.M., and M.P. Sheetz. 1997. Force effects on biochemical kinetics. *Ann. Rev. Biochem.* 66:785–805.
- Kuo, S.C., and M.P. Sheetz. 1993. Force of single kinesin molecules measured with optical tweezers. *Science.* 260:232–234.
- Matter, K., and I. Mellman. 1994. Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr. Opin. Cell Biol.* 6:545–554.
- Maxfield, F.R., and K.W. Dunn. 1990. In *Optical Microscopy for Biology*. B. Herman and K. Jacobson, editors. Wiley-Liss, New York. 357 pp.
- Pines, J. 1994. Protein kinases and cell cycle control. *Semin. Cell Biol.* 5:399–408.
- Pypaert, M., L.M. Lucocq, and G. Warren. 1987. Coated pits in interphase and mitotic A431 cells. *Eur. J. Cell Biol.* 45:23–29.
- Pypaert, M., D. Mundy, E. Souter, J.C. Labbe, and G. Warren. 1991. Mitotic cytosol inhibits invagination of coated pits in broken mitotic cells. *J. Cell Biol.*

- Rabito, C.A., and R. Tcho. 1980.  $^3\text{H}$ -ouabain binding during the monolayer organization and cell cycle in MDCK cells. *Am. J. Physiol.* 238:C43–C48.
- Riezman, H., P.G. Woodman, G. van Meer, and M. Marsh. 1997. Molecular mechanisms of endocytosis. *Cell* 91:731–738.
- Sager, P.R., P.A. Brown, and R.D. Berlin. 1984. Analysis of transferrin recycling in mitotic and interphase HeLa cells by quantitative fluorescence microscopy. *Cell* 39:275–282.
- Sanger, J.W. 1975. Changing patterns of actin localization during cell division. *Proc. Natl. Acad. Sci. USA* 72:1913–1916.
- Sanger, J.W., J.M. Sanger, T.E. Kreis, and B.M. Jockusch. 1980. Reversible translocation of cytoplasmic actin into the nucleus caused by dimethyl sulfoxide. *Proc. Natl. Acad. Sci. USA* 77:5268–5272.
- Sheetz, M.P., and J. Dai. 1996. Modulation of membrane dynamics and cell motility by membrane tension. *Trends Cell Biol.* 6:85–89.
- Sheetz, M.P., and S.J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA* 71:4457–4461.
- Sweitzer, S.M., and J.E. Hinshaw. 1998. Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* 93:1021–1029.
- Takei, K., V. Haucke, V. Slepnev, K. Farsad, M. Salazar, H. Chen, and P. De Camilli. 1998. Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. *Cell* 94:131–141.
- Terasaki, M. 1995. Visualization of exocytosis during sea urchin egg fertilization using confocal microscopy. *J. Cell Sci.* 108:2293–2300.
- Tuomikosi, T., M.A. Felix, M. Doree, and J. Gruenberg. 1989. Inhibition of endocytic vesicle fusion in vitro by the cell-cycle control protein kinase cdc2. *Nature* 342:942–945.
- Warren, G., C. Featherstone, G. Griffiths, and B. Burke. 1983. Newly synthesized G protein of vesicular stomatitis virus is not transported to the cell surface during mitosis. *J. Cell Biol.* 97:1623–1628.
- Warren, G., J. Davoust, and A. Cockcroft. 1984. Recycling of transferrin receptors in A431 cells is inhibited during mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2217–2225.
- Waugh, R.E., J. Song, S. Svetina, and B. Zeks. 1992. Local and nonlocal curvature elasticity in bilayer membrane by tether formation from lecithin vesicles. *Biophys. J.* 61:974–982.
- Wendell, K.L., L. Wilson, and M.A. Jordan. 1993. Mitotic block in HeLa cell by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and centrosomes. *J. Cell Sci.* 104:261–274.
- Zhelev, D.V. 1998. Material property characteristics for lipid bilayers containing lysolipid. *Biophys. J.* 75:321–330.