J. Biochem. 119, 887-897 (1996)

Wortmannin and Li^+ Specifically Inhibit Clathrin-Independent Endocytic Internalization of Bulk Fluid

Satoshi B. Sato,*,[1] Takahisa Taguchi,† Shohei Yamashina,‡ and Sakuji Toyama§

*Cell and Information, PRESTO, Research Development Corporation of Japan and Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606-01; †Department of Organic Materials, Osaka National Research Institute, Ikeda, Osaka 563; ‡Department of Anatomy, Kitasato University School of Medicine, Kitasato, Sagamihara, Kanagawa 228; and §Institute for Virus Research, Kyoto University, Kyoto 606-01

Received for publication, December 11, 1995

Incubation of a human fibrosarcoma cell line HT-1080 in Li^+-containing medium inhibited internalization of a fluid marker, horseradish peroxidase (HRP), by more than 80%. The ion inhibited the activity enhanced by Ca^{2+} or phorbol 12-myristate 13-acetate. We also found that wortmannin (WT), a potent inhibitor of phosphoinositide (PI) 3-kinase (PI 3-k), inhibited the non-stimulated and the two stimulated types of endocytosis to the same extent as Li^+. In contrast, neither WT nor Li^+ influenced the early internalization of transferrin (Tfn), EGF or platelet-derived growth factor. Neither targeting to early endosomes nor recycling of the once-internalized Tfn was influenced. When the cytoplasmic pH was lowered by chasing cells that had been preincubated with 25 mM NH_4Cl in an amiloride-containing Na^+-free medium, more than 90% of internalization of Tfn in HT-1080 cells was inhibited, while that of HRP was reduced by only 35%. In contrast, WT reduced the uptake of HRP by KB cells by 34%, while 60% of the activity was inhibited by the treatment for cytoplasmic acidification. Comparison of other cells *i.e.*, A-549 and a human diploid cell line Miyajima, indicated that cells showing higher sensitivity to WT were less sensitive to low cytoplasmic pH. These results suggest that, in all the cells studied, bulk fluid is internalized either *via* a clathrin-independent/PI 3-k-dependent route or *via* a clathrin-dependent/PI 3-k-independent one, though the ratio varied among them. We also found that internalization of a mAb directed toward the 116 (100)-kDa subunit of vacuolar ATPase [OSW2; Sato and Toyama (1994) *J. Cell Biol.* 127, 39-53] in the fluid phase was inhibited by WT, but the antibody was still internalized in a surface-bound form. Regardless of the treatment with WT, most of the antibody was transported to endosomes that were associated with Tfn receptor. These results suggest that both internalization routes are targeted to the same early endosomal compartments.

Key words: endocytosis, growth factor internalization, PI-3k, PI-turnover, transferrin.

Eukaryotic cells internalize physiologically important molecules by endocytosis. The process employing specific receptors in clathrin-coated pits and vesicles is essential for the internalization of many kinds of protein ligands (*1, 2*). With ligands, the bulk-fluid phase is opportunistically internalized by the vesicles. On the other hand, some toxins and plasma membrane components are internalized independently of clathrin (*3, 4*). The two surface routes appear to be targeted to the common early endosomes (*5, 6*). It has been shown that inhibition of clathrin-polymerization reduces endocytosis of transferrin by rat fetal fibroblasts while the internalization of HRP is much less affected (*7*). Damke *et al.* have demonstrated that induction of a mutant dynamin that lacks GTPase activity in HeLa cells specifically reduces most of the receptor-mediated internalization of protein ligands, but the cells still show a high degree of endocytosis of HRP (*8*). These results imply that the clathrin-independent mechanism(s) takes a large part in the internalization of the bulk fluid in some cells. However, in contrast to clathrin-dependent endocytosis, mechanistic details of the bulk-fluid endocytosis are still unclear (for review, Ref. *9*).

By using wortmannin, which inhibits phosphoinositide (PI) 3-kinase, recent studies have suggested that this enzyme is involved in endocytic activity (*10, 11*). Wortmannin was found to be inhibitory to the internalization of a fluid phase marker, but this was interpreted in terms of the mechanism of clathrin-dependent endocytosis (*10*). In apparent contradiction, Joly *et al.* indicated that a WT-sensitive mechanism is involved in post-endocytic step(s), where the reagent inhibited down-regulation of platelet-derived growth factor receptor (*11*).

We previously found that fluid phase endocytosis in a human fibrosarcoma cell line, HT-1080, was enhanced by Ca^{2+} or phorbol 12-myristate 13-acetate (PMA) (*12*). The fluid phase was transported to compartments that were localized by fluorescent transferrin. In the presence of okadaic acid (OKA) at 100 nM and 20 nM PMA, the fluid phase was targeted to compartments devoid of transferrin-labeling, suggesting that the cells potentially had a clathrin-independent alternate endocytic mechanism(s) (*12*). We

[1] To whom correspondence should be addressed. Tel: +81-75-753-4216, Fax: +81-75-791-0271

also found that Li^+ inhibited fluid-phase endocytosis activated by Ca^{2+}. In characterizing the effect of the ion, prolonged incubation in Li^+ also inhibited non-stimulated endocytic activity and that activated by PMA. We found that WT inhibits both the basal and activated endocytosis with identical magnitude. We used these two reagents for characterization of receptor-mediated internalization of transferrin (Tfn), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and observed no inhibition. Because both reagents interfere with the non-canonical PI-turnover, we suggest that the molecule(s) in the turnover is an essential element in the clathrin-independent endocytosis.

MATERIALS AND METHODS

Reagents—DME and Na phosphate-free MEM were obtained from Gibco BRL (Gaithersburg, MD). Wortmannin (Sigma Chemical, St. Louis, SO) and phorbol 12-myristate 13-acetate (Wako Pure Chemicals, Osaka) were purchased from the indicated distributors. A rabbit antiserum against the p85 subunit of PI 3-k was obtained from UBI (Lake Placid, NY). TRITC-dextran, TRITC-labeled human transferrin (Tfn) and FITC-Tfn were purchased from Molecular Probes (Eugene, OR). ^{125}I-labeled human Tfn, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF)-BB were obtained from Amersham (UK). Non-labeled human Tfn (Chemicon International, Temecula, CA), human recombinant EGF (Wako), and human recombinant PDGF (Genzyme, Cambridge, MA) were purchased from the indicated distributors. Fluorescently labeled antibodies against mouse IgG_1 and IgG_{2b} were obtained from Southern Biotechnology (Birmingham, AL). Other reagents were obtained from Wako.

Cells—HT-1080 clone 1 was as described (*12*). A-549 was obtained from ICN (Costa Mesa, CA). KB-100 was a subclone of KB cells (*13*). MDCK-II was kindly provided by Dr. T. Kobayashi of Tohoku University. A diploid human cell line, Miyajima, was established from a monolayer culture of donor cells. The cells were maintained in DME supplemented with 10% fetal bovine serum (FBS). They were used more than 2 days after culturing on small plastic dishes or on glass coverslips.

Treatment of Cells with Li^+ or Wortmannin—Cells were usually treated in DME-10% FBS containing 50 mM LiCl (>99.9%, from 1 M stock) or 50 nM wortmannin (WT, from ×1,000 stock in DMSO) at 37°C. For treatment with Ca^{2+}, cells were washed with prewarmed Na phosphate-free MEM containing 10 mM Hepes, 1 mM Na pyruvate, and 2% FBS (dialyzed against 150 mM NaCl) at pH 7.2, in order to avoid formation of Ca phosphate precipitates. They were incubated in the medium supplemented with 15 mM $CaCl_2$ (>99.9%, final 16.8 mM, high Ca^{2+}-medium) for 60 min at 37°C with gentle shaking in a humidified chamber. When Li^+- or WT-treated cells were further treated with Ca^{2+}, they were transferred to the high Ca^{2+}-medium containing the reagents 1 h before the assay.

Assay of Fluid-Phase Endocytosis—Duplicate cultures of cells in DME- or MEM-2% FBS ($6-8\times10^5$ cells/small dish) were supplemented with horseradish peroxidase to a final concentration between 0.5-2 mg/ml. They were incubated in a humidified chamber placed in a shaking incubator at 35°C. At intervals, cells were washed with ice-cold PBS (145 mM NaCl, 10 mM Na phosphate, pH 7.2) 3 times, scraped into Eppendorf tubes, and washed with PBS 3 times by centrifugation at $3,000\times g$ at 4°C for 5 min. KB and Miyajima, to which slight background binding of HRP at 0°C was detectable, were treated with 0.01% pronase and sedimented through 0.5 ml of 15% sucrose-PBS. The washed cells were solubilized in 0.45 ml of TBS (150 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing 0.2% Triton X-100 and 10 μg/ml pepstatin A and 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF), at 0°C for 1 h. After centrifugation at $10,000\times g$ for 10 min, the supernatant was analyzed in triplicate for protein (*14*) and the peroxidase activity using tetramethylbenzidine as a substrate. The results were normalized for cellular protein and represented as relative uptake to the control cells. Cells were also treated with 25 mM NH_4Cl (Fluka, Switzerland) for 20 min, followed by incubation in K^+-buffer (150 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM K Hepes, pH 7.0) containing 500 μM amiloride in a similar manner to that described (*15*). Control cells were incubated in DME-10 mM Na Hepes (DME-Hepes) pH 7.2 without serum. After 5 min, cells were incubated with HRP for 10 min and the uptake of the enzyme was determined as described above. The treatments did not change the mechanical stability against the scraping procedure as assessed by membrane permeability to trypan blue.

Assay of Receptor-Mediated Endocytosis—For assay of internalization of transferrin (Tfn), cells were first incubated in serum-free DME for 1.5 h at 37°C, then with 50 nM WT for 30 min. They were incubated in 1 ml of serum-free DME containing 0.5 μg/ml non-labeled Tfn and 1.85 kBq (0.05 μCi)/ml ^{125}I-Tfn at 37°C. Cells were washed with ice-cold DME-Hepes, pH 7.2, 6 times. After removal of surface-bound Tfn by treatment with 50 mM glycine, 100 mM NaCl pH 3.0 for 2 min, 2 times, cell-associated radioactivity was recovered in 2×0.8 ml of 1 N NaOH. When recycling of the ligand was measured, cells depleted of the intrinsic ligand as described above were first incubated with Tfn (1.85 kBq/ml, 0.1 μg/ml) for 1 h at 37°C. WT was added at 50 nM during the next 30 min. The cells were washed and chased in DME containing 50 μg/ml non-labeled Tfn. At intervals, 100 μl aliquots of the chase medium were collected and the radioactivity was determined. For the assay of internalization of EGF or PDGF, cells pretreated with 50 nM WT for 60 min were incubated in DME-5 mM Na Hepes containing 2% dialyzed FBS, 5-10 ng/ml EGF or PDGF and 1.85 kBq/ml ^{125}I-labeled corresponding ligands. At intervals, cells were washed, acid-treated and solubilized in 1 N NaOH. In order to test the effect of WT on the lysosomal degradation, cells were incubated with 2 ng/ml EGF/^{125}I-EGF in DME-2% FBS at 20°C for 1 h. Cells were then treated with WT in the presence of the ligand for 20 min, washed 6 times at 0°C, and chased at 37°C. At intervals, 100 μl of the medium was collected and 100% TCA-0.25% Na deoxycholate was added to give a final concentration of 20%. After incubation in ice for 1 h, the samples were centrifuged at $10,000\times g$ for 5 min and the TCA-soluble count was measured. The radioactivity was determined in a Clinigamma counter (Pharmacia LKB, Uppsala, Sweden).

Fluorescence Microscopy of Endosomes—For labeling of early endosomes with Tfn, cells on coverslips were depleted

of the ligand by incubation in serum-free DME-Hepes pH 7.2 at 37°C for 60 min. FITC-Tfn (100 μg/ml) was then added for 1 h. The cells were further incubated with 50 nM WT in the presence of the ligand for 30 min. After having been washed with the medium containing WT over 5 min, the cells were incubated with TRITC-Tfn (50 μg/ml) for 10 min at room temperature (25°C). The coverslips were mounted in DME-5 mM Na Hepes containing 20 mM methylamine for visualization of otherwise quenched FITC-fluorescence. Cells were also incubated with a mAb directed toward the 116(100)-kDa subunit of the vacuolar-type proton pump, OSW2 (IgG_{2b}) (*16*), at 100 or 3 μg/ml for 10 min at 37°C. The cells were washed and fixed with 3% paraformaldehyde-PBS for 10 min then permeabilized with 0.05% Triton X-100 in PBS for 1.5 min. After incubation with an anti-human Tfn mAb (IgG_1: Chemicon), the antibodies were visualized by fluorescent subtype-specific antibodies. Cells were observed by using a confocal laser scanning system (MRC-600, Biorad, Hercules, CA) mounted on an Axiophoto fluorescence microscope (Carl Zeiss, Germany).

Electron Microscopy—Cells in 8-well Lab-Tek chamber slides (Japan InterMed, Tokyo) were treated with 10 mg/ml HRP at 37°C for 30 min. They were washed with MEM-Hepes pH 7.2, then chased in the presence or the absence of 50 nM WT for 20 min. Cationized ferritin (Sigma) was added to a final concentration of 100 μg/ml for 20 min. Cells were fixed with 0.5% glutaraldehyde in 7% sucrose-PBS at room temperature for 30 min. For visualization of HRP, fixed cells were treated with 0.5 mg/ml diaminobenzidine in TBS and 0.03% H_2O_2. The samples were further fixed in 1% OsO_4. Cells were also stained with ruthenium red in a similar manner to that described (*8*). The cells preincubated with 50 nM WT at 37°C were washed with 150 mM Na cacodylate pH 7.6 and fixed with 1% glutaraldehyde in 100 mM Na cacodylate containing 0.5 mg/ml ruthenium red at room temperature for 1 h. They were then washed with 150 mM Na cacodylate 5 times and further treated with 0.5 mg/ml ruthenium red and 1% OsO_4 in 75 mM Na cacodylate for 3 h. Samples were dehydrated with a graded series of ethanol, and finally embedded in Epon. Ultrathin sections were examined with or without counter-staining by uranyl acetate and lead citrate.

RESULTS

Inhibition of Fluid-Phase Endocytosis by Li^+—Fluid-phase endocytosis by HT-1080 was measured by using a non-specific marker, HRP. The activity was 0.081 ± 0.005 μl/h/10^6 cells, which is comparable to that in hepatocytes (*17, 18*). When cells were incubated with 50 mM Li^+ for increasing periods, the ion reduced not only the basal endocytosis, but also the activation by Ca^{2+} or phorbol 12-myristate 13-acetate (PMA) (Fig. 1a). When cells preincubated with Li^+ for 3 h were returned to a normal medium, the inhibition was reversed nearly to the original level within 3 h (Fig. 1b). Prolonged incubation in Li^+ did not induce any evident cytopathic effect. The ion did not change the activity of the marker. The cells remained fully extended even after 18 h. At that time point, the number of cells was 80% of the control, suggesting slight inhibition of multiplication. However, the cells grew normally upon removal of the ion (data not shown).

Fluid-Phase Endocytosis Is Inhibited by WT—Li^+ inhibits dephosphorylation of inositol di- and monophosphate, resulting in reduction of a phosphatidylinositol pool. It therefore interferes with both the canonical and non-canonical phosphoinositide (PI)-turnovers (*19-21*). Since Li^+ reduced enhancement of fluid-phase endocytosis by Ca^{2+} or PMA, which can mimic some parts of the canonical turnover, we tested the effect of inhibition of non-canonical PI-turnover on the fluid-phase endocytosis by using a potent inhibitor of PI 3-k, wortmannin (WT) (*22, 23*). When cells were incubated with the reagent for 1 h, not only the basal activity, but also that stimulated by Ca^{2+} or PMA were very similarly reduced (Fig. 2a). The concentrations giving 50% inhibition of the original activities were 2 nM for all three cases, which was similar to that inhibiting the partially purified enzyme. In contrast to these effects, incubation of cells with 2,3-dihydroxybenzaldehyde at 5 μg/ml, which inhibits PI 4-k (*24*), did not change the activity.

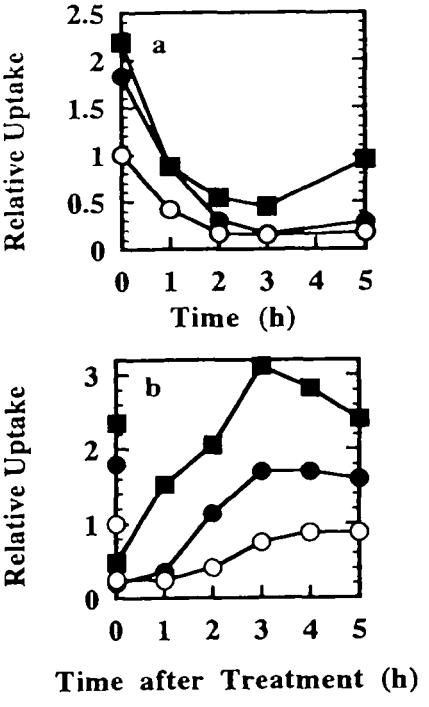

Fig. 1. **Inhibition of fluid phase endocytosis by Li^+.** a: Cells were incubated with 50 mM LiCl for indicated periods. During the last 1 h, they were treated with either none (○, 1.8 mM Ca^{2+}), 16.8 mM Ca^{2+} (●) or 20 nM PMA (■) in Na phosphate-free MEM-2% FBS dialyzed against 150 mM NaCl. For treatment with Li^+ for longer than 1 h, incubation was initially done in DME-10% FBS. Internalization of HRP was determined by incubating cells with the enzyme at 1 mg/ml at 37°C for 20 min. The uptake at 0 h denotes treatments without Li^+. b: Reversibility of the inhibition. Cells previously incubated with 50 mM LiCl in DME-10% FBS for 3 h were chased in the Li^+-free medium for indicated periods. During the last 1 h of the chase, medium was replaced with Na phosphate-free MEM (○) or either in the presence of 16.8 mM Ca^{2+} (●) or 20 nM PMA (■). 0 h indicates incubation in Li^+-containing DME for 2 h, then in Li^+-containing Na phosphate-free MEM for 1 h. Symbols without lines at 0 h indicate the amounts in cells without the Li^+-treatment. The results are averages of at least three experiments. Errors, which were less than 15% of each value, are not indicated.

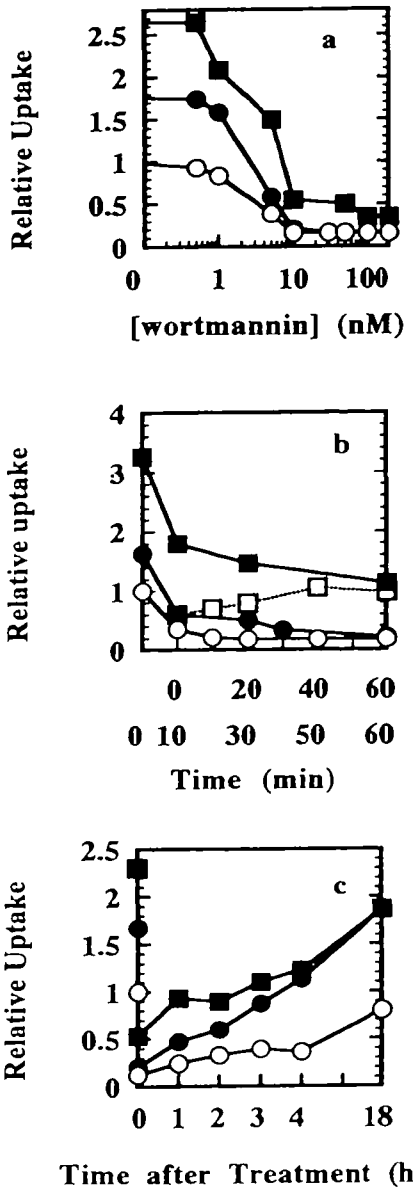

Fig. 2. **Inhibition of endocytosis by wortmannin (WT).** a: Cells were incubated with indicated concentrations of WT for 1 h either in Na phosphate-free MEM (1.8 mM Ca^{2+}, ○), or in the MEM containing 16.8 mM Ca^{2+} (●) 20 nM PMA (■). Endocytosis was then assayed in terms of the uptake of HRP for 20 min. b: Cells preincubated in Na phosphate-free MEM (1.8 mM Ca^{2+}, ○) or in the MEM containing 16.8 mM Ca^{2+} (●) or PMA (20 nM, ■) were treated with 50 nM WT and incubated for the periods indicated in the upper row. They were then assayed for endocytosis by adding HRP (1 mg/ml) for 10 min. The lower row depicts the duration of contact with WT. Open squares with dotted lines denote the internalization of HRP in cells simultaneously treated with PMA and WT. c: Cells were treated with 50 nM WT in DME-10% FBS at 37°C for 1 h. The medium was then replaced with fresh medium lacking WT and incubated for the indicated periods. During the final 1 h, cells were incubated in phosphate-free MEM (1.8 mM Ca^{2+}, ○) or with Ca^{2+} (16. 8 mM, ●) or PMA (20 nM, ■). Cells at the time point of 0 h were directly treated with Ca^{2+} or PMA in WT-containing phosphate-free MEM for 1 h, without subsequent chase. Data points without lines at 0 h indicate internalization without treatment with WT. Endocytosis was assayed for 15 min. The results are averages of at least three experiments. Errors were less than 5% of each value.

Inhibition by WT occurred very rapidly, and more than 60% of the activity was lost when cells made contact with 50 nM WT during the uptake of HRP for 10 min (Fig. 2b). When cells were pretreated with the reagent for 10 min, full inhibition was achieved, suggesting that the reagent inhibited the earliest step. When cells first treated with Ca^{2+} were incubated with WT, the inhibition similarly occurred rapidly. In contrast, when WT was added to cells pretreated with 20 nM PMA for 1 h, the reduction of the full activation by about 65% was significantly lower than the 80% reduction of the activation of WT-treated cells. More inhibition occurred when cells were treated simultaneously with PMA and WT, but the activity then slightly increased. These results suggest that a large part of the PMA-enhanceable process is dependent on a WT-sensitive mechanism, but the cells are able to recruit a certain less sensitive mechanism.

When cells incubated with WT for 1 h were returned to a normal medium, the basal activity was slowly restored. It was 30-40% at 4 h and 80% of the original activity at 18 h

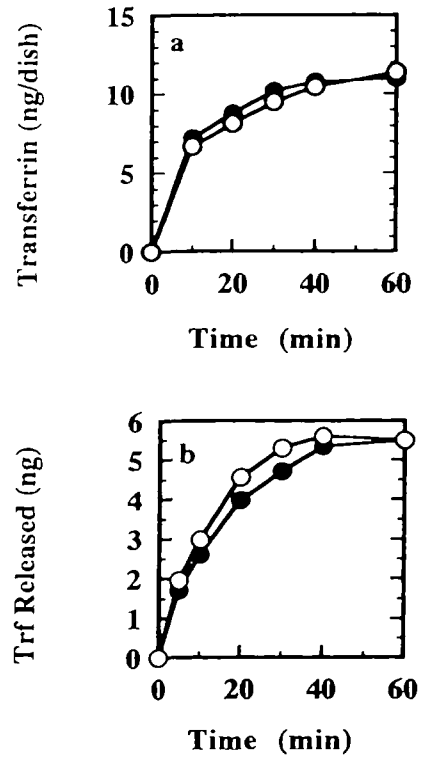

Fig. 3. **Wortmannin does not reduce the internalization (a) or recycling (b) of transferrin.** a: Cells preincubated in a serum-free medium for 1.5 h were further incubated for 30 min in the absence (○) or presence (●) of 50 nM WT. Internalization was assayed by incubating the cells with 0.5 μg/ml Tfn/^{125}I-Tfn in the absence (○) or presence (●) of 50 nM WT at 37°C. At indicated time points, cells were washed with DME and further with 50 mM glycine, 100 mM NaCl (pH 3.5) at 0°C. The cell-associated radioactivity recovered in 0. 1 N NaOH was determined. b: Cells preincubated in a serum-free medium were incubated with 0.1 μg/ml Tfn/^{125}I-Tfn for 1 h and for additional 30 min in the absence (○) or presence (●) of 50 nM WT. After having been washed, they were chased in the presence of non-labeled Tfn at 50 μg/ml. At indicated time points 100 μl of the medium was collected and assayed for radioactivity. The amounts represent the sum of the released protein at the time points. The errors were all within 5% and are not shown.

(Fig. 2c, compare to Fig. 1b). The reagent did not inhibit cell multiplication at up to 750 nM. WT did not induce any sizable change in the amount of the 85 kDa subunit of PI-3k in the post-mitochondrial endomembrane fraction analyzed by western blotting (data not shown). This agrees with the published result (*22*).

Receptor-Mediated Internalization and Recycling of Transferrin Are Not Inhibited by WT and Li$^+$—We tested the effect of WT and Li^+ on the clathrin-dependent receptor-mediated endocytosis. We first used transferrin (Tfn) whose receptor clusters in clathrin-coated pits irrespective of the ligand binding (*25*). In contrast to the effect on the fluid-phase endocytosis, treatment of cells with 50 nM WT for 1 h had no effect on the internalization of ^{125}I-Tfn (Fig. 3a). A similar result was obtained in cells pretreated with 50 mM Li^+ for 3 h (data not shown). When cells pre-loaded with the labeled ligand were chased with excess non-labeled Tfn (50 μg/ml), release of the radioactivity occurred indistinguishably to the control (Fig. 3b). These results indicate that WT has no effect on the internalization and recycling of Tfn.

WT did not change the targeting of TRITC-Tfn. Early endosomes were first labeled with FITC-Tfn (100 μg/ml) and the cells were treated with 50 nM WT for 20 min at 37°C. Inclusion of an excess of non-labeled Tfn (1 mg/ml) inhibited the fluorescence, indicating that the labeling was receptor-dependent. When the cells were chased with TRITC-Tfn (50 μg/ml) for 10 min and subsequently treated with 20 mM methylamine for visualization of FITC-fluorescence, TRITC-fluorescence was almost exclusively colocalized with vesicular FITC-fluorescence regardless of the presence of WT (Fig. 4). Separate experiments on the distribution of the receptor using murine mAb indicated that WT induced no sizable difference (data not shown).

WT Does Not Interfere with Internalization of EGF and PDGF—We next studied the effect of WT on the internalization of epidermal growth factor (EGF), which does not recycle, but is transported to lysosomes. The ligand is also of interest because its binding to the receptor increases the amount of $PI(3,4)P_2$ (*26*). When cells pretreated with 50 nM WT were incubated with ^{125}I-EGF, surface binding and internalization were not affected during the first 20 min (Fig. 5a). In the following 40 min, cell-associated radioactivity rather decreased in the presence of WT. In the control, this kind of change occurred only at around 90 min. A similar result was obtained when cells were pretreated with 50 mM Li^+ for 3 h (data not shown).

Since the reduction of cell-associated radioactivity suggested release of the degraded ligands, we examined the kinetics of release of the TCA-soluble products. Cells were firstly incubated with ^{125}I-EGF at 20°C for 1 h. We did not detect TCA-soluble radioactivity under this condition. After having been treated with 50 nM WT for 20 min and then washed at 0°C, cells were chased in a fresh medium at 37°C. In control cells, TCA-soluble radioactivity was detectable at 40 min and increased linearly (Fig. 5b). Inclusion of 10 mM NH_4Cl, which inhibits lysosomal degradation by elevating the pH there, substantially reduced the release of the radioactivity. When cells were treated with WT, the onset of detection of TCA-soluble radioactivity was again at 40 min. However, the increase of the soluble radioactivity was then enhanced twofold. These results indicate that release of the degraded ligand was enhanced by WT, but this did not change the targeting of endosomes to lysosomes.

We also examined the endocytosis of platelet-derived growth factor (PDGF). This ligand has also been shown to be internalized *via* coated pits and delivered to lysosomes (*27*). The ligand was of particular interest because PI 3-k binds to specific phosphotyrosine-containing sequences in the cytoplasmic tail of the receptor which is induced by binding of the ligand (*28, 29*). When cells pretreated with WT for 1 h were incubated with 5 ng/ml BB homodimer of PDGF, the cell-associated radioactivity was indistinguishable from the control (Fig. 5c). The radioactivity releasable by acid-treatment was also very similar. These results indicate that WT also does not inhibit the binding and internalization of PDGF.

A mAb Directed toward the Vacuolar H$^+$-ATPase Is Internalized Both in Membrane-Bound Form and in the Fluid Phase, and Is Delivered to the Same Early Endosomes—We previously found that incubation of cells with a mAb directed toward the 116(100)-kDa subunit of the vacuolar H^+-ATPase, OSW2, results in its internalization in endosomes (*16*). Since the antibody could be internalized in the surface-bound form and in the fluid phase, we

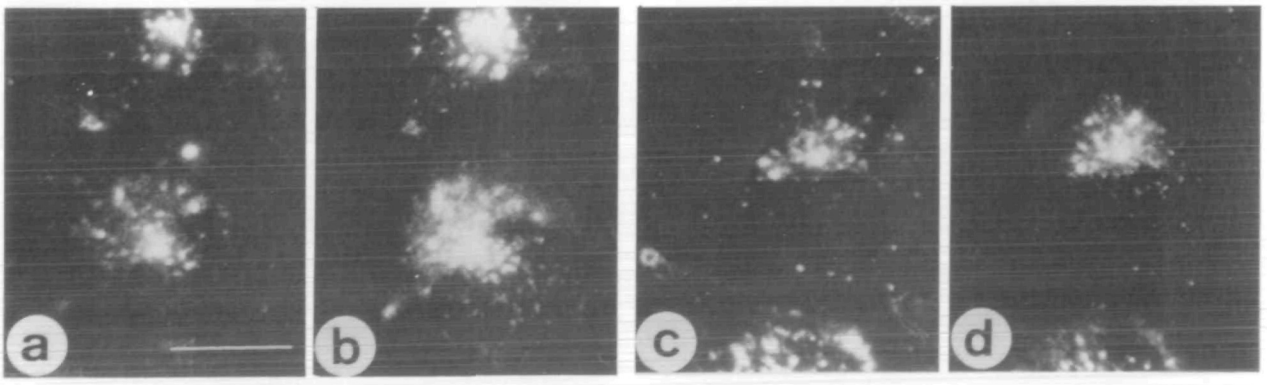

Fig. 4. **Wortmannin does not change the targeting of transferrin.** Cells whose early endosomes were labeled with FITC-Tfn (100 μg/ml) for 1 h were further incubated without (a, b) or with (c, d) 50 nM WT for 20 min. They were washed and chased with TRITC-Tfn (50 μg/ml) in the same media at 25°C for 10 min. They were then washed in a medium containing 10 mM methylamine for the visualization of FITC fluorescence. In both cases, TRITC-Tfn (a, c) was transported to compartments localized with FITC-Tfn (b, c). Bar, 20 μm.

addressed the effect of WT on the internalization of OSW2. When non-treated HT-1080 cells were incubated with OSW2 at 100 μg/ml at 37°C for 10 min, the internalized antibody was localized in compartments with vesicular appearance, as noted in a previous report on other cells (*16*) (Fig. 6a). When the cells were treated with 50 nM WT, immunofluorescence for the internalized antibody was much reduced but still significant (Fig. 6b). In contrast, the difference in the intensity became smaller when OSW2 was added at 3 μg/ml, (Fig. 6, c and d). A separate experiment for quantification of the bound antibody at 0°C using HRP-anti mouse IgG indicated that WT did not change the surface-associated antigen (not shown). These results indicate that OSW2 is internalized both in the surface-bound form and in the fluid phase. The latter mechanism provides a major route at high concentration of the antibody and is inhibited by WT.

In order to assess the identity of the compartments to which the surface-bound and fluid-phase routes were targeted, cells pretreated with WT for 1 h were incubated with 100 μg/ml OSW2 for 10 min. In control cells, most of the compartments associated with the internalized antibody were colocalized with Tfn receptor (Fig. 7, a and b). Some OSW2-immunofluorescence without or with very little Tfn receptor-immunofluorescence was recognizable near the cellular periphery (arrows in Fig. 7b). When cells were treated with WT, almost all the compartments were doubly localized by OSW2 and a mAb against Tfn receptor (Fig. 7, c and d) (no difference in the OSW2-immunofluorescence intensity was demonstrated). These results suggest that the antibody molecules in a surface-bound form and in the fluid phase are transported by different compartments but they are targeted to the same early endosomes.

Electron-Microscopy of WT-Treated Cells—In contrast to Li^+, the rapid effect of WT enabled us to study its effect on the surface internalization in cells which had previously internalized a fluid phase marker by electron microscopy. Cells which had previously internalized HRP (10 mg/ml) in

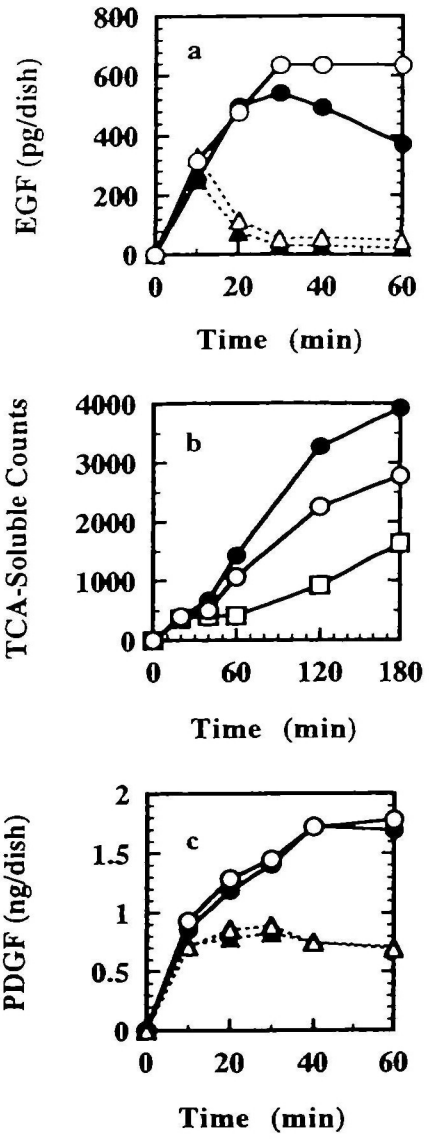

Fig. 5. **Effect of WT on the endocytosis of EGF and PDGF.** a: Cells pretreated with 50 nM WT for 1 h were incubated with 5 ng/ml EGF/^{125}I-EGF at 37°C. At intervals, surface-bound ligand was released by acid-treatment (triangles) and cell-associated radioactivity (circles) was determined by solubilizing cells in 1 N NaOH. Treatment with WT (filled symbols) did not influence the surface-bound amount. b. WT enhanced intracellular degradation of EGF. Cells were incubated with 2 ng/ml EGF/^{125}I-EGF at 20°C for 1 h. After treatment with 50 nM WT in the same medium for 20 min, cells were washed with a fresh medium at 0°C. They were then chased at 37°C in the presence of none (○), 50 nM WT (●), or 10 mM NH_4Cl (□). At indicated time points 100 μl of medium was collected and TCA-soluble radioactivity was determined. c: Cells were treated in the same manner as in a, and incubated with 5 ng/ml PDGF(BB)/^{125}I PDGF(BB). Symbols are the same as in a. Each result varied by less than 8% of the value, so error bars are not shown.

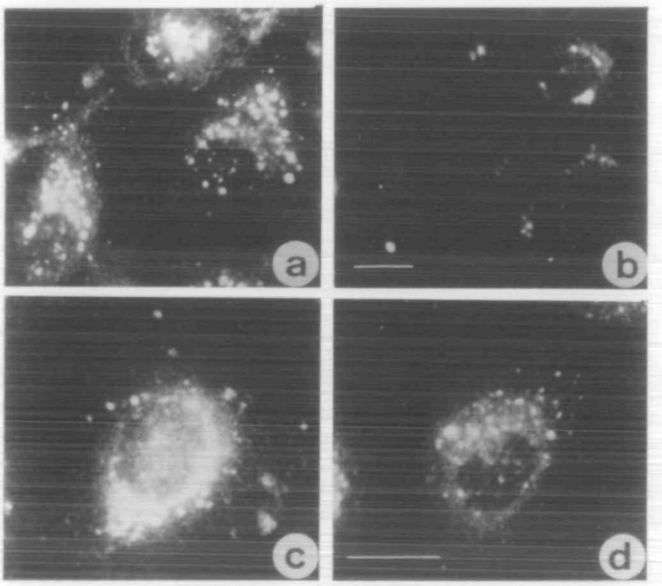

Fig. 6. **Internalization of a monoclonal antibody OSW2 directed toward the 116 kDa subunit of the vacuolar type H^+-ATPase in the absence (a, c) and the presence (b, d) of pretreatment with 50 nM WT for 1 h.** Cells were incubated with 100 μg/ml (a, b) or 3 μg/ml (c, d) OSW2 at 37°C for 20 min and the antibody was localized by immunofluorescence. In a and b, and c and d, respectively, photographs were taken with couples at identical detection sensitivities of the confocal laser scanning microscope to demonstrate the difference in the amount of cell-associated antibody. Bars, 20 μm.

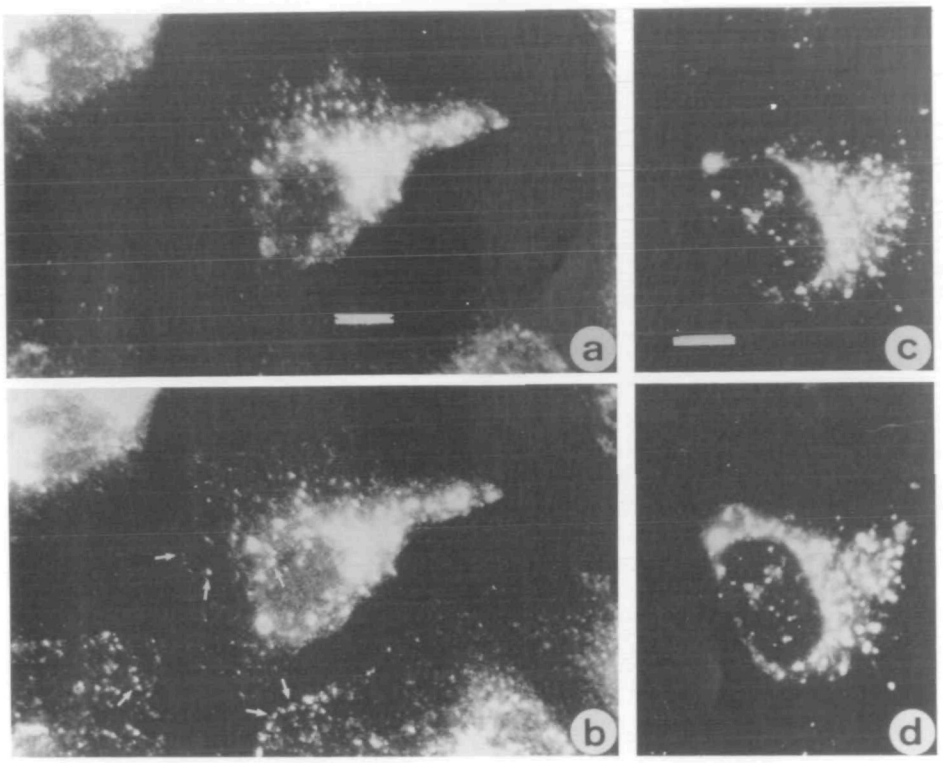

Fig 7 **Most OSW2 was targeted to early endosomes localized by Tfn receptor.** Non-treated cells (a, b) and those pretreated with 50 nM WT for 1 h (c, d) were incubated with 100 μg/ml OSW2 at 35°C for 10 min They were processed for immunofluorescence for human Tfn receptor (a, c) and OSW2 (b, d). Regardless of the treatment with WT, most of the internalized OSW2 was colocalized in compartments associated with Tfn receptor. In control cells, some compartments were associated mainly with OSW2 immunofluorescence (arrows in b) OSW2-immunofluorescence in d, which was as weak as in Fig. 6b, was enhanced to indicate the detail. Bars, 10 μm

the endocytic pool for 30 min were chased in the presence or absence of WT for 20 min and then cationized ferritin (100 μg/ml) was added as a relatively large surface marker for another 20 min. In control cells, HRP was localized mostly in vesicular compartments distributed in the deeper part of the cytoplasm (Fig. 8a). Below the cell surface were localized vesicular compartments that contained ferritin particles. In contrast, only a small number of ferritin paticles was accumulated in vesicles in the presence of WT (Fig. 8b). The ferritin particles usually remained on the cell surface or surrounded by a non-coated membrane at the cell surface. Some particles were observed in coated pits (Fig. 8c). Accumulation of aberrant coated structures was not evident. No effect of WT could be detected on other cell organelles including Golgi apparatus.

Damke *et al.* have employed the small, membrane-impermeant, electron dense stain ruthenium red to demonstrate tubular membranes that continued to the surface of HeLa cells overexpressing GTPase-deficient dynamin (*8*). Electron micrographs of non-treated HT-1080 cells fixed in the presence of ruthenium red showed a smooth surface with a few invaginations (Fig. 9a). The appearance was very similar to that of wild-type HeLa cells (*8*). In cells treated with 50 nM WT for 1 h, we detected connected vesicular invaginations and some tubules that were stained dark by ruthenium red (Fig. 9, b and c). The diameter of vesicular parts was 40 to 120 nm. In contrast, we did not observe the very long tubular membranes or multivesicular bodies that were found in mutant HeLa cells (*8*). We found neither elongated tubular structures nor increased number of multivesicular bodies in conventionally stained specimens (see Fig. 8).

Internalization of HRP in Various Cell Lines is Differently Influenced by WT and by Inhibition of Receptor-Mediated Endocytosis—We next addressed the effect of inhibition of receptor-mediated endocytosis on the fluid-phase uptake (Fig. 10). We treated cells preincubated in a medium containing 25 mM NH_4Cl for 20 min with Na^+-free buffer containing 500 μM amiloride. This procedure inhibits formation of clathrin-coated vesicles, thereby impairing receptor internalization (*15*). In addition to HT-1080, we studied epithelial (KB100) and two fibroblastic (A-549 and a normal human fibroblast, Miyajima) cells. Compared to HT-1080, whose activity was as high as that of hepatocytes, the internalization of HRP per cellular protein was 4.5, 5.5, and 2.5-fold lower in A-549, KB100, and Miyajima cells, respectively. In contrast, all these cells internalized a similar amount of ^{125}I-Tfn after depletion of the intrinsic ligands by incubation in a serum-free medium for 2 h. Internalization of Tfn was inhibited by more than 90% in all the cells when the cytoplasmic pH was lowered

In contrast to the effect of WT, internalization of fluid phase in HT-1080 cells whose cytoplasm was acidified was inhibited by only 30%. In contrast, WT inhibited the internalization in KB100 cells by only 34% while cytoplasmic acidification reduced it by more than 60%. Comparison of four different human cells indicated that cells showing higher sensitivity to WT were less sensitive to the treatment for cytoplasmic acidification. We also examined a non-human epithelial cell line MDCK-II. WT inhibited the endocytosis by only 30% while the treatment for the cytoplasmic acidification resulted in as much as 75% loss. These results strongly suggest that the WT-sensitive bulk-fluid internalization is significant, but is substantially smaller in other cells. When WT-treated cells were loaded with NH_4Cl and chased in the amiloride-containing K^+-buffer, internalization of HRP was lost by 80 and 84% in KB100 and A-549 cells, respectively. The same treatment

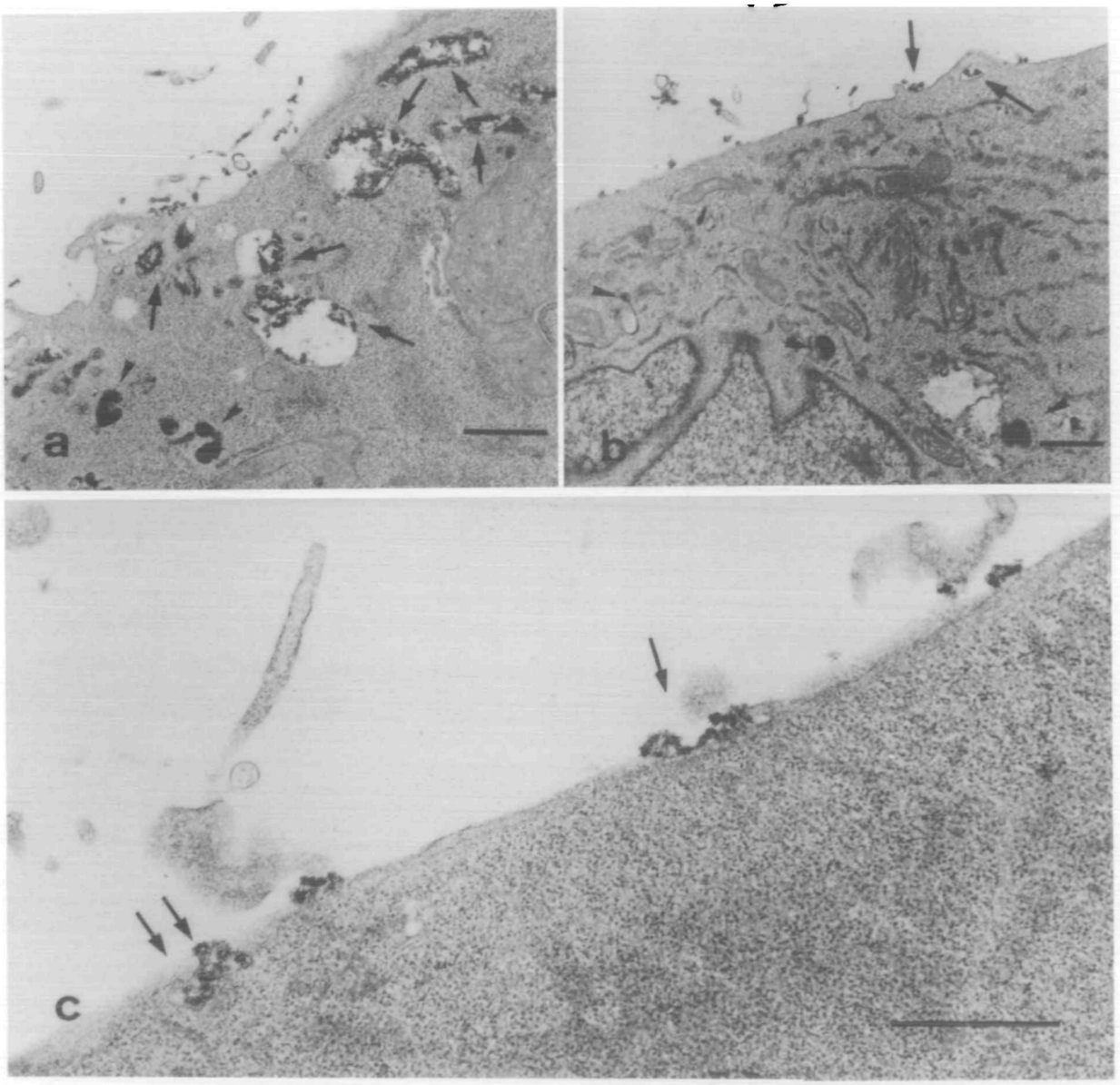

Fig. 8 **Electron micrographs of cells incubated with HRP and subsequently with cationized ferritin in the absence (a) or presence (b and c) of WT.** Cells which had previously internalized HRP (10 mg/ml) for 30 min were chased in the presence or absence of 50 nM WT for 20 min They were then exposed to cationized ferritin (100 μg/ml) for 20 min In a, endosomes were intensely labeled with the HRP (arrowheads) and ferritin particles (arrows). In contrast, treatment with WT resulted in association of ferritin particles only on the cell surface (b, c). Examination at higher magnification (c) indicates the particles on a coated pit-like structure (single arrow) or in a vesicle near the cell surface (double arrows). Bars, 1 μm

induced cellular detaching of HT-1080 and Miyajima cells of more than 60%. The activities in remaining cells, normalized by protein amount, were about 30% of the control in both cases.

DISCUSSION

Internalization of the fluid-phase marker HRP in HT-1080 cells was highly inhibited by Li^+ (Fig. 1). The ion also inhibited the endocytosis activated by Ca^{2+} or PMA, indicating the presence of a control mechanism that is independent of Ca^{2+}-signaling and canonical PI-turnover. We were therefore prompted to test the involvement of non-canonical phosphoinositide (PI) turnover that employs PI 3-kinase (PI 3-k). We found that wortmannin (WT), a potent inhibitor of PI 3-k (*19, 20*), suppressed all three types of endocytosis to the same degree as in the case of Li^+ (Fig. 2). PI 3-k consists of two tightly associating subunits, p85 and p110. The reaction products of PI 3-k, *i.e.*, PI(3)P, PI(3,4)P_2, and PI(3,4,5)P_3, are rapidly dephosphorylated. PI(3,4,5)P_3 is converted to PI(3,4)P_2, then to PI(3)P and ultimately to phosphatidylinositol (*30*). In the presence of WT or Li^+, loss of the supply of one or some of the phosphoinositides probably results in rapid depletion of them by the undisturbed dephosphorylation. Stephens *et al.* have shown that, although WT does not inhibit the

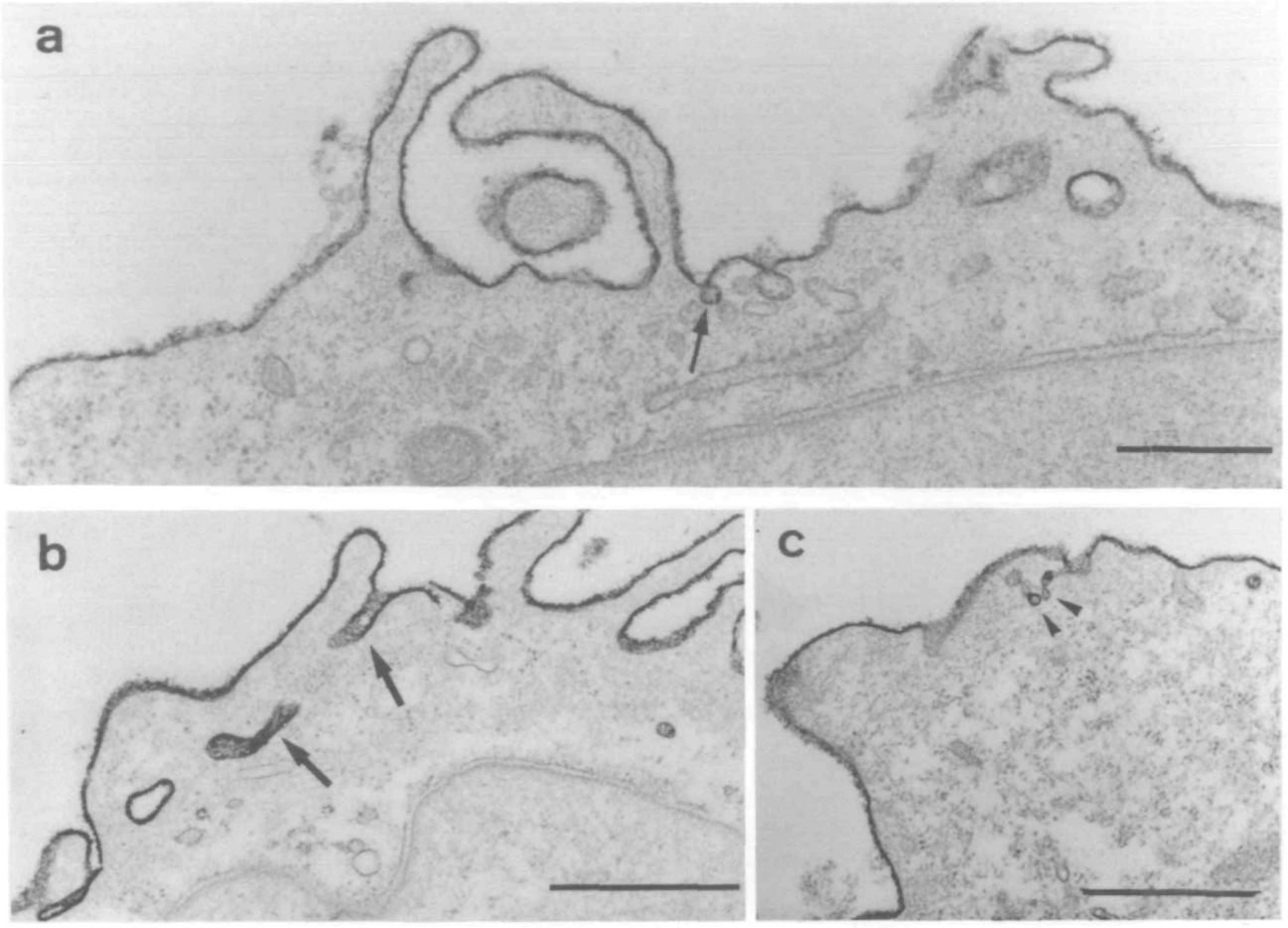

Fig 9. **Electron micrographs of cells fixed and stained with ruthenium red.** The surface of non-treated cells (a) was smooth and only a few vesicular invaginations connected to the cell surface (an arrow) were observed In cells treated with 50 nM WT for 1 h (b, c) tubular invaginations (arrows) and those with connected vesicular appearance (arrowheads) were often found. Bars, 0.5 μm for a and 1 μm for b and c, respectively.

partially purified phosphatidylinositol-specific 3-k, it reduces the amount of PI(3)P in mammalian cells to 10 to 30% (*31*). The concentration dependence is very similar to that in abolishing synthesis of PI(3,4,5)P_2 and is also similar to that for the inhibition of the fluid-phase endocytosis in HT-1080 cells. WT has been shown to inhibit, albeit not fully, phospholipase A_2 in activated neutrophils (*32*). However, the slow effect of Li^+ can be consistently understood in terms of the indirect nature of the inhibition through reducing the regeneration of phosphatidylinositol by dephosphorylation of inositol phosphates (*21*). The present results can be best explained by the involvement of molecule(s) in non-canonical PI-turnover in the bulk-fluid endocytosis in HT-1080 cells.

In contrast to the strong inhibition of fluid-phase uptake, neither WT nor Li^+ had any significant effect on the internalization of protein ligands that are internalized *via* coated pits (*3, 8, 27*) (Figs. 3, 4, and 5). The result of internalization of PDGF is in agreement with that using cells transfected with a panel of receptor mutants (*11*). In HT-1080 cells, fluid-phase endocytosis occurs at the rate of 0.081 μl/h/10^5 cells. WT reduced 80% of the internalization of bulk fluid, while a treatment for inhibiting clathrin-dependent endocytosis reduced the activity by only 35%. On the other hand, comparison of the effects in a variety of cells strongly suggests that less PI 3-k dependent endocytosis occurs in other cells (Fig. 10). In particular, internalization of HRP in KB100 cells was reduced by WT by only 34%, while it was inhibited by more than 60% by acidification of cytoplasm. Interestingly, the fluid-phase endocytosis per protein amount was 5.5- to 2.5-fold higher in HT-1080 than in other cells (*i.e.*, Miyajima, A-549 and KB100) while the magnitude of fluid-phase internalization by the mechanism sensitive to cytoplasmic acidification is only 20 to 30% smaller. If 20% (*i.e.*, uninhibited internalization minus WT-sensitive internalization) of the fluid to be internalized in HT-1080 cells is imported *via* clathrin-coated vesicles in the range of 50–100 nm in internal diameter, the number of internalized clathrin-coated vesicles is estimated to be 4,160–520/min/cell. This estimation is comparable to the experimentally determined value in BHK-21 cells using Semliki Forest virus (3,000/min/cell) (*33*). These results suggest that a large part of the difference in the internalization of the fluid phase among the cells in this study is due to the differential magnitude of the WT-sensitive mechanism.

Without the inhibition by WT, the fluid phase containing a mAb against the 116 kDa subunit of the V-ATPase

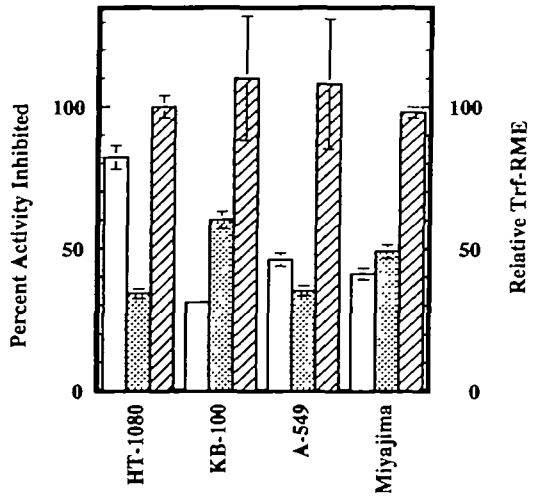

Fig. 10. **Effect of treatment with 50 nM WT (open bars) and that of chase of cells preloaded with 25 mM NH_4Cl in Na^+-free buffer containing 500 μM amiloride (shaded bars) on the internalization of HRP (0.5 mg/ml) at 37°C for 10 min.** The results are expressed as percent of activity lost after the treatments. Cells were also examined for receptor-mediated endocytosis of Tfn/^{125}I-Tfn (Tfn-RME) for 10 min. The results were normalized for the uptake by 8×10^5 cells and are expressed using striped bars as relative activities compared to that of HT-1080.

(OSW2) (*16*) reached endosomes associated with Tfn receptor in 5 to 10 min (Fig. 6). We have also observed that FITC-dextran is similarly transported (*12*). In the absence of WT, some OSW2 was found in small vesicular structures lacking Tfn receptor. These results suggest that, as described in different cells (*4*), the presence of preendosomal intermediate vesicles used for clathrin-independent endocytosis is also the case in HT-1080 cells. Although the vesicles are yet to be characterized *in vitro*, slow recovery from the effect of Li^+ and WT would allow isolation and characterization of vesicles derived from isolated plasma membrane from the treated cells. We are currently addressing the *in vitro* reconstitution of the budding.

In addition to the inhibition of internalization of fluid phase, Li^+ and WT affected the postendocytic transport of EGF. EGF accumulated in cells at 20°C began to be degraded at a similar time point during the chase at 37°C regardless of the treatment with WT (Fig. 5b). In contrast, the degradation thereafter increased in the presence of WT. These results indicate that WT did not change the delivery of the ligand to lysosomes, while the transport of degradation products outside the cell was accelerated. The results are in contrast to the effect of WT on the intracellular transport of PDGF receptor reported by Joly *et al.* (*11*). In that report, a large part of the receptors was exposed to the cell surface even after incubation with the ligand for 90 min in the presence of WT, when the receptors were normal in terms of the PI 3-k binding site. In contrast, treatment with WT slightly reduced the number of mutant receptors that lacked the motif. Although clarification of the reason for the difference between the present result on EGF and that on PDGF receptors awaits further characterization, it is interesting to note that post endocytic traffic of EGF and that of its receptor appear different with respect to the modification of the receptor kinase (*34, 35*).

The present results indicate that WT-sensitive and non-specific fluid-phase endocytosis occurs distinctly from clathrin-dependent and receptor-mediated endocytosis. Although WT has no evident effect on the survival of cells in culture (*22*), the reagent is very toxic to the whole animal (*36*). Because both the clathrin-dependent and -independent endocytosis can be expected to represent non-specific sampling mechanisms, a study on the process involved in internalization of bulk fluid should provide a coherent understanding of the role of fluid-phase internalization in cellular physiology. PI 3-k has been mainly studied in relation to oncogenesis and signal transduction (*22, 23, 37-39*). WT has been mainly employed in relation to signal transduction or cell activation. In contrast, endocytosis is a constitutive process which can be observed in non-stimulated cells. Further study should reveal the functional link through membrane traffic between the resting and stimulated cellular states.

S.B.S. is grateful to the stimulating lecture on "Cell and Information" and encouragement by Dr. Fumio Oosawa, who orginized the research project with the same title. S.B.S. also thanks Dr. Masayuki Otsu for helpful discussions, the PRESTO office of JRDC for generous encouragement and Ms. Yoshiko Iida for her excellent technical help.

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