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ATP and cytosol requirements for transferrin recycling in intact and disrupted MDCK cells

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We have developed an *in vitro* system for studying membrane transport during receptor-mediated endocytosis. Using nitrocellulose disruption to permeabilize selectively the apical domain of filter-grown MDCK cells, the recycling of receptor-bound transferrin (Tfn) from an intracellular pool was reconstituted *in vitro* with a rate and efficiency similar to that of intact cells. Tfn and Tfn receptor recycling from endosomes back to the cell surface was dependent on added ATP and cytosol-derived proteins. Thus, incubation of intact cells under conditions of ATP depletion resulted in the clearance of Tfn receptors from the basolateral membrane, this was reversible upon removal of the energy poisons. Reappearance of previously internalized receptors could also be obtained in disrupted cells but required the addition of both ATP and cytosol to the assay mixture. Similarly, when intact cells were allowed to internalize labeled Tfn prior to disruption, efficient and rapid release of ligand back into the medium was markedly stimulated by ATP and cytosol. Recycling was judged to be both selective and vectorial since only the expected small fraction of a previously internalized horseradish peroxidase was released after addition of ATP and cytosol, and release was primarily into the basal medium. While the cytosol contributed one or more protein factors, none was sensitive to *N*-ethylmaleimide. Alkylation of the disrupted cells, however, did inactivate recycling.

Key words: endocytosis/*in vitro* assay/membrane traffic/receptor recycling/transferrin

Introduction

While the general pathway of receptor-mediated endocytosis is well understood (Kornfeld and Mellman, 1989), there is relatively little information concerning the mechanisms controlling the transport and functions of endocytic organelles. It is clear from studies of intact cells that endocytosis usually begins with the accumulation of receptors at clathrin-coated pits which then pinch off to form coated vesicles. These vesicles rapidly uncoat and fuse with early endosomes whose acidic internal pH facilitates the dissociation of receptor–ligand complexes. Internalized receptors typically recycle back to the plasma membrane while discharged ligands are transferred from early to late endosomes and finally to lysosomes where they are degraded. Not only are the factors governing the formation and interactions of endocytic vesicles unknown, but even their ATP requirements in intact cells remain poorly defined.

It is clear from the study of the biosynthetic pathway that understanding the biochemistry of membrane transport during endocytosis will require the reconstitution of individual steps *in vitro* and, optimally, the development of genetic approaches. The few endocytosis mutants thus far identified have not yielded information similar to that obtained for the yeast secretory pathway. However, there has recently been progress towards analyzing at least some of the events on the endocytic pathway in cell-free systems (Gruenberg and Howell, 1989). For example, the assembly of coated pits and the formation of clathrin cages has been reconstituted *in vitro*, although neither the energy requirements nor the possible involvement of accessory proteins are completely clear (Mahaffey *et al.*, 1989; Moore *et al.*, 1987). Evidence for coated vesicle formation has also been obtained and seems to exhibit at least a partial requirement for energy and cytosol proteins (Smythe *et al.*, 1989). ATP has also been shown to be required for the uncoating of coated vesicles *in vitro* (Rothman and Schmid, 1986).

Although the fusion of uncoated coated vesicles with early endosomes has not yet been accomplished, several groups have reconstituted homotypic fusion between endosomes (Braell, 1987; Davey *et al.*, 1985; Diaz *et al.*, 1989; Gruenberg and Howell, 1986). Endosome–endosome fusion requires ATP and one or more cytosolic factors. Recent evidence also suggests that the mechanism may be related to fusion events on the secretory pathway for which more extensive biochemical and genetic data are available. This work has suggested that one or more steps of biosynthetic membrane traffic involve an *N*-ethylmaleimide (NEM) sensitive factor (NSF) (Block *et al.*, 1988) and *ras*-like GTP-binding proteins (Bacon *et al.*, 1989; Beckers and Balch, 1989; Melançon *et al.*, 1987) such as the sec4 gene product of yeast (Goud *et al.*, 1988; Salminen and Novick, 1987). In at least one cell-free system, endosome fusion—like ER to Golgi or intra-Golgi transport—can be inhibited by GTPγS (Mayorga *et al.*, 1989) and antibodies to NSF (Diaz *et al.*, 1989).

Other steps on the endocytotic pathway remain poorly characterized *in vitro* or have yet to be reconstituted. While fusions between early and late endosomes have not been accomplished, it has been possible to reproduce interactions of late endosomes with heavy density lysosomes (Mullock *et al.*, 1989) and with glycosyl transferase-containing Golgi vesicles (Goda and Pfeffer, 1988). Reconstitution of recycling vesicle fusion with the plasma membrane, the final step on the endocytic pathway, has not yet been achieved. Since any cell-free system must involve the disruption of the plasma membrane, reconstitution of recycling *in vitro* may be compromised by the unavoidable damage to the fusion target. Similar considerations may explain why the formation of coated vesicles in disrupted cell preparations has proved to be inefficient relative to that of unbroken cells (Smythe *et al.*, 1989). In this paper, we have adapted a

method for the 'perforation' of polarized filter-grown Madin–Darby canine kidney (MDCK) cells that limits disruption of the plasma membrane to the apical surface (Simons and Virta, 1987). By following the recycling of receptor-bound transferrin (Tfn) from an intracellular pool to the undamaged basolateral surface, this late step on the endocytotic pathway was reconstituted and its features compared with those of intact cells.

Results

Binding of Tfn at the basolateral surface of MDCK cells

The internalization and recycling of receptor-bound Tfn has been characterized in a variety of cells (Ciechanover *et al.*, 1983; Hopkins and Trowbridge, 1983). The Tfn–receptor complex is internalized via clathrin-coated pits and coated vesicles and then delivered to early endosomes (Dautry-Varsat *et al.*, 1983; Schmid *et al.*, 1988; Van Renswoude *et al.*, 1982) in which the acidic pH causes the discharge of its bound iron. Apo-Tfn remains bound to the receptor and recycles back to the plasma membrane where it is released into the extracellular medium.

In MDCK cells, Tfn receptors are expressed predominantly on the basolateral surface (Fuller and Simons, 1986). Since previous work did not characterize the kinetics of [^{125}I]Tfn endocytosis in this cell line, it was necessary to establish the features of Tfn internalization and recycling prior to using this probe as a marker in a cell-free system. As in earlier studies (Fuller and Simons, 1986), we first used [^{125}I]human Tfn (hTfn) but found that the amount of specific binding at 4°C at the basolateral membrane was too low to permit detailed analysis. The binding of homologous canine Tfn (cTfn) on the other hand, was saturable at much lower concentrations and produced a >10-fold greater signal. Both species of Tfn bound to the same receptors since unlabeled cTfn competed with the binding of [^{125}I]hTfn. However, Scatchard analysis suggested that cTfn bound to a 10-fold greater number of sites ($4.04 \times 10^4 \pm 2.38$ per cell; $K_D = 1.04 \times 10^{-8} \pm 0.38$), >99% of which were restricted to the basolateral surface. Due to the increased signal, we were able to follow a single round of Tfn uptake and recycling.

Kinetics of transferrin internalization and recycling in intact MDCK cells

To determine the kinetics of Tfn internalization and recycling by intact cells, [^{125}I]cTfn was pre-bound at 0°C, unbound Tfn washed away, and the cells warmed up to 37°C for different periods of time. As in many previous studies, internalization was determined from the amount of [^{125}I]cTfn that became resistant to removal by low pH (Ciechanover *et al.*, 1983; Hopkins and Trowbridge, 1983; Klausner *et al.*, 1983); surface-bound Tfn was determined by subtracting acid-resistant [^{125}I]cTfn from total cell associated radioactivity. Since surface-bound [^{125}I]apo-cTfn rapidly exchanged with the excess of unlabeled Tfn included in the assay medium, recycling was detected as the release of radiolabel into the apical and/or basal media. As shown in Figure 1, [^{125}I]cTfn was rapidly internalized, intracellular Tfn reaching a maximum within 2.5 min of warming and accounting for ~60% of the total cell-associated radioactivity. After 2.5 min, there was a decrease in internalized [^{125}I]cTfn accompanied, after a brief lag, by an increase in the amount of [^{125}I]cTfn released (recycled) into the medium. Most (>75%) of this Tfn was released into the basolateral medium with a $t_{1/2}$ of ~10 min. Although somewhat less polarized than shown for a different strain of MDCK cells (Fuller and Simons, 1986), it is clear that cTfn endocytosis and recycling occurs predominantly at the basolateral surface.

ATP is required to complete one transferrin cycle

To determine whether Tfn recycling was sensitive to a decrease in cytosolic ATP, we next measured endocytosis of bound [^{125}I]cTfn in intact cells incubated in energy poisons. Cells were treated for 1 h at 4°C in binding medium containing NaCN and 2-deoxyglucose (dGlc), conditions which reduced ATP levels to ~10% of control. Upon warming to 37°C, cytosolic ATP was further reduced to ~5% of control after 2.5 min and to <1% after 15 min.

After warming to 37°C for 15 min, ~25% of the [^{125}I]cTfn in controls cells (+ ATP) was found in an acid resistant compartment with almost none remaining on the cell surface (accessible to release by low pH) (Figure 2). Most of the remaining Tfn (~70%) was released into the medium presumably due to the recycling of previously internalized ligand (Figure 1). In contrast, in the ATP-depleted cells (−ATP), almost all of the cell-associated radioactivity was intracellular (resistant to removal by low pH) with little radiolabel released into the medium (Figure 2). Thus, while we were unable to demonstrate an ATP

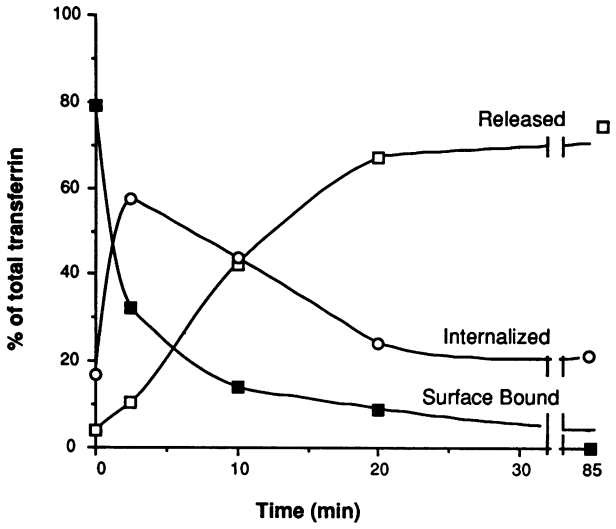

Fig. 1. One cycle of endocytosis and polarized recycling of transferrin in MDCK cells. Confluent filters of MDCK cells were allowed to bind 1 μg/ml [^{125}I]cTfn added to the basolateral compartment for 1 h at 4°C. Unbound radioactivity was removed by washing, and the cells were quickly warmed to 37°C by transferring the plates to a circulating water bath. At the indicated times, the apical and basolateral media were removed and the cells rapidly cooled on ice and rinsed with cold PBS$^+$. One set of two filters was acid washed to remove surface-bound [^{125}I]Tfn, excised from the filter units, and counted to determine internalized (acid-resistant) Tfn. A second set of filters was washed further with PBS$^+$ and used to determine total cell-associated radioactivity. Surface bound Tfn was calculated by subtracting acid-resistant from total cell-associated ^{125}I. Each point was corrected by subtracting non-specific binding as described in Materials and methods, and represents the mean of duplicate filters (<10% variation).

dependence of [^{125}I]cTfn uptake into an acid-resistant compartment, Tfn recycling appeared to be inhibited significantly.

Tfn receptor recycling is ATP-dependent in intact and disrupted cells

If ATP is required for Tfn receptor recycling, maintaining cells under conditions of ATP depletions at 37°C should result in the disappearance of receptors from the basolateral plasma membrane. To test this possibility, monolayers were incubated in medium containing NaCN and dGlc for 1 h at 4°C followed by 15 min at 37°C. Surface receptors were then determined by measuring [^{125}I]cTfn binding at 0°C. As shown in Figure 3A, cells incubated in energy poisons at 37°C (as opposed to 4°C) were devoid of surface Tfn receptors. The receptor loss was reversible, however. Upon removal of NaCN and dGlc from the medium, [^{125}I]cTfn binding activity on the basolateral surface returned to control levels after 15 min at 37°C (Figure 3B). Since preliminary electron microscopy suggests that biotin-labeled cTfn accumulates in endosomes even in ATP-depleted cells, this reappearance presumably reflected the recycling of internalized receptors from one or more populations of endosomes back to the cell surface.

Importantly, the reappearance of Tfn receptor activity also occurred in disrupted cells. As shown in Figure 3B, ATP-depleted cells were first disrupted using nitrocellulose prior to warming to 37°C in ICT buffer containing an ATP-regenerating (+ATP) or ATP-depleting (−ATP) system in the presence or absence of MDCK cell cytosol. Although the kinetics were slower than those of intact cells, up to 50% of the initial [^{125}I]cTfn binding activity was found to reappear on the basolateral surface, but only when the disrupted cells were supplemented with both ATP and cytosol. The amount of receptor reappearance reached a plateau after 30 min at 37°C. No increase was observed if ATP and/or cytosol were omitted, or if the cells were incubated at 4°C. These results suggested that the functional

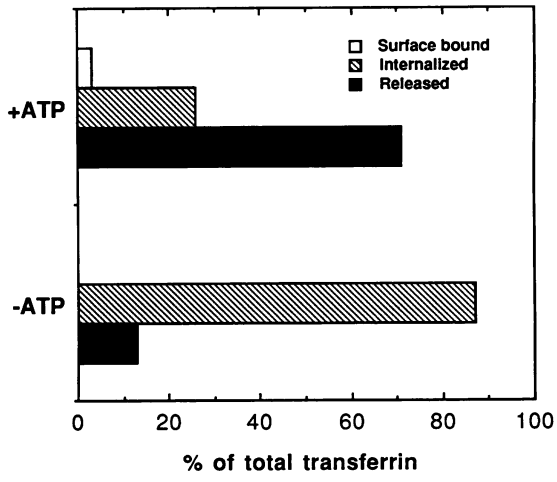

Fig. 2. ATP is required to complete one transferrin cycle. Confluent monolayers of filter-grown MDCK cells were allowed to bind [^{125}I]cTfn (5 μg/ml) in DMEM/BSA/HEPES (+ATP) or in glucose-free medium containing 20 mM dGlc and 10 mM NaCN (−ATP) added to the basolateral compartment for 1 h at 4°C and then washed to remove unbound radioactivity. Washed cells were incubated in the presence or absence of dGlc/NaCN (±ATP) for 15 min at 4°C. The filters were then warmed to 37°C for 15 min and surface-bound, internalized, and released [^{125}I]Tfn determined as in Figure 1. Treatment with energy poisons reduced ATP concentrations to ~10% of control after the 4°C incubation and to <0.5% of control after 15 min at 37°C. In control cells (+ATP), almost 100% of [^{125}I]Tfn was internalized and/or released into the medium (presumably due to recycling of previously internalized ligand). In ATP-depleted cells (−ATP), ~85% of the cell-associated [^{125}I]Tfn was internalized, but only 13% was released into the medium.

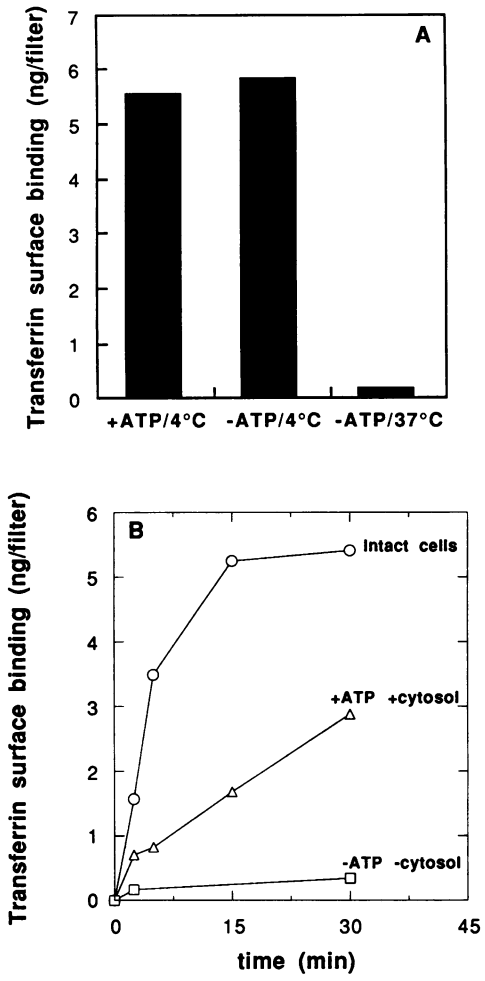

Fig. 3. Recycling of Tfn receptors in intact and disrupted cells: reversibility of the ATP block. (**Panel A**) Effect of ATP depletion on expression of surface Tfn receptors. Cells were incubated in the presence or absence of dGlc/NaCN (±ATP) for 15 min at 4°C or 37°C, washed with cold PBS, and returned to ice. Specific basolateral binding of [^{125}I]Tfn was then determined as above. Incubation in energy poisons at 4°C had no effect on [^{125}I]cTfn binding. However, there was no binding to cells incubated in dGlc/NaCN at 37°C, suggesting that in the absence of ATP, surface receptors were internalized but not recycled back to the plasma membrane. The specific activity was 1.47 × 10^3 c.p.m./ng. (**Panel B**) ATP-dependent re-appearance of Tfn receptors. Cells were incubated in DMEM/BSA for 30 min at 37°C to deplete serum Tfn and then in dGlc/NaCN to deplete cytosolic ATP (as above). After washing with cold PBS, the depleted cells were then re-cultured in DMEM/BSA/glucose at 37°C for 4°C (intact cells). Alternatively, the cells were disrupted using nitrocellulose and incubated in ICT buffer containing an ATP regenerating system and 3 mg/ml cytosol (+ATP +cytosol) or an ATP-deleting system and 6 mg/ml BSA (glucose-hexokinase; −ATP, −cytosol). Specific basolateral binding of [^{125}I]Tfn was then determined at various times as described in Materials and methods. The binding obtained before warming up (0 min) was subtracted from each point and corresponded to 0.96 ng (intact cells) and 0.17 ng (disrupted cells). The specific activity was 3.81 × 10^3 c.p.m./ng.

recycling of previously internalized Tfn receptors could be reconstituted *in vitro*.

[^{125}I]cTfn recycling in intact cells is ATP-dependent

Since reappearance of Tfn receptors in intact and/or disrupted cells was ATP- and cytosol-dependent, it was likely that the recycling of internalized Tfn would exhibit similar characteristics. Accordingly, we developed an assay to determine the release of previously internalized [^{125}I]cTfn to provide a more convenient measure of recycling activity. Conditions were first optimized to pre-load MDCK cell endosomes with labeled ligand. Intact cells were incubated in medium containing [^{125}I]cTfn and the fraction of internalized radiolabel was determined after various periods of time. After 40–60 min, acid-resistant [^{125}I]cTfn reached a plateau corresponding to 4 times the amount of surface-bound Tfn; thus, like other cell types, MDCK cells appear to contain a significant intracellular pool of Tfn receptors (Ajioka and Kaplan, 1986).

To measure recycling selectively, intact cells were first allowed to internalize [^{125}I]cTfn for 1 h at 37°C. The cells were then washed on ice and warmed briefly (2.5 min, 37°C) in medium containing 10 μg/ml unlabeled Tfn to internalize or dissociate remaining surface-bound ligand. Control experiments showed that <1% of the total cell [^{125}I]cTfn remained on the plasma membrane after this incubation (see Figure 1). Finally, the cells were fed with fresh medium containing unlabeled Tfn and incubated for various times at 37°C. As shown in Figure 4A, [^{125}I]cTfn began appearing in the medium without a lag and with a $t_{1/2}$ of ~11 min. The rate of release was linear for the first 10–20 min and then proceeded at a slower rate which was somewhat variable between experiments. Tfn release was mostly (>90%) into the basolateral medium (Figure 4B) and was blocked at 4°C.

The release of pre-internalized Tfn by intact cells was also inhibited by depletion of cytosolic ATP. Cells were loaded with [^{125}I]cTfn as above, washed, and re-cultured at 37°C in the presence of NaCN and dGlc. As shown in Figure 5, incubation in energy poisons decreased by at least three-fold the initial rate and extent of Tfn release into the basal medium. The inhibition was reversed if the NaCN and dGlc were removed within 30 min after addition (not shown).

Tfn recycling in permeabilized cells requires both ATP and cytosol

We next determined whether ATP-dependent Tfn recycling could be reconstituted in disrupted cells. As above, intact MDCK cells were loaded with [^{125}I]cTfn prior to permeabilization. The disrupted cells were then incubated for 1 h at 4°C or 37°C in ICT buffer (Burke and Gerace, 1986) in the presence or absence of ATP and dialyzed MDCK (or CHO) cell cytosol. As shown in Figure 6A, [^{125}I]cTfn was released efficiently from disrupted cells, but only when the reaction mixture was supplemented with ATP and cytosol at 37°C. The rate of release was most rapid for the first 20 min but proceeded at a slower rate for at least 40–60 min. While the slower rate was somewhat variable between experiments, after 1 h, the total amount of [^{125}I]cTfn released accounted for >70% of the internalized ligand. This amount corresponded to 60–80% of the recycling activity exhibited by intact cells over the same time course (e.g., Figures 4A, 5). In the absence of both ATP and cytosol, or in the presence of cytosol without ATP, Tfn release was not significantly different from background ([^{125}I]cTfn release at 4°C), and was 5–7 times less than that observed in the presence of both components. While some activity was stimulated by ATP alone (with 8 mg/ml BSA substituting for cytosol), the rate and extent of [^{125}I]cTfn release were still 3- to 4-fold lower than those obtained using the complete system.

We next compared the initial rates of Tfn release by intact versus disrupted cells in greater detail (Figure 6B). Recycling rates were calculated for the linear period of release and found to be similar for intact and disrupted cells. In intact cells, 1042 Tfn molecules were released/min/cell; in permeabilized cells with ATP and cytosol, 866 Tfn molecules were released/min/cell. Permeabilized cells incubated with

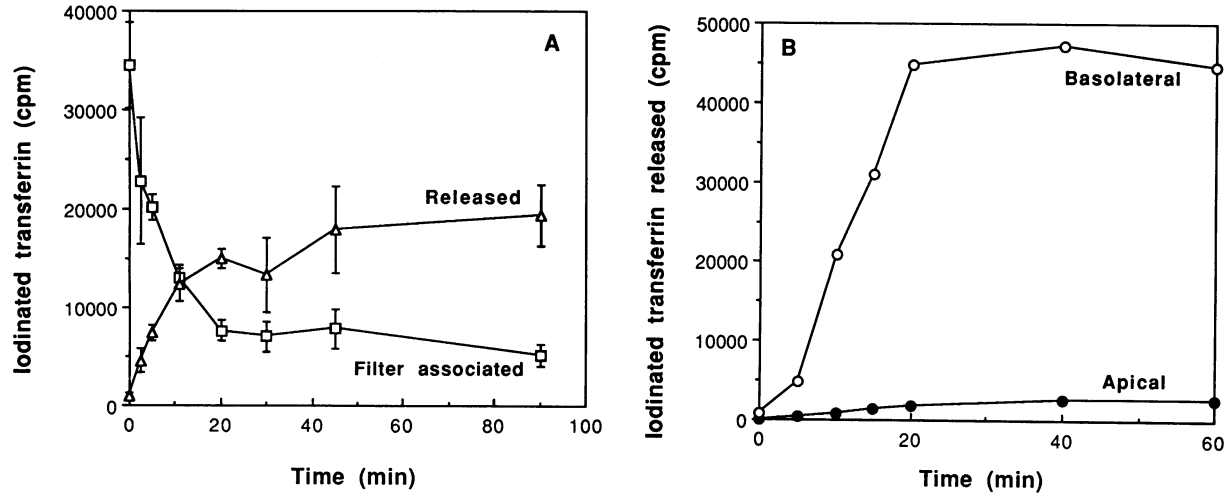

Fig. 4. Recycling of pre-internalized transferrin in intact cells. **(Panel A)** Filter grown MDCK cells were allowed to internalize 12 μg/ml [^{125}I]cTfn in the basolateral medium for 1 h at 37°C. The filters were washed at 4°C in PBS, and then chased in medium containing 12 μg/ml unlabeled Tfn at 37°C for the indicated periods of time. The medium was then removed and the cells washed with ice cold PBS. Cell-associated and released [^{125}I]Tfn were determined in a γ-counter. The means of specific radioactivities from triplicate filters are shown. **(Panel B)** The release of [^{125}I]Tfn into the apical or basolateral compartments was quantified from 100 μl aliquots collected at different times and corrected to the total volume. The total amount of [^{125}I]Tfn internalized prior to the chase in medium containing unlabeled Tfn was 5.4 × 10^4 c.p.m. Non-specific uptake, determined in the presence of a 100-fold excess of unlabeled Tfn, was 3.6 × 10^3 c.p.m. Panels A and B were derived from different experiments.

an ATP depleting system had a rate of only <120 Tfn molecules released/min/cell. Thus, during the first 20 min both intact and disrupted cells released 40–60% of their initial internal pool of Tfn, indicating that recycling activity proceeded with comparable efficiencies both with and without nitrocellulose disruption.

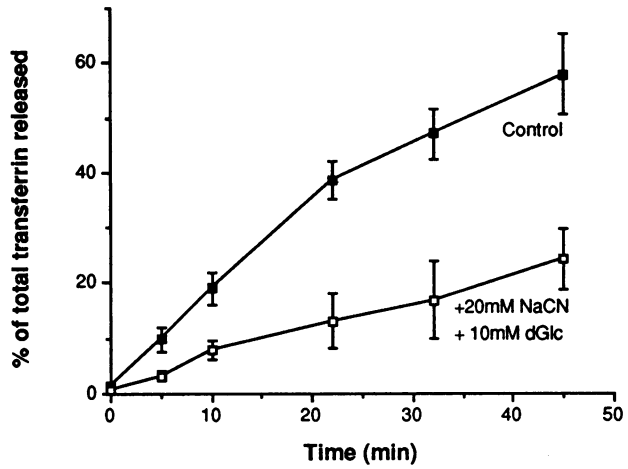

Fig. 5. Transferrin recycling in intact cells requires ATP. Cells were allowed to internalize 5 μg/ml [^{125}I]Tfn for 1 h at 37°C, washed in PBS, and then incubated an additional 2.5 min at 37°C in the presence of 10 μg/ml unlabeled Tfn to allow remaining surface-bound [^{125}I]Tfn to be internalized or to dissociate. The cells were washed again in PBS and then incubated for the indicated periods of time at 37°C in the presence or absence of dGlc/NaCN (as above). At each time point, the amount of radiolabel released into the medium was determined. Each point represents the mean of four filters (counted separately) and is expressed as the percentage of cell-associated [^{125}I]Tfn at the beginning of the final chase period (total radioactivity was 6849 ± 1284 for control cells, 5129 ± 365 for ATP-depleted cells).

Several observations further demonstrated that the ATP- and cytosol-dependent release of [^{125}I]cTfn was primarily due to activity exhibited by disrupted cells. Disruption routinely permeabilized 80–90% of the cells to small molecules (trypan blue, propidium iodide) and antibodies (anti-myosin, anti-clathrin) while [^{125}I]cTfn release was up to 80% of that of intact cell controls. Moreover, the ability of intact cells to recycle [^{125}I]cTfn in the high K^+ ('ICT') buffer used after disruption was reduced to ~40% of that of intact cell controls in growth medium or PBS (not shown). Thus, even 20% non-disrupted cells would contribute only 8–10% of the signal observed in ICT buffer.

[^{125}I]cTfn released to the media was TCA-precipitable, protease sensitive, and migrated with authentic Tfn on SDS–PAGE. The radiolabel also remained in the supernatant after centrifugation at 250 000 × *g* for 2 h, indicating that the released radioactivity was not contained within membrane vesicles, as would be expected if recycling vesicles fused with the basolateral plasma membrane. Low pH stripping of disrupted cells incubated in the presence or absence of ATP and cytosol did not elute additional [^{125}I]cTfn (not shown).

Selective retention and recycling of fluid phase markers in intact and disrupted cells

The observations that ATP and cytosol could stimulate the release of [^{125}I]cTfn as well as the reappearance of internalized Tfn receptors strongly suggested that recycling activity *in vitro* reflected receptor recycling in intact cells. To provide further evidence, we next examined the behavior of a second marker for endocytosis and recycling after nitrocellulose disruption.

Bomsel *et al.* (1989) have recently shown that a fraction of the fluid phase marker horseradish peroxidase (HRP) is

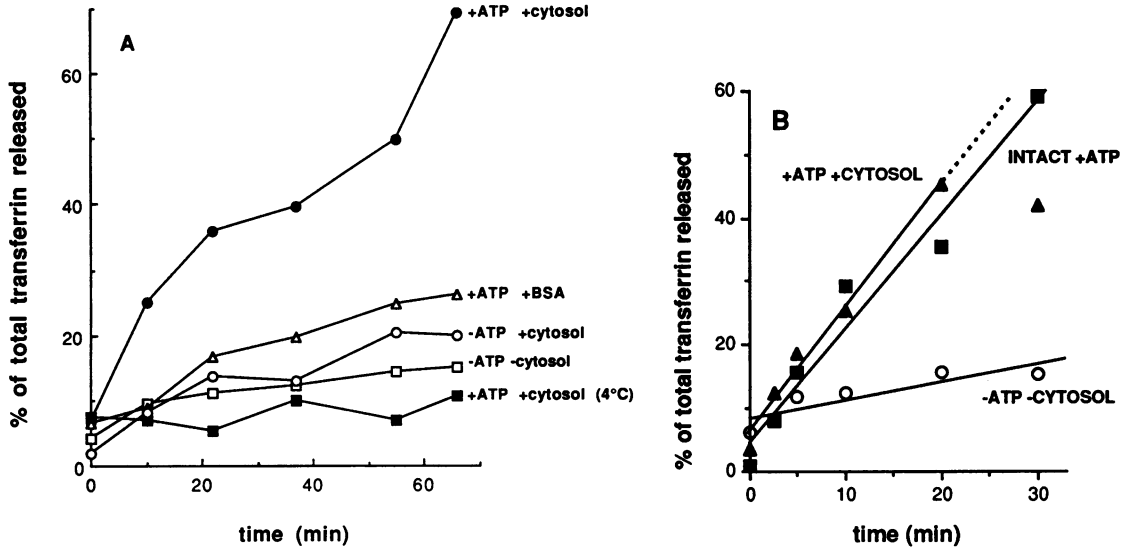

Fig. 6. Transferrin recycling in disrupted cells is dependent on ATP and cytosol. Intact cells were allowed to internalize 2.5 μg/ml [^{125}I]cTfn from the basolateral compartment for 1 h at 37°C, washed, and then incubated for an additional 2.5 min in medium containing 10 μg/ml unlabeled Tfn to deplete remaining surface-bound [^{125}I]cTfn. After washing with cold PBS, the cells were disrupted using nitrocellulose (see Materials and methods). In Panel A, but not in panel B, the disrupted cells were washed at 4°C in 150 mM KCl/50 mM HEPES/KOH (pH 7) for 30 min to further remove remaining cytosol. The filters were cut in halves or quarters and incubated for 15 min at 4°C under the indicated conditions: +ATP = 1 mM ATP, 8 mM creatine phosphate and 32 IU/ml creatine phosphokinase; +cytosol = 4 mg/ml (Panel A) and 3 mg/ml (Panel B) dialyzed CHO cytosol; +BSA = 8 mg/ml; −ATP = 25 U/ml hexokinase, 5 mM glucose. In **Panel A**, the release of [^{125}I]cTfn into the media was quantified from 50 μl aliquots collected at different times and corrected to the total remaining volume. The total amount of [^{125}I]cTfn/filter internalized prior to disruption was ~3 × 10^4 c.p.m. Non-specific uptake, determined in the presence of a 200-fold excess of unlabeled Tfn, was 1.2 × 10^3 c.p.m./filter. In **Panel B**, the initial rate of [^{125}I]cTfn release from disrupted cells incubated in the presence (▲) or absence (○) of ATP and cytosol was compared with the time course of release from intact cells (■). All points represent the mean of duplicate or triplicate filters and varied by <10%.

recycled back to the medium after basolateral endocytosis by MDCK cells. Unlike Tfn, however, the bulk of internalized HRP is sorted from the recycling pathway and retained intracellularly. While HRP is thus a less efficient marker of recycling activity than Tfn, it provides an internal control for endosome lysis after disruption. To determine whether HRP retention and recycling could be reconstituted *in vitro*, MDCK cells were allowed to take up HRP from the basal medium (10 min, 37°C), washed in the cold, and then re-cultured with or without prior nitrocellulose disruption. In both intact and disrupted cells, most (80–90%) of the internalized HRP remained cell-associated even after incubation for 1 h at 37°C. Thus, incubation of disrupted cells in the presence of ATP and cytosol did not result in the efficient lysis of HRP- (and presumably Tfn-) containing endosomes.

The fraction of HRP released by the disrupted cells at 37°C, however, was slightly stimulated by the addition of both ATP and cytosol. Ten percent of the initial cell-associated HRP was released after 30 min whereas only 4% was released from disrupted cells incubated in the absence of ATP and cytosol (Figure 7); no HRP appeared in the medium of cells re-cultured at 4°C. Although ATP and cytosol stimulated HRP release by two- to three-fold, the disrupted cells were still only about half as efficient as intact cells over the same time course (Figure 7). This is in contrast to Tfn release which occurred at comparable efficiencies in intact and disrupted cells (Figure 6B). Since the amount of signal due to HRP was relatively low, however, it is difficult to interpret the significance of the quantitative difference between the two markers.

That the small amount of HRP release reflected physiological recycling activity, however, was supported by the fact that it was released in a polarized fashion. In intact cells, 85% of the total HRP released was basolateral after 2.5 min, 72% after 10 min and 60% after 20 min at 37°C. Similarly, in permeabilized cells, 76% of total HRP released was in the basal medium after 20 min. The vectorial release of HRP suggests that the disrupted monolayers represent at least a

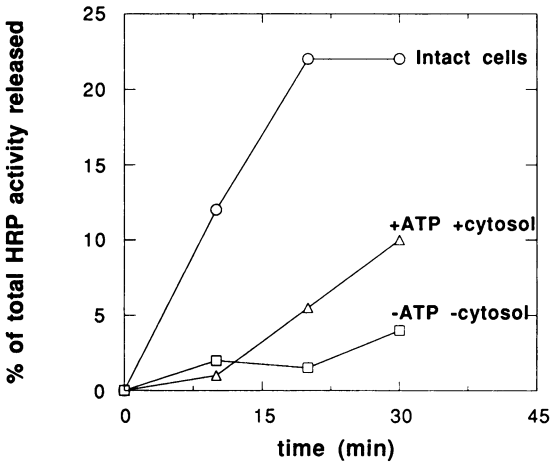

Fig. 7. Recycling of a fluid phase marker from early endosomes *in vivo* and *in vitro*. Horseradish peroxidase (HRP, 10 mg/ml in DMEM/BSA containing 20 mM HEPES) was internalized from the basolateral side for 10 min at 37°C or 4°C. Filters were then placed on a shaker on ice in a cold room and washed with six changes of PBS containing 50 mg/ml BSA over 30 min. After washing, the cells were either permeabilized using nitrocellulose or left intact and re-cultured in ICT buffer or medium at 37°C or 4°C. At the indicated times, the incubation media were collected and the cells washed with cold PBS. HRP released into either the apical or basolateral medium was determined, as was the remaining cell-associated HRP (following solubilization of the cells in 1% Triton X-100) as described (Bomsel *et al.*, 1989). The total HRP internalized at 37°C prior to the chase period (100%) was equivalent to 4 nl/filter; <5% of this amount was found associated with cells initially incubated with HRP at 4°C. The HRP activity in the media before warming up (0 min) was subtracted from each point and corresponded to ~2.5%. Each point is the mean of two filters which varied by <20%.

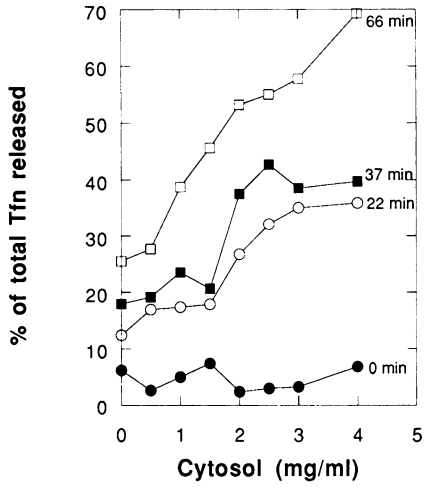

Fig. 8. Cytosol dependence of transferrin recycling *in vitro*. Cells were allowed to internalize [^{125}I]cTfn (2.5 μg/ml) from the basolateral medium for 1 h at 37°C and permeabilized using nitrocellulose as in Figure 6A. The disrupted cells were washed in KCl and then incubated for 15 min at 4°C in the presence of the indicated concentrations of dialyzed CHO cytosol in the presence of an ATP regenerating system. The filters were then warmed to 37°C for 66 min and the fraction of [^{125}I]cTfn released into the medium was determined by taking 50 μl aliquots at different times. Each point is the mean of two filters which varied by <10%; non-specific binding was subtracted as in Figure 6A.

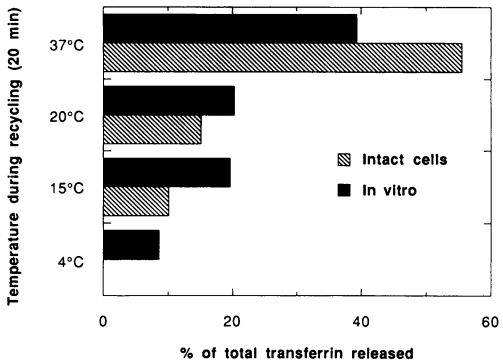

Fig. 9. Temperature dependence of tranferrin recycling in intact and disrupted cells. After pre-internalization of [^{125}I]cTfn (5 μg/ml) for 90 min at 37°C, the cells were washed and processed as above to remove remaining surface-bound [^{125}I]cTfn. One set of cells was then returned to culture at the indicated temperatures. A second set of cells were first disrupted with nitrocellulose, and returned to culture in ICT buffer containing 3 mg/ml dialyzed CHO cytosol and an ATP-regenerating system. Filters were harvested after 20 min, and the fraction of [^{125}I]cTfn released into the medium determined. The amount of [^{125}I]cTfn initially cell-associated was ~6 × 10^3 c.p.m. Each bar is the mean of two filters (<10% variation); ~10% non-specific binding was routinely subtracted.

partial barrier to diffusion, possibly because many intracellular tight junctions remain intact (Simons and Virta, 1987). More importantly, these results further indicate that HRP release reflects basolateral recycling as opposed to endosome lysis.

Requirements of transferrin recycling in vitro

We next characterized some of the basic features of recycling in disrupted cells. First, the cytosol requirement for [^{125}I]cTfn release in the presence of ATP was found to be concentration-dependent. Maximum activity was obtained with between 3–4 mg protein/ml of dialyzed CHO cell cytosol, whether [^{125}I]cTfn release was determined after brief or long incubations at 37°C (Figure 8). Cytosol prepared from CHO cells was as effective as MDCK cell cytosol. Cytosol from the yeast *Saccharomyces cerevisiae* could substitute for MDCK or CHO cell cytosol, as for *in vitro* transport using mammalian Golgi membranes (Wilson *et al.*, 1989). While 80% as efficient, higher concentrations of yeast cytosol (>8 mg/ml) were required. The temperature-dependence of Tfn recycling was next found to be similar in both intact and disrupted cells. As shown in Figure 9, the release of [^{125}I]cTfn pre-internalized at 37°C was similarly reduced at 20°C and 15°C.

Thus, disrupted cells released free [^{125}I]cTfn into the medium in an ATP- and cytosol-dependent fashion and returned internalized Tfn receptors to the plasma membrane. Since the efficiency, kinetics, polarity, energy- and temperature-dependence of Tfn and HRP release and reappearance of Tfn receptors were similar in disrupted and intact MDCK cells, it is likely that [^{125}I]cTfn release by disrupted cells reflected the *in vitro* fusion of recycling vesicles with the basolateral plasma membrane.

Both cytosol and membrane protein components are required for Tfn recycling in vitro

To determine whether cytosol-derived or membrane associated protein components were needed for recycling *in vitro*, disrupted cells were treated with various agents prior to assaying the release of pre-internalized [^{125}I]cTfn. To ensure accurate estimates, inhibition was determined from the effect of each agent or treatment on the linear initial rate of [^{125}I]cTfn release. These results are summarized in Figure 10A which illustrates the percentage inhibition of cytosol-dependent Tfn release due to each treatment. Thus, by definition, the complete system (+ATP +cytosol) resulted in 0% inhibition (1.42% of total Tfn released/min; lane a) while omission of cytosol resulted in a 100% inhibition (this corresponded to 0.29% Tfn released/min; lane b). As shown earlier, non-specific protein did not

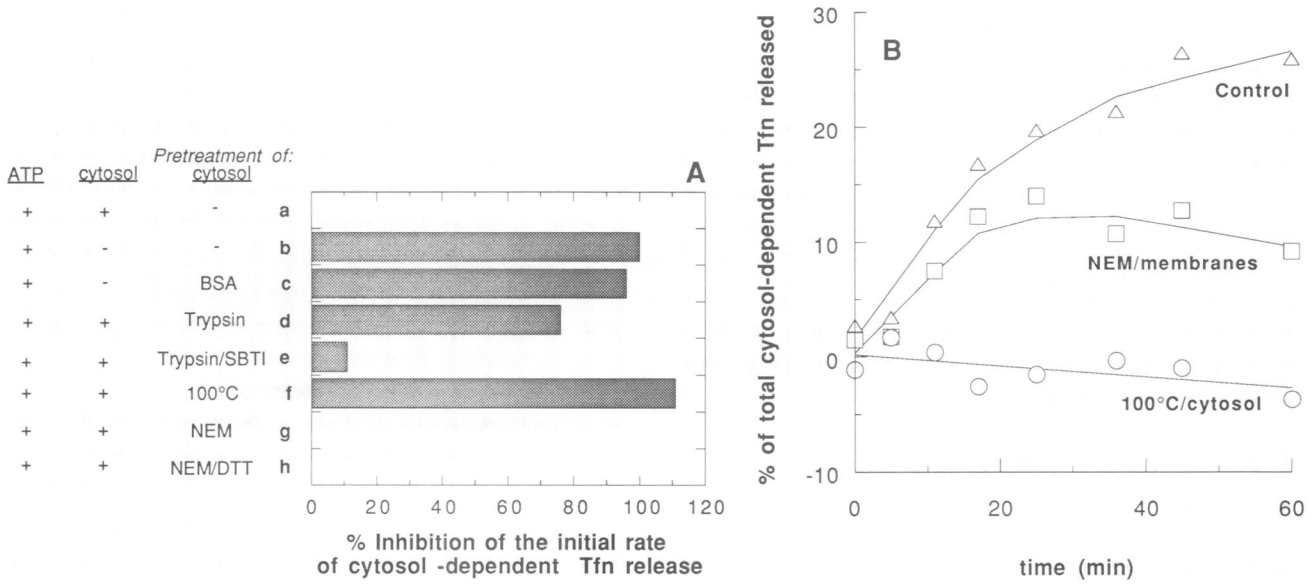

Fig. 10. Inhibition of transferrin recycling by inactivation of cytosolic or membrane components. Intact cells were allowed to internalize [^{125}I]Tfn for 90 min at 37°C and processed for nitrocellulose disruption as in Figure 6A. The permeabilized cells were washed with 150 mM KCl/50 mM HEPES–KOH pH 7 for 30 min before the incubations in ICT buffer with ATP and treated cytosol to wash away most of the cytosol. Cells were then warmed to 37°C for 1 h in the presence of an ATP-regenerating system in the presence or absence of cytosol and aliquots were collected after 0, 5, 11, 17, 25, 36, 45 and 60 min. Before warming, the cytosol or membranes (i.e. disrupted cells) were incubated or pre-treated as indicated: **Panel A** (a) no pre-treatment of cytosol (3 mg/ml); (b) no cytosol added; (c) BSA (6 mg/ml) added instead of cytosol; (d) cytosol incubated with trypsin 100 μg/ml for 30 min at 37°C and then inactivated with 1 mg/ml soybean trypsin inhibitor (SBTI) prior to use; (e) cytosol incubated simultaneously with trypsin/SBTI; (f) cytosol heated at 100°C for 30 min and then centrifuged to remove precipitate (15 000 × *g*); (g) cytosol incubated with 1 mM *N*-ethylmaleimide (NEM) for 15 min at 4°C and then quenched with 2 mM dithiothreitol (DTT); (h) cytosol pre-incubated for 15 min at 4°C with 1 mM NEM in the presence of 2 mM DTT. Data are presented as the percentage inhibition of the initial rate of cytosol-dependent [^{125}I]cTfn release. This was calculated by defining the initial rate of [^{125}I]cTfn release in the presence of ATP and cytosol as 0% inhibition (a). Accordingly, the initial rate of [^{125}I]cTfn release obtained in the absence of added cytosol was thus defined as 100% inhibition (b). All other values were determined relative to these two numbers. The slope for (b) was subtracted from all the other values. **Panel B** illustrates the kinetics of inhibition due to NEM treatment of the disrupted cells. Disrupted cells were treated in the cold with NEM and quenched with DTT as in Panel A. The treated cells were then incubated at 37°C in ATP and cytosol for the indicated times. Although the initial rate of [^{125}I]cTfn release was similar to untreated controls, after 15–20 min release was completely inhibited. In contrast, even at early times after warm-up, no [^{125}I]cTfn release was observed in cells incubated with ATP in the presence of heat inactivated (100°C) cytosol. Data are presented as the percent of total cytosol-dependent Tfn release. This was determined by subtracting from each value the release obtained in the absence of added cytosol.

substitute for cytosol; thus addition of BSA in the absence of cytosol also resulted in ~100% inhibition (lane c). At least one of the required cytosol components must be a protein since a treatment with trypsin (100 μg/ml) inhibited cytosol-dependent recycling by 84% (lane d). The effect of trypsin was blocked by simultaneous incubation with excess soybean trypsin inhibitor (SBTI; lane e). The cytosol-derived components were also completely inactivated by heating to 100°C for 30 min (lane f).

Pre-treatment of cytosol with NEM (0.1–3.0 mM, 15 min, 4°C) had no effect on the initial rate of [^{125}I]cTfn release (lanes g and h) and gave only a slight inhibition of recycling after 60 min (not shown). This was in contrast to the inhibition obtained when the membranes were pre-treated with NEM. The inhibition was irreversible and could not be overcome by the addition of untreated cytosol (prepared with or without ATP), suggesting that the inactivated components) were not present as soluble cytosolic factors. Interestingly, the NEM-mediated inhibition of the membranes did not affect the initial rate of [^{125}I]cTfn release, but completely blocked the subsequent slower rate of release (Figure 10B). These inhibition kinetics suggest that Tfn recycling involves two reactions, one NEM-insensitive the other NEM-sensitive.

Discussion

In spite of considerable interest in establishing *in vitro* approaches to studying endocytosis, it has been difficult to reconstitute efficiently events other than fusion among endosomes (Gruenberg *et al.*, 1989). Part of the difficulty may reflect the fact that many transport steps during endocytosis involve membrane fission or fusion at the plasma membrane, the one organelle which must be disrupted in any *in vitro* system. In this paper, we show that disrupted MDCK cells (Simons and Virta, 1987) provide an efficient means to reconstitute one or more late steps during receptor-mediated endocytosis. Probably because disruption was limited to the apical plasma membrane, recycling of Tfn at the undamaged basolateral surface occurred at efficiencies approaching that of intact cells.

Features of the Tfn recycling assay

Since accumulation of Tfn into an acid-resistant compartment was not blocked by ATP depletion, further study of internalization in disrupted MDCK cells was not pursued. We therefore concentrated on characterizing the recycling of Tfn and Tfn receptors from early endosomes back to the plasma membrane, a step which was dependent on added ATP and cytosol. Recycling was selectively monitored by using cells that had been allowed to internalize ligand at 37°C prior to disruption. Thus, by definition, at the start of each experiment Tfn was localized to the early endosomes that comprise the constitutive pathway of Tfn recycling in MDCK cells. It is likely that these endosomes are located in the basolateral cytoplasm (Hughson and Hopkins, 1990; Parton *et al.*, 1989). Recycling was conveniently monitored by the release of labeled Tfn into the medium, since the inclusion of excess unlabeled, holo-Tfn in the medium ensured complete dissociation of [^{125}I]cTfn from surface receptors without further treatment.

Several lines of evidence strongly suggest that Tfn release reflected the fusion of recycling vesicles with the basolateral plasma membrane and not rupture of Tfn-containing endosomes: (i) not just Tfn, but previously internalized Tfn receptor could be returned to the cell surface in disrupted cells in an ATP- and cytosol-dependent fashion. The functional reappearance of receptor in the membrane was most likely to have occurred as a consequence of a fusion event between recycling vesicles and the basolateral surface. (ii) A fraction of HRP internalized at the basolateral surface was also found to be released by the disrupted cells in an ATP- and cytosol-dependent fashion (Bomsel *et al.*, 1989). The bulk (85–90%) of the HRP remained cell-associated, indicating that there was not extensive endosome lysis and that the HRP was effectively sorted from internalized Tfn, most of which (up to 70%) was released. Furthermore, most of the recycled HRP was vectorially released into the basal medium, consistent with the selective fusion of recycling vesicles with the basolateral plasma membrane. Vesicle lysis would have been expected to release HRP preferentially into the apical medium. (iii) Only free Tfn was released, not Tfn within membrane vesicles. This distinguishes the behavior of Tfn-containing recycling vesicles from transport vesicles derived from the *trans*-Golgi network (TGN). Under the same conditions, TGN-derived vesicles containing newly synthesized membrane proteins are efficiently released into the medium (Bennett *et al.*, 1988). Presumptive transcytotic transport vesicles containing [^{125}I]IgA internalized at the basolateral surface also appear to be released from disrupted MDCK cells transfected with the polymeric Ig receptor (W.Hunziker, unpublished results). Thus, Tfn recycling vesicles are constrained from escaping from the disrupted cells. (iv) The facts that Tfn release was dependent on time and temperature, and occurred with an initial rate and efficiency similar to that exhibited by intact cells argues that both the *in vitro* and intact cell assays measure similar processes. It is further unlikely that vesicle lysis contributes appreciably to [^{125}I]cTfn release since even crude preparations of Tfn-containing endosomes are well known to remain stable and capable of ATP-dependent acidification for hours after isolation (Fuchs *et al.*, 1989; Schmid *et al.*, 1988).

Requirements for Tfn recycling

An important feature of these experiments is that it was possible to correlate at least one key requirement for Tfn recycling *in vitro* and *in vivo*. Thus, in both intact and disrupted cells, we were able to show that Tfn and Tfn receptor recycling was dependent on added ATP. *In vitro*, other XTP's did not substitute for ATP, nor did the non-hydrolyzable ATP analogs AMP-PNP, or ATPγS (data not shown). Unlike many other cell-free systems for membrane transport, the ATP block *in vitro* was found to be reversible (as in intact cells). ATP could be depleted from the complete incubation mixture using hexokinase and glucose at 37°C and recycling activity could be restored upon addition of ATP. In the presence of GTPγS (5–50 μM), recycling activity was slightly stimulated, particularly at sub-optimal ATP concentrations (<0.5 mM).

As found for other transport or fusion asssays of endocytotic or biosynthetic vesicles, Tfn recycling was also found to exhibit a requirement for one or more cytosol-derived proteins. These were found to be sensitive to both heat and protease treatment, and their activity was strictly concentration-dependent and saturable. In addition, these proteins appeared to be highly conserved in that cytosol from diverse

sources was found to support recycling activity. Unlike several other transport assays, however, Tfn recycling was not dependent on a pool of cytosol-derived NSF. The role of NSF has been extensively characterized for transport within the Golgi, where this protein—homologous to the yeast *sec18* gene product (Wilson *et al.*, 1989)—is involved in the fusion of transport vesicles with their target membranes (Block *et al.*, 1988). Under these conditions, NSF is usually a required soluble component of the added cytosol. For Tfn recycling *in vitro*, NEM treatment of the cytosol under conditions known to inactivate NSF activity exhibited little if any effect. In contrast, treatment of the disrupted cells with NEM resulted in an inhibition of recycling. While the membrane-associated targets of NEM are unknown, at least one may be immunologically related to NSF. In our preliminary results, inclusion of a monoclonal anti-NSF IgM found previously to inhibit membrane transport within the Golgi, between ER and Golgi, and between isolated endosomes (Beckers and Balch, 1989; Diaz *et al.*, 1989), was also shown to inhibit almost completely the initial rate of release of [^{125}I]cTfn from disrupted cells. It will be necessary to demonstrate that the inhibition can be reversed by excess NSF protein before concluding an involvement of NSF in Tfn recycling, however.

An interesting aspect of the kinetics of NEM inhibition is the suggestion that the mechanism of Tfn release involves NEM-sensitive and -insensitive steps. This observation indicates either that recycling reflects two parallel reactions or that recycling endosomes lie on two sides of a single block. Only Tfn-containing vesicles that have not yet progressed through the NEM-sensitive step at the time of disruption are susceptible to inhibition. The two steps also correlate with the apparent biphasic kinetics of Tfn recycling *in vitro*. The initial rate is linear for 20 min and is highly dependent on added ATP and cytosol. It is also not blocked by pre-treatment of the disrupted cells with NEM. The second phase in the reaction seems to involve a variably slower rate of ATP-dependent release that is substantially inhibited by NEM (Figure 10B).

ATP and the transferrin cycle

As mentioned above, intact MDCK cells mediate the efficient uptake of Tfn into an acid-resistant compartment in the presence or absence of energy poisons. Accordingly, the disrupted MDCK cells will not readily permit the type of biochemical analysis of Tfn endocytosis as can now be accomplished for recycling; thus, we did not further pursue the mechanism of Tfn uptake. The ATP-independence of Tfn uptake was surprising since the formation of Tfn-containing coated vesicles in disrupted A431 cells has recently been shown by electron microscopy to require added ATP (Smythe *et al.*, 1989). Although the efficiency of Tfn uptake in broken A431 cells was low (12% of total bound Tfn) and the stimulation due to ATP only 2- to 2.5-fold, it is possible that our criterion for Tfn internalization—resistance to removal by low pH wash—did not reflect endocytosis but sequestration of Tfn in deep invaginations of the plasma membrane (Smythe *et al.*, 1989). In the absence of electron microscopy (thus far difficult in MDCK cells due to the low number of Tfn receptors), we cannot be certain whether Tfn internalization is dependent on ATP or not. While it is also possible that our conditions of ATP-depletion were insufficient to block endocytosis in intact cells, a similar ATP-independence of uptake into an acid-resistant compartment was observed in disrupted cells.

On the other hand, it is conceivable that MDCK cells are already 'primed' for receptor-mediated endocytosis of Tfn, while recycling of internalized Tfn requires the contribution of additional cytosolic components and ATP. This, in turn, suggests that the regulation of endocytic membrane traffic may be exerted primarily at the level of recycling. Indeed, Tfn uptake occurs at 3–4 times the rate of recycling (4000 Tfn/min/cell internalized versus 1000 Tfn/min/cell recycled). This difference may reflect the fact that internalization of a receptor already localized at coated pits requires only a single event, fission to form a coated vesicle, while recycling must involve several distinct steps and/or a rate limiting step. That one or more steps during recycling may serve as the site of regulation of endocytic membrane transport has been suggested by several observations in intact cells. For example, it is well known that endocytosis ceases during mitosis in animal cells. The inhibition of Tfn internalization, however, occurs only after Tfn recycling stops (Warren *et al.*, 1984). In addition, at least two polypeptide hormones are known to regulate the rate of Tfn recycling without affecting Tfn endocytosis (Davis *et al.*, 1987). Since Tfn recycling *in vitro* is strictly dependent on added energy and cytosol, we should be able to identify both the sites at which regulation occur, and the molecules involved in the formation and transport of recycling vesicles.

Materials and methods

Cells and cell culture

MDCK cells (strain II) were maintained on plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 20 mM HEPES pH 7, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. The cells were split 1:5 every week and used for < 15 passages. For experiments, 1.5×10^6 cells were plated on 24.5 mm filter units (0.4 μM pore size; Transwells, Costar, Cambridge, MA). Confluent monolayers were used on the third day in culture.

Preparation of transferrin

Human and canine apotransferrin (>98% iron free; Sigma, St. Louis, MO) were converted to fully saturated, chelate-free iron(III)-transferrin (holotransferrin) using nitrilotriacetic acid (Bates and Schlabach, 1973). Holo-Tfn was desalted by chromatography on Sephadex G-25 (Pharmacia, Piscataway, NJ) in 0.1 M sodium perchlorate followed by dialysis (or a second column) in 20 mM HEPES pH 7, and concentrated using Centricon microconcentrators (10 000 mol. wt cutoff; Amicon, Danvers, MA). Holo-Tfn concentrations were obtained by determining absorption at 457 nm (human holo-Tfn, $E^{1\%} = 0.56$; canine holo-Tfn $E^{1\%} = 0.72$). Aliquots were frozen and stored at −80°C. Tfn was iodinated ($1-5 \times 10^6$ c.p.m./μg) using Iodogen (Pierce, Rockford, IL) as described (Mellman *et al.*, 1983).

Binding and elution of [^{125}I]Tfn

Filter-grown MDCK cells were washed twice with PBS containing divalent cations (PBS^+) and incubated for 45 min at 37°C in DMEM supplemented with 0.5% BSA and 20 mM HEPES pH 7 ('binding medium') to deplete bovine Tfn. The cells were then transferred to ice, and binding medium containing 1–5 μg/ml [^{125}I]holo-canine Tfn (cTfn) was added to the apical or basal compartments for 1.5 h. < 1% of the label diffused to the opposite compartment under these conditions. Both sides of the filter were washed with cold PBS^+, the filters cut from the Transwell unit and counted. Identical results were obtained if the cells were harvested by scraping, indicating that no radioactivity was associated with the filter. Greater than 85% of the radiolabel bound at 4°C could be eluted from cells by alternating PBS^+ and low pH washes (25 mM acetic acid, 150 mM NaCl pH ~3) over 35 min for 5 min each. Non-specific binding and internalization was routinely determined by subtracting the amounts of radiolabel bound to parallel filters incubated in the presence of a 100-fold excess of unlabeled

Tfn; non-specific binding was <10% of the specific binding. Experiments were performed in duplicate and differed by <10%. For Scatchard analysis, cells were incubated in a constant amount of [^{125}I]Tfn (0.4 μg) and increasing concentrations of unlabeled Tfn (0.4–80 μg/ml; 5–1000 nM).

Internalization and recycling of pre-bound transferrin

After incubation with [^{125}I]Tfn on ice, both sides of the filters were washed seven times with 2 ml of cold PBS$^+$. The filters were transferred to 6 well dishes that contained 1 ml of binding medium/well on the basal compartment; 1 ml was then added to the apical compartment. The cells were incubated (with or without permeabilization, see below) at 37°C and rapidly cooled to 4°C at various times by adding ice-cold PBS$^+$ and immediately transferring the cells to an ice bath. Aliquots of the apical and basal media were collected and released [^{125}I]Tfn measured. Internalized [^{125}I]Tfn was determined from the amount resistant to release from the cell surface by acid-washing, as described above. A parallel set of filters was washed with ice cold PBS$^+$ and used to determine total cell-associated radioactivity. Surface bound radioactivity was calculated by subtracting the acid-resistant radioactivity from the total cell associated. In some experiments, surface-bound radioactivity was determined directly by measuring the amount of [^{125}I]Tfn released by the acid wash. Specific binding, release, and internalization on duplicate filters differed by <10%.

Recycling of pre-internalized Tfn

Filter-grown cells were incubated in binding medium containing 5 μg/ml [^{125}I]cTfn in the basal medium for 1 h at 37°C. Both sides of the filters were then washed with ice-cold PBS$^+$ to remove unbound Tfn and incubated in binding medium containing 10 μg/ml unlabeled cTfn for 2.5 min at 37°C to internalize remaining surface bound [^{125}I]cTfn. After this treatment, virtually 100% of the cell-associated [^{125}I]cTfn was resistant to removal by low pH. The cells were then either permeabilized (see below) or incubated at 37°C in binding medium containing 10 μg/ml unlabeled cTfn to allow internalized [^{125}I]cTfn to recycle and be released into the medium. After various times, the medium was harvested and replaced with ice-cold PBS$^+$, and the filters transferred to ice. Cell-associated [^{125}I]cTfn and radioactivity in the apical and the basal media were determined. Non-specific counts (<10% of total) were determined using parallel filters allowed to internalize [^{125}I]cTfn in the presence of excess unlabeled Tfn (500 μg/ml). For convenience, filters were cut into 2–4 equal pieces after Tfn uptake (and/or disruption) and placed in separate vials for the second 37°C incubation.

Permeabilization of the apical membrane

The perforation of MDCK cells (Simons and Virta, 1987) was optimized for cells grown on polycarbonate filters. Monolayers on filter units were washed twice with 2 ml ice cold PBS$^+$ on the apical and the basal compartments and once with 1 ml of 'intracellular transport' buffer (ICT; 78 mM KCl, 4 mM $MgCl_2$, 8.37 mM $CaCl_2$, 10 mM EGTA, 1 mM DTT, 50 mM HEPES–KOH pH 7) (Burke and Gerace, 1986). The apical medium was then aspirated and replaced by a 24 mm diameter nitrocellulose filter (0.45 μm pore size; Schleicher and Schuell, Inc., Keene, NH); before use, nitrocellulose filters were presoaked for 1 min in deionized water, then for 1 min in ICT buffer, and blotted for 1 min between filter paper under a 1 l bottle. A 25 mm glass microfiber filter was placed on top of the nitrocellulose filter to remove excess ICT buffer. The basal buffer was removed and uniform gentle pressure was applied for 10 s using a 24.5 mm rubber stopper attached to a 20 ml syringe. After 1 min, 1 ml of ICT buffer was added to the apical and the basal sides. After an additional 1 min, the microfiber filter was removed, the polycarbonate filter was excised from the Transwell unit with a razor blade, and the nitrocellulose filter was slowly ripped off using forceps. Permeabilized cells could be kept in ICT buffer on ice for up to 6 h without loss of activity.

The cytosol-dependent transferrin release was optimized by a mild salt wash of the permeabilized cells just after nitrocellulose ripping. When indicated, the disrupted cells were incubated for 30 min at 4°C in 150 mM KCl, 50 mM HEPES–KOH pH 7 and then transferred back to the ICT buffer (Figures 6A, 8, 10).

The efficiency of permeabilization was routinely assessed by trypan blue (0.04% exclusion, the increased ability of disrupted cells to accumulate 10 μg/ml propidium iodide in their nuclei, and the accessibility of cytoplasmic myosin and clathrin to antibodies added to unfixed cells as determined by immunofluorescence. To quantify disruptions, the fraction of propidium iodide-stained nuclei (n ~600) surrounded by fluorescent staining due to anti-myosin were counted. Monolayers were routinely judged to be >85% permeable by these criteria. Approximately 80% of the total lactic dehydrogenase (LDH) activity was found in the media after permeabilization at 4°C. LDH was determined as described (Bennett *et al.*, 1988).

Incubation of intact and permeabilized cells

After removal from the Transwell units, filters containing disrupted cells were placed cell side up in 6-well dishes on 80 μl droplets of ICT buffer containing the indicated additions; 160 μl of the identical buffer were added to the apical side. The assay was supplemented with 0.5–8 mg/ml desalted cytosol or 3–8 mg/ml ovalbumin or BSA. Cytosol was prepared in CIT buffer (with or without 1 mM ATP) from scraped MDCK cells passed 4 times through a 22 gauge syringe needle, or from CHO cells or yeast essentially as described (Balch *et al.*, 1984; Ruohola *et al.*, 1988). Cytosol preparations were dialyzed, frozen in liquid nitrogen, and stored at −80°C. For incubations in the presence of ATP, an ATP regenerating system was included: 1 mM ATP pH 7, 8 mM creatine phosphate (CP) and 32 IU/ml creatine phosphokinase (CPK). To deplete ATP, either 25 U/ml hexokinase and 5 mM glucose, or 50 U/ml apyrase (Sigma), was added.

To reduce internal ATP pools, cells were incubated in Hanks' buffer or DMEM without glucose containing 40 mM HEPES (pH 7), 20 mM dGlc and 10 mM NaCN for 1 h at 4°C. Inhibitors were present during subsequent incubations at 37°C. ATP concentrations were determined on neutralized perchloric acid extracts (Weigel and Englund, 1975) using an ATP Bioluminescence kit (Boehringer–Mannheim) and quantified using a spectrofluorometer (SLM Instruments, Inc., Urbana, IL). Proteins were determined as described (Bradford, 1976).

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