**Isolation by Fluorescence-activated Cell Sorting of Chinese Hamster Ovary Cell Lines with Pleiotropic, Temperature-conditional Defects in Receptor Recycling**

(Received for publication, December 27, 1990)

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We have isolated several Chinese hamster ovary cell lines with temperature-sensitive defects in the recycling of receptors after endocytosis. These cell lines were selected using fluorescence-activated cell sorting for retention of a pulse of labeled transferrin after a chase in the presence of unlabeled transferrin. One of these cell lines, TTT1.11, was selected for further characterization. In TTT1.11 the trapping of transferrin within the cells is paralleled by a loss of cell surface transferrin receptors. Within 4 h after the shift from 33 to 41 °C the surface binding of transferrin is reduced to 18% of parental cells at 41 °C. The trapping of transferrin and the loss of transferrin receptor from the cell surface are caused by a temperature-conditional 5.5-fold decrease in the initial rate of transferrin recycling. TTT1.11 cells also rapidly lose 89% of their ability to take up α2-macroglobulin after the temperature shift to 41 °C. These data indicate that the TTT1.11 cell line has a pleiotropic defect in receptor recycling.

Several early observations indicated that there is extensive recycling of internalized membrane and protein to the cell surface after endocytosis (reviewed in Refs. 1 and 2). During extended periods of endocytic activity cells internalize a large percentage of their plasma membrane and a large volume of fluid although the dimensions of the cells do not change. In addition, for many molecules the total amount of ligand internalized greatly exceeds the number of cell surface receptors, however, Tf escapes lysosomal degradation by recycling vesicles and endosomes may account for the segregation of free ligand from receptor during recycling (21). There are several membrane-bound proteins, including epidermal growth factor receptor, IgFc domain receptor, and mannose 6-phosphate receptor, which are retained and carried further by recycling to occur. Although the mechanism for dissociation of the ligands and receptors has been determined, very little is known about the mechanism for segregation and recycling. It has been suggested that the morphology of the endosome may be responsible for the segregation of receptors and ligands (20). Although the relative surface area to volume ratios of the recycling vesicles and endosomes may account for the segregation of free ligand from receptor during recycling (21), there are several membrane-bound proteins, including epidermal growth factor receptor, IgFc domain receptor, and mannose 6-phosphate receptor, which are retained and carried further by recycling vesicles and endosomes may account for the segregation of free ligand from receptor during recycling (21). There are several membrane-bound proteins, including epidermal growth factor receptor, IgFc domain receptor, and mannose 6-phosphate receptor, which are retained and carried further by recycling vesicles and endosomes may account for the segregation of free ligand from receptor during recycling (21).

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To begin to identify molecules involved in this process we selected cell lines defective in receptor recycling using labeled transferrin (Tf) as a marker for its receptor. Tf, a major iron transport protein, binds with high affinity to surface receptors and is internalized during endocytosis. Unlike most other ligands, however, Tf escapes lysosomal degradation by recycling back to the cell surface bound to its receptor (31, 32). A single cycle of internalization and recycling is completed from receptor is coupled to endocytic acidification (for review, see 10). Segregation of most receptor-ligand complexes occurs in the endosome, an early, prelysosomal endocytic compartment with a low buoyant density (11–15). The pH of early endosomes is regulated in many cell types to approximately pH 6 (16–18), a pH sufficient to drive the dissociation of many ligands and receptors. This allows recycling to occur before exposure to the degradative environment of the lysosomes. Once ligands have dissociated from their receptors, segregation largely becomes a problem of the relative retention of fluid during the recycling of membrane and membrane-bound material (19).

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within 10–15 min (4, 5, 16). To isolate recycling defective mutants cells were selected for retention of labeled Tf after a chase with excess unlabeled Tf. In this paper we describe the isolation of these cell lines and the initial characterization of the clone TffT1.11, which shows a temperature-dependent, pleiotropic defect in receptor recycling.

**EXPERIMENTAL PROCEDURES**

Tissue culture supplies were purchased from Gibco, and other materials were purchased from Sigma, unless otherwise noted.

**Preparation of α2-Macroglobulin—α2-Macroglobulin (α2M) was prepared from fresh human serum. A 7–26% polyethylene glycol fraction (33) was dialyzed against distilled water, centrifuged (40,000 × g), and dialyzed against buffer A (10 mM NaCl, 0.02 M NaPO₄, pH 6.0). The solution was then fractionated by zym affinity column chromatography (34). The α2M bound to the affinity column (2.5 × 27 cm) was eluted as described (35) except that 50 mM acetate, pH 4.5, was used for elution. The eluted protein was then dialyzed against buffer A and fractionated by gel permeation on a Sephacryl S-300 column (2.5 × 90 cm). The purified α2M was then activated with methylamine as described (33).**

**Synthesis of Fluorescent Conjugates—FITC-dextran (70,000 daltons) was prepared by the dibutyltin-dilaurate method with the modifications described previously to reduce free dye contamination (36).**

(human dextran, Miles Scientific, Naperville, IL) was labeled with Cy5.18-OSu (Cy5) (52) using the protocol described previously for Tf conjugated with lissamine rhodamine sulfonyl chloride (16). Cy5.18-OSu has properties similar to those of Cy5-12-OSu (37) and was a generous gift from Dr. Alan Waggoner, Carnegie Mellon University. The resulting dye to protein ratios were 2.75 for the conjugate used in mutant isolation experiments and 5.0 for the conjugate used in the characterization experiments, α2M was labeled with Cy5, with a resulting dye to protein ratio of 7.1. All of the Cy5 protein conjugates had binding specificities of greater than 90%. Typical ratios of Cy5 fluorescence to background fluorescence from unlabelled cells were 130 ± 1 for Tf (10 μg/ml) and 45 ± 1 for α2M (10 μg/ml).

**Flow Cytometry—A dual laser FACS 440 flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with argon and krypton lasers was used for all analyses. FITC fluorescence (488 nm excitation, 400 milliwatts) was collected using a 530 nm band pass filter (30 nm band width), and Cy5 fluorescence (647 nm excitation, 200 milliwatts) was collected using a 670 nm band pass filter (13.5 nm band width). A FACStarPLUS equipped with argon and helium-neon lasers was used for sorting.**

**Isolation of Recycling Defective Cell Lines—CHO WT cells (obtained from Dr. Brian Storrie, Virginia Polytechnic Institute and State University) were maintained at 33 °C in α-MEM supplemented with 10% calf serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 g/liter proline (α-MEM), and 10% calf serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 g/liter proline (α-MEM), and 10% calf serum (HyClone Laboratories, Logan, UT). Cells were plated as described for Tf binding experiments, shifted to 41 °C for various times and washed with α-MEM salts, and incubated for 30 min at the appropriate temperature in 0.5 ml of α-MEM containing 1 mg/ml bovine serum albumin and various concentrations of Cy5-Tf (2–200 μg/ml, three samples/concentration). The samples were then washed and analyzed as above. Mean fluorescence values were corrected for nonspecific binding, determined at each concentration by competition using 5 μg/ml unlabeled Tf. For Scatchard analysis the concentration of Tf added was used as an estimate of free Tf (since the fraction of input Tf bound to cells was negligible under the conditions used).**

**Transferin Externalization—Cells were plated as described for Tf binding experiments. Samples of TffT1.11 and WT were washed two times with α-MEM salts and incubated for 30 min at the appropriate temperature in 0.5 ml of α-MEM containing 1 mg/ml bovine serum albumin and 10 μg/ml Cy5-Tf. The cells were then chilled quickly to 4 °C by washing twice with ice-cold α-MEM salts. To remove surface-bound Tf cells were incubated at 4 °C in 0.5 ml of stripping buffer (150 mM NaCl, 100 mM dithiothreitol, 10 mM N-ethylmaleimide, 0.5% bovine serum albumin, 0.05% NaN₃, 50 mM MESS) for 10 min. The cells were then washed two times with α-MEM salts and incubated at 41 °C for 0.5, 1, 1.5, or 2 h and labeled for 30 min with 2.5 μg/ml Cy5-Tf in α-MEM. The cells were then incubated for 1 h in α-MEM with 1 mg/ml unlabeled human dextran Tf and 2 mg/ml FITC-dextran at 4 °C (to determine the initial amount of Tf internalized) or at 41 °C (for samples to be sorted). After labeling, the cells were washed six times with ice-cold α-MEM salts (116 mM NaCl, 5.4 mM KCl, 0.2 mM CaCl₂, 0.8 mM MgSO₄, 10 mM NaH₂PO₄, pH 7.4) and suspended by scraping into α-MEM salts. Mean fluorescence values were corrected for nonspecific binding, determined at each concentration by competition using 5 μg/ml unlabeled Tf. After various times the cells were again chilled quickly to 4 °C with two washes of ice-cold α-MEM salts. The cells were maintained at 4 °C until all samples had been collected, washed five times with α-MEM salts, and suspended by scraping immediately prior to analysis by flow cytometry. The data were fit by one or two exponentials using a nonlinear least squares fitting program (38).**

Nonlinear fits to all data were made using four models: a single exponential (two free parameters), a single exponential with a nonrecycled component (three free parameters), two exponentials (four free parameters), and two exponentials with a nonrecycled component (five free parameters). The root mean square error (normalized to a percent error by division by the mean Y value for each data set) was used to judge goodness of fit. For the 33 °C data the first model yielded significantly higher error values (11.6 and 16.8% for WT and TffT1.11, respectively) than the other three. Since the error values for these three models were within 0.03% of each other, the second model (single exponential with a nonrecycled component) was chosen for these data. The error values were 6.5 and 14.5% for WT and TffT1.11, respectively. For the 41 °C data, the first two models gave higher error values (21.2 and 14.5% for WT, 12.9 and 10.2% for TffT1.11) than the last two (9.6–9.7% for both WT and TffT1.11). The decrease in error of less than 0.1% between the third and fourth models was not considered to justify the addition of a free parameter, so the third model was chosen. Results are also shown for the second model for TffT1.11 at 41 °C since the difference in error between the second and third models was only 0.6%.

To determine how similar the pathways in TffT1.11 and WT were at 41 °C, an additional fit was made using the third model with the parameters fixed to those obtained with WT cells (by this time only two free parameters). The error obtained (14.2%) was significantly larger than the others for this data set (including the first model, which also had only two free parameters); the results are shown in Table IV for comparison only.

For all of the samples there was a significant but variable fraction of the Tf which was released very rapidly from the cells (t₁/₂ < 30 s). This may represent the "fast" external compartment described by McKinley and Wiley (39) for adherent cells, which is caused by trapping of ligand between the cells and the plates at low temperature. An attempt at fitting this component was made by including a second exponential (the second component for WTB and TffT1.11) with a long half-time of release was shorter than the first point (30 s). To estimate the amount of cell-associated Tf excluding this external compartment, the nonlinear fits were extrapolated to 0 min. The raw data were then converted to a percentage of this value.

α2-Macroglobulin_Uptake—Cells were plated as described for the Tf binding experiments, shifted to 41 °C for various times, and labeled
for 6 min at 41 °C in α-MEM with 10 μg/ml Cy5-αM. To clear receptors of unlabeled αM from the serum, the cells were incubated without serum at either 33 or 41 °C for 30 min prior to labeling. After labeling the cells were chilled quickly to 4 °C, washed twice with α-MEM salts, suspended by scraping into α-MEM salts, and analyzed by flow cytometry. Short-term uptake was measured instead of surface binding because very little binding could be detected at 4 °C even after a 1-h incubation. The temperature dependence of αM binding has been noted for other cell types as well (40). Measurements of cell-associated αM reflect both surface bound and internalized ligand because the off rate, like the on rate, is quite slow.

**Cellular ATP Levels**—Cells were plated as for the Tf binding experiments. The cells were shifted to 41 °C for 4 h or maintained at 33 °C prior to harvesting by scraping at 4 °C. The amount of ATP/sample was determined using a luciferin/luciferase ATP determination kit for somatic cells (Sigma) and a Thorn EMI PMT (800 V) coupled to a Thorn EMI photon counter (Thorn EMI Gencom Inc., Fairfield, NJ). The average number of cells/sample was determined from parallel samples.

**Protein Synthesis**—Cells were plated as for the Tf binding experiments. The plates were shifted to 41 °C for 4 h or maintained at 33 °C, washed with α-MEM salts, and incubated at the appropriate temperature for 15 min in assay medium (deficient Dulbecco's modified Eagle's medium supplemented with 10%! calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.292 g/liter L-glutamine, 0.105 g/liter L-leucine, 0.146 μg/liter L-lysine, 4 μg/liter proline, and 0.3 μg/liter L-methionine). 8 μCi of Tran35S-label (ICN; Irvine, CA) was added halfway through of the wells, and the incubation continued for 45 min. The cells were then washed three times with α-MEM salts. The cells that did not receive radioactivity were suspended by trypsin treatment and counted with a Coulter Counter (Coulter Corp; Hialeah, FL). The cells receiving radioactivity were lysed in 85 μl of lysis solution containing 0.1%! sodium dodecyl sulfate, 0.1 mg/ml deoxyribonuclease I, 1 mM CaCl2, and 1 mM MgCl2. The lysate from each well was transferred to 2.5-cm squares of Whatman 3MM, soaked for 20 min in 5% trichloroacetic acid containing 0.5 μg/ml L-methionine, and washed once in 95% ethanol. The filter papers were dried, and the radioactivity was measured in a liquid scintillation counter using Ecolume (ICN) as the scintillant.

**RESULTS**

**Cell Sorting**—To isolate cells with temperature-conditional defects in receptor recycling we explored the feasibility of using fluorescence-activated cell sorting to select cells that were unable to recycle labeled Tf administered after a shift to a higher temperature. A continuous incubation in the presence of labeled Tf during the temperature shift could not be used to label the cells, because normal WT cells were observed to retain some labeled transferrin after chase. The accumulation of labeled Tf occurred at a rate of approximately 5% of the total internal Tf for each h of labeling and was inhibited by the addition of excess unlabeled Tf during the pulse (data not shown). This phenomenon was observed for both Cy5- and FITC-Tf and was also observed in CHO K1 and Swiss 3T3 cells (data not shown). The retention of a small fraction of the internal Tf has been described in other cell lines as well (27, 41). To avoid this problem cells were incubated at 41 °C for various times to allow expression of heat-sensitive lesions, pulse labeled with Cy5-Tf for 30 min, and chased for 1 h with excess unlabeled Tf. Cy5 was used as the fluorescent marker because it gave very high signal to noise ratios (typically 130:1 for the initial labeling; see “Experimental Procedures”). To ensure that the sorted cells were capable of internalization, FITC-dextran was included in the chase media as a marker of fluid phase endocytosis. The desired mutants were selected to be positive for Cy5-Tf (caused by failure to recycle it during the chase) and also positive for FITC-dextran (normal for internalization). Examples of the sort conditions are shown in Fig. 1. Double-positive cells were sorted from the samples chased at 41 °C (Fig. 1C, quadrant II).

There was no detectable enrichment for double positives after the first sort (data not shown). After the second sort there was an enrichment of large cells (as judged by increased light scattering) which had a concomitant increase in the number of receptors/cell (data not shown). In the larger cells, the Cy5 fluorescence after chase was higher than in the control cells (Fig. 1, C and D, quadrant IV), which accounts for their selection; however, the higher fluorescence represented the same percent of the internalized Tf as in the unselected population (not shown). The apparent increase is caused by an increase in the total number of receptors rather than a difference in receptor recycling. In addition to the large cells present after the second sort there was a distinct subpopulation of double-positive cells in the 2-h sample, comprising approximately 9% of the total cells observed (Fig. 1D, quadrant II). This subpopulation was sorted and cloned by limiting dilution.

Failure to recycle Tf (and TIR) would be expected to result in a decrease in cell surface TIR. If this is the case, expression time (time at 41 °C) would be a critical variable in this selection protocol. At early times no Tf would be trapped because of the lack of expression of the defect whereas at late times the number of surface TIR would be decreased, reducing initial Cy5-Tf labeling and decreasing the potential signal. This effect was demonstrated by examining the kinetics of expression of the trapping phenotype for the population of double-positive cells obtained after the third sort (Fig. 2). The trapping reaches a maximum at 3.5 h and then decreases. After 5 h at 41 °C, the uptake of Tf during the 30-min pulse is only 35% of the uptake at 33 °C. Although there appears to be an optimal time at which to select for Tf trapping mutants, the expression time may be different for each recycling mutant isolated.

**Initial Characterization of Clones**—To verify that the clones had temperature-conditional defects in receptor recycling clones were screened for temperature-dependent loss of surface TIR. The results are shown in Table I. Since these clones were derived from a single mutagenesis and carried as a bulk population for several generations after sorting it is possible that they may be derived from a single mutation. However,

![Fig. 1](image-url)
Fig. 2. Kinetics of expression of the Tf trapping phenotype. WTB cells (●) and cells sorted from the double-positive population shown in Fig. 1D (▲) were incubated at 41 °C for the indicated times, labeled with Cy5-Tf, and chased as in Fig. 1. The fluorescence remaining cell associated after the chase is expressed as a percentage of the amount taken up during the pulse (means of duplicate samples).

Table I

Screening of TfT clones for loss of surface transferrin receptors

| Relative Tf Binding | 33 °C | 41 °C |
|---------------------|-------|-------|
| WTB                 | 1.00  | 1.00  |
| TfT1.2              | 0.68  | 0.18  |
| TfT1.11             | 1.02  | 0.28  |
| TfT1.13             | 0.93  | 0.26  |
| TfT1.14             | 0.99  | 0.29  |
| TfT1.16             | 0.90  | 0.24  |

Fig. 3. TfT1.11 cells trap Tf. WTB (A and C) and TfT1.11 (B and D) cells were incubated for 2 h at 41 °C, labeled for 30 min with Cy5-Tf (A and B), and chased for 1 h with FITC-dextran and excess unlabeled Tf (C and D) as in Fig. 1. Contours are drawn at 20, 40, 60, and 100 events/bin (20,000 events total).

one of the clones, TfT1.2, appears to be different from the others, as it shows a 30% reduction in Tf binding (relative to parental) even at the permissive temperature. The clone TfT1.11 was selected because it had normal levels of TfR at the permissive temperature and showed a significant loss of TfR at the nonpermissive temperature.

After a 2-h incubation at 41 °C, TfT1.11 shows a small reduction in the amount of Tf internalized in a 30-min pulse (compare Fig. 3, A and B), and the majority of the cells are clearly positive for both dextran accumulation and retention of Cy5-Tf (Fig. 3D). However, TfT1.11 shows some decrease in the extent of dextran labeling (Fig. 3D). This defect in dextran accumulation is not caused by a difference in the initial rate of dextran internalization but by a difference in the efflux of fluid after endocytosis. The retention of Cy5-Tf in TfT1.11 is temperature dependent, as there is a significant amount of Tf retained after chase at 41 °C (Fig. 4B) but little trapping at 33 °C (Fig. 4C). TfT1.11 is similar in size to WTB (Fig. 4A), in contrast to the majority of the cells after the second sort (see above). The phenotype of TfT1.11 is not caused by a gross alteration in cellular metabolism. There is no significant decrease in the levels of ATP in TfT1.11 cells and no significant difference in the relative rates of protein synthesis after temperature shift (Table II).

Loss of Surface Transferrin Receptors—Fig. 5 shows the kinetics of loss of apparent cell surface Tf binding sites from TfT1.11 cells during incubation at 41 °C. Within 4 h after the shift to 41 °C the binding decreased to less than 20% of the initial binding and less than 25% of the increased binding to WTB at the elevated temperature. The loss of receptors occurred with a t1/2 of approximately 2 h and began almost immediately after shifting the temperature. Scatchard analysis (Table III) indicates that the loss of binding is caused by a decrease in the number of surface receptors rather than a change in receptor affinity. After a 4-h incubation at 41 °C there was no difference in the TfR affinity of WTB and TfT1.11 cells; the calculated Kd for WTB is in good agreement with values reported previously (42). Under these conditions the number of surface receptors in TfT1.11 is reduced to 18% of WTB.

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COMPONENTS are 2.5-3 times slower in Tffl.11 cells than in WTB. Immediately prior to analysis the cells were washed and suspended by scraping. Showed are the means and standard deviations for triplicate samples from three experiments (n = 9) normalized to the WTB 33°C average value.

**TABLE III**

Transferrin receptor properties of WTB and Tffl.11

Scatchard analysis was performed on Cy5-Tf binding data obtained as described under "Experimental Procedures." Mean and standard deviations of deviations from two experiments are shown. The estimated errors in the linear fit parameters for each experiment were propagated during averaging to yield the estimated error of the average.

|        | Relative no. of receptors/cell |
|--------|--------------------------------|
| WTB    | 18.6 ± 3.6                     |
| Tffl.11| 18.5 ± 3.5                     |

a Cy5-Tf fluorescence was normalized such that the relative number of receptors/WTB cell was one in each experiment.

**Kinetics of Transferrin Externalization**—Receptor loss from the cell surface could be the result of either an increase in the rate of receptor internalization or a decrease in the rate of receptor recycling. To address this point the kinetics of clearance of a 30-min pulse of Cy5-Tf were measured for WTB and Tffl.11 at permissive and nonpermissive temperatures. At 33°C the kinetics of loss of labeled Tf were similar for WTB and Tffl.11 although the fraction of Tf which is not recycled (at least over 90 min) is higher in Tffl.11 (Fig. 6A). First-order rate constants and maximum fraction lost were estimated by nonlinear least squares fitting of a single exponential (Table IV). The products of these values (the initial rate) were similar for WTB and Tffl.11. At 41°C the loss of Tf is accelerated in WTB whereas it is slowed in Tffl.11 (Fig. 6B). When cells are shifted to 41°C the initial rate of recycling increases by a factor of 3 in the WTB cells, but a significant fraction of the Tf is cleared from the cells through a slow pathway (t1/2 = 27 min). In Tffl.11 cells recycling through the fast pathway is either absent or altered dramatically. When the data are fit using the same model as for WTB the results suggest that a shift to 41°C redirects the bulk of the Tf through the slow component (63% in Tffl.11 cells compared with 38% in WTB cells). In addition, the rate constants for clearance through both the fast and slow components are 2.5-3 times slower in Tffl.11 cells than in WTB cells at 41°C. Since the error values obtained when the data are fit with the model used for the 33°C data are only slightly higher than for the two-component model, it is possible that only a single component is present at 41°C in Tffl.11 with a rate more than seven times slower than WTB. Regardless of which model is used, the analysis leads to the conclusion that the Tffl.11 mutant retains Tf and TfR at the nonpermissive temperature because of an alteration in the rate of receptor externalization.

**Reduced a2-Macroglobulin Uptake**—To determine whether the Tf recycling defect in Tffl.11 is caused by a general defect in the recycling pathway, α2M accumulation was measured for Tffl.11 and WTB cells after the temperature shift. Cells were incubated for various times at 41°C and labeled with Cy5-α2M for 6 min at 41°C. Differences in α2M uptake in these short incubations are presumed to reflect differences in the number of surface receptors, as there is no difference between the two cell lines in the amount of dextran internalized in a 6-min pulse at either 33 or 41°C. a Interestingly,
TT1.11 cells have 1.4 times as many α2M receptors as WTB cells at 33 °C (Fig. 7). α2M uptake is lost rapidly after the temperature shift (t½ of approximately 1.5 h) and declines by greater than 90% by 4 h. The loss of surface α2M receptor indicates that the defect in TT1.11 is not specific for the TfR but is a pleiotropic defect in receptor recycling.

**DISCUSSION**

Most endocytosis-defective mutants described previously have been isolated by selection against some aspect of the endocytic pathway (16, 43–46). In all of these selection schemes mutant cells were isolated by killing normal cells. We have isolated cell lines with temperature-conditional defects in receptor recycling during endocytosis using the trapping of labeled Tf as a marker selectable by fluorescence-activated cell sorting. There are several advantages of using cell sorting for selection of mutants. Positive selection by sorting allows the isolation of new classes of mutants which would be difficult to isolate using negative selection techniques. Mutants are isolated under conditions that are not lethal, and the selection does not require the perturbation of the endocytic pathway in normal cells. Flow sorting has been used recently to select CHO cell lines with temperature-conditional defects in the expression of membrane glycoproteins on the cell surface (47).

After screening several clones for a temperature-conditional loss of surface TfR, TT1.11 was chosen for further analysis. This cell line shows temperature-dependent trapping of labeled Tf at 41 °C (Figs. 3 and 4) and a concomitant loss of surface Tf binding (Fig. 5). Scatchard analysis shows that this loss of binding is caused by a decrease in the number of surface receptors at the nonpermissive temperature (Table III). Analysis of the clearance of internalized Tf showed that TT1.11 has a temperature-sensitive defect in the rate of receptor recycling (Fig. 6). After temperature shift, the initial rate of clearance of internalized Tf from TT1.11 cells was reduced 5.5-fold compared with WTB cells, and 63% of the internal Tf was released through a very slow component (t½ = 67 min; Table IV). It is not currently known if the Tf released through the slow process was intact or degraded.

O'Keefe and Draper (44) described a mutant, AF192, isolated from mouse L cells by selecting for resistance to a diphtheria toxin-Tf conjugate, with an aberrant Tf cycle. Like TT1.11, this cell line shows a reduction in Tf surface binding which is caused by a shunting of 25% of the TfR to a pathway with a very slow rate of return to the cell surface (t½ greater than 100 min). However, the loss of TfR from the cell surface in AF192 is not as severe as the loss in TT1.11 at 41 °C (a 25% reduction compared with an 82% reduction in TT1.11 after the temperature shift). Like AF192 cells, TT1.11 cells at 41 °C show an increase in the amount of Tf released through a slow pathway. Unlike AF192, however, there is also a 2.5–3-fold difference in the rate constants for recycling from both the fast and the slow pathways in TT1.11 (at 41 °C there is a fraction of Tf which recycles slowly in WTB cells as well). When the TT1.11 data are fit using the WTB rate constants, 94% of the Tf appears to recycle through the slow pathway (Table IV). It is possible that these two cell lines have the same defect, with the difference in severity caused by differences between temperature-conditional and nonconditional alleles. However, it was not determined if the defect in AF192 was specific for Tf or affected other ligands as well.

There is considerable evidence that intrinsic properties of receptors affect recycling. A point mutation in the cytoplasmic domain of the epidermal growth factor receptor which eliminates kinase activity causes the receptor and a significant portion of the ligand to be redirected from the lysosomal pathway to the recycling pathway (25) whereas proteolytic processing of the insulin receptor at the cell surface results in redirection of the receptor from the recycling to the degradative pathway (48). Deletion of the extracellular growth factor homology region of the low density lipoprotein receptor inhibits acid-dependent ligand dissociation and receptor recycling (9). In addition, aggregation of receptors by multivalent ligands or antibodies causes a redirection of receptors to the lysosome (11, 49). These data suggest that a defect in the TfR which causes aggregation or a change in conformation could result in the loss of TfR from the surface. However, the fact that at 41 °C TT1.11 shows a loss in surface α2M-receptor as well as TfR indicates that the defect in TT1.11 is not specific for TfR but is a more general defect in the pathway of receptor recycling.

Although there are intrinsic properties of receptors which determine whether they will be recycled, once that decision has been made there seems to be little distinction among receptors. The rates of externalization of several receptors, including Tf, α2M, and mannose-6-phosphate receptors, are identical, suggesting that the receptors are moving as a single cohort (50). Treatment with growth factors and phorbol esters causes a rapid redistribution of many receptors including TfR, α2M receptor, mannose-6-phosphate receptor, and mannose receptor from an intracellular pool to the plasma membrane (26, 27, 51), because of an increase in the rate constant for receptor externalization (26, 27). It is possible that TT1.11 contains a defect in one of the components involved in regulating this rate. Isolation and further characterization of pleiotropic mutants defective in receptor recycling should lead to insights into the general mechanism of receptor recycling and permit identification of molecules required for this process.

**Acknowledgments**—We thank Greg LaRocca for technical assistance and Drs. Alan Wagoner and Lauren Ernst for the gift of Cy5-α2M and mannose-6-phosphate receptor. We also thank Dr. Phil McCoy of the Pittsburgh Cancer Institute for assistance with the cell sorting.

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