Recovery of Function in Chinese Hamster Ovary Cell Mutants with Temperature-sensitive Defects in Vacuolar Acidification

Calvin F. Roff, Clara W. Hall, and April R. Robbins

Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract. After 4 h at 41°C, B3853 and M311, temperature-sensitive Chinese hamster ovary cell End1 and End2 mutants, respectively, are pleiotropically defective in endocytosis and trans-Golgi network–associated activities (Roff, C. F., R. Fuchs, I. Mellman, and A. R. Robbins. 1986. J. Cell Biol. 103:2283–2297). We have measured recovery of function after return to the permissive temperature. Based on return of normal transferrin-mediated Fe uptake and sensitivity to diphtheria toxoid both mutants had restored endosomal function at 10 h; based on delivery of endocytosed lysosomal enzymes to lysosomes and normal sensitivity to modeccin both had functional late endocytic organelles at 10–12 h; and based on retention of newly synthesized lysosomal enzymes and sialylation of secreted glycoproteins both had functional trans-Golgi network at 6 h. At 10 h, M311 had recovered almost all of its ability to endocytose lysosomal enzymes; B3853 required 30 h to recover fully its ability to endocytose lysosomal enzymes.

Slow recovery of mannose 6-phosphate-dependent uptake in B3853 reflected altered trafficking of cation-independent mannose 6-phosphate receptors. Although B3853 had normal amounts of receptor at 6–8 h, it had greatly diminished amounts of receptor at the cell surface. Altered trafficking was also suggested by the finding that B3853 rapidly degraded receptor that had been present before the shift to the nonpermissive temperature.

Four classes of mutant Chinese hamster ovary (CHO) cells (Endl-4) exhibiting temperature-sensitive (ts)

defects in acidification of organelles have been defined by genetic complementation (7, 8, 36). Endl-3 mutants were originally characterized as defective in ATP-dependent endosomal acidification (26, 27, 35, 36, 44, 54, 55). Acidification of other organelles is also affected in End1 and End2 mutants; indirect evidence has been presented suggesting altered acidification of the trans-Golgi network (TGN; 35, 36), and a recent study demonstrates impaired lysosomal acidification in both of these classes (Robbins, A. R., and C. F. Roff, manuscript in preparation). To date, evidence that the primary lesion is defective acidification exists only for an End3 mutant: ATP-dependent acidification of a light membrane fraction was ts (49), as were both ATP hydrolysis and ATP-dependent acidification in a coated vesicle preparation (47).

We have examined a variety of activities in End1 and 2 mutants (34, 35, 36) and have shown that both classes of mutants are impaired in receptor-mediated accumulation of lysosomal enzymes, α2-macroglobulin, and Fe presented as diferric transferrin. In addition, these mutants are resistant to diphtheria and Pseudomonas toxins and modeccin and are hypersensitive to ricin. They also show defective segregation of newly synthesized lysosomal enzymes out of the secretory pathway and decreased sialylation of some glycoproteins. Given that pH exerts a threshold effect on endocytic functions it is not surprising that “leaky” mutants showed little or no impairment of some of the functions listed above; e.g., a nonconditional End2 mutant (DTF-151) with residual endosomal acidification (35) exhibited normal sensitivity to modeccin and Pseudomonas toxins, but was resistant to diphtheria toxin (34). However, the pH threshold must reflect more than the pH in a single compartment (e.g., the early endosome) because at the nonpermissive temperature a ts End2 mutant (I223) is resistant to modeccin but not to diphtheria and Pseudomonas toxins (36).

On comparisons of the kinetics of phenotypic changes after shifting B3853 and M311 (ts End1 and End2 mutants, respectively, exhibiting similar severity of defects in endosomal acidification) to the nonpermissive temperature, little difference was observed between the mutants (36). Only one phenotypic change distinguishing End1 and End2 as separate classes has been described. Ryser and colleagues have shown that End2 mutants exhibit enhanced resistance to methotrexate polylysine conjugates (39), whereas End1 mutants do not (24); resistance correlates with decreased degradation of the polylysine moiety (25). Although the degree of resistance among End2 mutants increased proportionately to

1. Abbreviations used in this paper: M6P, mannose 6-phosphate; CD-M6PR, cation-dependent M6P receptor; Cl-M6PR, cation-independent M6P receptor; TGN, trans-Golgi network; ts, temperature-sensitive.
the severity of the defect in endosomal acidification, a "leaky" End2 mutant was more resistant than severely affected End1 mutants.

After shifting B3853 and M311 to the nonpermissive temperature, all of the phenotypic changes listed above occurred quite synchronously over a period of <4 h (36). An order of events did emerge regarding loss of mannose 6-phosphate (M6P) receptor-dependent uptake of lysosomal enzymes and compartmentalization of endogenous enzymes, suggesting that multiple stages of transport are affected. (a) Transfer of endocytosed enzymes from a buoyant (presumably endosomal) to a denser ("light lysosomal") compartment ceased within minutes in B3853. (b) Loss of enzyme uptake and of segregation of endogenous enzymes from the secretory pathway occurred in 1-2 h. The former activity is solely dependent on the cation-independent mannose 6-phosphate receptor (CI-M6PR; 9, 13, 22, 32, 45, 52), identical to the IGF II receptor (28), whereas the latter appears to depend on activities of both the CI-M6PR and the cation-dependent mannose 6-phosphate receptor (CD-M6PR; 9, 13, 21, 32). (c) Depletion of surface binding activity required 4 h; this loss did not reflect loss of CI-M6PR from the surface but rather loss of activity of CI-M6PR on the cell surface.

In this study, we examine recovery of function in B3853 and M311. We demonstrate a large difference between these mutants with respect to the kinetics of recovery of CI-M6PR dependent binding and uptake, because of different rates of restoration of receptor molecules to the cell surface.

**Materials and Methods**

Culture (31) of mutants (33) and WTB (48) were as previously described. All endocytosis assays, measurement of toxin sensitivities, fractionation of cell extracts on Percoll gradients, isotopic labeling, immunoprecipitations, PAGE, fluorography, and scintillation counting of radioactive bands were as previously described (36). Any additional details will be presented in figure legends.

Pseudomonas toxin was provided by Dr. Stephen Leppla (United States Army Medical Research Institute of Infectious Diseases, Frederick, MD), ricin by Dr. Richard J. Youle (National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD), 56Fe-transferin by Dr. Richard Klausner (National Institute of Child Health and Development, Bethesda, MD), goat anti-beta-glucuronidase antisera by Dr. Richard T. Swank (Roswell Park Memorial Institute, Buffalo, NY), rabbit anti-CI-M6PR and anti-major excreted protein (cathepsin L) antisera by Dr. G. Gary Sahagian (Tufts University School of Medicine, Boston, MA) and rabbit anti-CI-M6PR used for immunoblotting by Dr. Leonard H. Rome (University of California, Los Angeles, CA).

**Quantitation of Surface M6P Binding Activity**

Binding to intact cells was measured as previously described (36) with two modifications of the published procedure: (a) incubation was with 35S-labeled secretions at 1 x 10^6 cpm/ml rather than 7.5 x 10^6; (b) before displacement of bound 35S-labeled secretions with M6P, cells were washed seven, rather than five, times with medium; the fifth and seventh washes were left on the cells for 10 min. With this procedure all of the bound ligand whose binding was inhibited in the presence of 10 mM M6P could be displaced by addition of 10 mM M6P.

**Quantitation of CI-M6PR**

To measure total CI-M6PR, cells in 12 well trays were solubilized in 100 μl of 3% SDS containing 10% glycerol; extracts were heated at 96°C for 2 min, centrifuged for 5 min in a table top centrifuge (model 5414; Eppendorf made by Brinkmann Instruments, Inc., Westbury, NY), and the supernatants were stored at −20°C for up to 3 d. Freezing samples did not change the amount of receptor detected on immunoblots. Samples were thawed immediately before use, vortexed vigorously, and protein levels were determined by the method of Lowry (23). Samples were then adjusted to 0.125 M Tris, pH 6.8, 10 mM DTT, and heated at 96°C for 1 min. Aliquots containing 15 μg of protein were electrophoresed on preparative SDS-4-12% gradient gels (Novex, Encinitas, CA), and transferred to nitrocellulose (50) at 300 mA for 1.5 h. After fixation for 5 min with 100 ml 50% methanol containing 10% acetic acid, the nitrocellulose was rinsed briefly with water and blocked with 5% defatted powdered milk in TBS (20 mM Tris, pH 7.5, 0.5 M NaCl). Blotting with rabbit anti-CI-M6PR (1:500 dilution) in TBS containing 2% milk was for 2 h, and with alkaline phosphatase coupled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., Aosdale, PA; 1:3,330 dilution) in TBS containing 2% milk was for 12.5 h. After blocking and after incubation with both primary and secondary antibodies, the nitrocellulose was rinsed twice with TBS containing 0.5% Tween-20 (10 min/rinse), and then twice with TBS (7 min/rinse). Detection (3) was with the Protoblot System (Promega Biotech, Madison, WI). Quantitation of immunoblots was by densitometric scans of the nitrocellulose paper using an Ultrascan laser densitometer (model 2202; LKB Instruments Inc., Bromma, Sweden). Receptor absorbance increased linearly in immunoblots of 5-35 μg total protein from lysates of WTB grown at 34°C. All blotted samples had absorbances within this range.

To determine degradation of old CI-M6PR, cells in 6-well trays were rinsed two to three times with methionine free medium, then incubated for 13 h at 34°C with [35S]methionine (New England Nuclear, Boston, MA; 50 μCi/ml, 1 μg/ml methionine, 3 ml/well), rinsed three times with 3 ml medium, and incubated at 34°C for 2-3 h. Cells were then shifted to 4°C for 4 h, then returned to 34°C for various times before harvesting; one set of cells was harvested at the time of shift to 4°C, and the amount of receptor in these cells was set at 100%. Solubilization in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.02% NaN3, 1% deoxycholate, 0.1% SDS, and 1% Triton X-100) containing 10 μg/ml BSA and 10 mM M6P, immunoprecipitation, and SDS-PAGE of CI-M6PR were as previously described (36). Quantitation was by densitometric scans of the fluorogram and/or by determining the amount of radioactivity in the excised receptor band.

To determine simultaneously degradation of old receptor and the total amount of immunoprecipitated receptor, cells were labeled, temperature-shifted, and harvested just as for determination of degradation of old receptor. The immunoprecipitated receptor was heated in 60 μl of SDS-PAGE sample buffer containing 10 mM DTT at 96°C for 5 min and centrifuged for 5 min to pellet Immunoprecipitin (Bethesda Research Laboratories, Gaithersburg, MD). Aliquots of 25 μl were electrophoresed and transferred to nitrocellulose just as for determination of total receptor. The nitrocellulose paper was immunoblotted to determine total receptor then exposed to film to determine 35S-labeled receptor.

**Analysis of Secreted Proteins by Two-Dimensional Gel Electrophoreses**

Cells in 12-well trays were pulse for 2 h with 0.5 ml [35S]methionine (200 μCi/ml) in serum free medium. Medium was collected, centrifuged at 1000 g for 10 min, and secreted proteins were precipitated overnight at −20°C in 6 vol of a 1:1 mixture of ethanol and acetone. Protein, solubilized in 100 μl of focusing sample buffer, was focused on pH 4.0-6.5 prefocussed isoelectric tube gels, and then electrophoresed in a second dimension on 9% SDS-polyacrylamide gels all as originally described by O’Farrell (30, 56).

**Results**

**Graphical Presentation**

To examine recovery of function after return to the permissive temperature (34°C), cells were first shifted to the non-permissive temperature (41°C) for 4 h (previously shown to effect maximal alteration of phenotype). We express "time of temperature shift" with respect to the initiation of the assay; i.e., 0 h indicates that ligand was added at the same time cells were shifted to 34°C; positive times indicate shifts to 34°C before addition of ligand or radiolabeled metabolite. Thus, increasing values for "time of temperature shift" correspond to increasing times spent at the permissive temperature.
Transferrin-mediated Fe Uptake

Accumulation of Fe from dimeric-transferrin was used to monitor recovery of endosomal function (51). M311 showed increased Fe accumulation when shifted to 34°C at the start of incubation with [59Fe2]transferrin (Fig. 1, 0 h). B3853 required 1 h at 34°C before it showed any increase (Fig. 1). Maximal uptake in both mutants was attained after 6–8 h. Increases in uptake after brief periods at 34°C likely reflected uptake occurring near the end of the 2-h incubation with ligand. Based on the ability to support Fe accumulation, early endosomes regained partial function at 2–3 h and were fully restored by 6–8 h.

Response to Toxins

Diphtheria toxin, modeccin, and Pseudomonas toxin all require entry into or passage through an acidic endocytic compartment for release of toxic subunits into the cytosol (11, 12, 43); diphtheria emerges from endosomes (11, 43), modeccin from a postendosomal compartment (11, 43), and the organelle whence Pseudomonas emerges is unknown. Restored sensitivity to these toxins likely reflects the mutants’ recovered ability to acidify one or more endocytic compartments. After return to 34°C for 1 h M311 showed normal sensitivity to Pseudomonas toxin (Fig. 2 A), whereas B3853 had a sensitivity intermediate between normal and that at 41°C (Fig. 2 D). After 3 h, B3853 recovered near normal sensitivity. When modeccin was added after 1 h of recovery, the mutants remained resistant to the highest dose tested (Fig. 2, B and E); 10 h at 34°C was required to restore near normal sensitivity. Recovery of sensitivity to diphtheria toxin differed in the two mutants; M311 (Fig. 2 C) recovered at a rate similar to that seen with Pseudomonas toxin; B3853 (Fig. 2 F) recovered at a rate similar to that seen with modeccin. Based on toxin sensitivity, restoration of endocytic activity began early and was maximal by 10 h.

M6P-dependent Endocytosis

Recovery of toxin sensitivity and transferrin-mediated Fe uptake may only require recovery of functional endosomes. Uptake via the Cl-M6PR with ultimate delivery of ligands to lysosomes involves both early and late endocytic compartments (53). It may also involve compartments not used by other ligands; e.g., vesicles cycling between the Golgi and organelles of the endocytic pathway.

M311 recovered uptake concomitantly with other endocytic activities. Return to 34°C for 2 h was sufficient to restore some uptake (Fig. 3). During the first 10–12 h uptake increased to 85% of normal at ~5%/h. In some experiments (see Fig. 5), M311 recovered 60–80% of uptake at 5%/h during the first 10–12 h, and then recovered the remainder at a slow rate, <2%/h. Recovery in B3853 differed from that in M311 in two aspects: a 4–6-h lag was observed before uptake began to increase (Fig. 3, inset), and then restoration occurred at <3%/h, requiring 24–30 h for full recovery (Fig. 3). Recovery of M6P uptake in I223, another ts End2 mutant isolated independently from M311 (36), was also determined. In I223, always kept at 34°C, M6P uptake and surface binding activity are 55–65% of parental levels (36). I223 returned to 34°C after a 4-h incubation at 41°C recovered M6P.

Figure 1. Transferrin-mediated 59Fe accumulation. After a 4-h incubation at 41°C (41°) or return to 34°C for the indicated times WTB (solid squares), M311 (open circles), and B3853 (solid circles), were incubated with [59Fe2]transferrin (10 µg/ml, 3.8 × 10^4 cpm/µg) for 2 h, then cell-associated radioactivity was determined. Results are averages of duplicate assays, normalized to cell protein, corrected for nonspecific 59Fe accumulation (880 cpm/100 µg), and expressed as percent specific uptake of cultures always kept at 34°C (WTB, 7,960 cpm/100 µg; M311, 6,360 cpm/100 µg; and B3853, 7,620 cpm/100 µg). In WTB cell associated 59Fe because of binding of [59Fe2]transferrin to its receptor accounts for 30% of cell-associated radioactivity in a 2-h assay.

Figure 2. Response to toxins. M311 (A–C) and B3853 (D–F) were kept at 34°C (solid circles) or incubated at 41°C for 4 h (open circles), then returned to 34°C for 1 h (solid squares), 3 h (open squares), 6 h (solid triangles), or 10 h (open triangles) before treatment with Pseudomonas toxin for 3 h (A and D); modeccin for 2 h (B and E), or diphtheria toxin for 2 h (C and F). Results are expressed as percent of protein synthesis measured in parallel cultures not treated with toxin.
uptake at a rate similar to that seen with M311. Thus, relatively rapid recovery of M6P uptake appears common to End2 mutants.

After 4 h of recovery, M311 appeared to have a complete endocytic pathway, as defined by delivery of ligand to lysosomes. After return to 34°C for 4 h WTB and M311 were incubated with 35S-labeled secretions at 34°C (WTB, 3,000 cpm/100 μg; M311, 3,220 cpm/100 μg; B3853, 3,260 cpm/100 μg). Inset: WTB, M311, and B3853 were shifted to 41°C for 4 h, returned to 34°C for 4 h, and then incubated with 35S-labeled secretions for varying times before determinations of cell-associated radioactivity.

To determine whether some residual acidification activity at 41°C was responsible for the difference between M311 and B3853 in recovery of M6P uptake, cells were incubated at 41°C for 4 h then returned to 34°C for 5.5 h, all in the presence of 10 mM NH4Cl. Immediately before incubation with 35S-labeled secretions for 1 h, monolayers were washed four times with 3 ml of medium to remove NH4Cl. During the last wash, cells were incubated at 34°C for 5 min.

* Results are averages of duplicate assays, normalized to cell protein, corrected for nonspecific uptake (1,310 cpm/100 μg), and are expressed as percent uptake in untreated cells always kept at 34°C (WTB, 12,160 cpm/100 μg; M311, 8,950 cpm/100 μg; and B3853, 9,130 cpm/100 μg).

## Table I. Effect of NH4Cl on Recovery of M6P Uptake

| Cell  | NH4Cl | Uptake* (percent control) |
|-------|-------|---------------------------|
| WTB   | -     | 83                        |
| WTB   | +     | 45                        |
| M311  | -     | 69                        |
| M311  | +     | 35                        |
| B3853 | -     | 17                        |
| B3853 | +     | 6                         |

Parallel cultures were incubated at 41°C for 4 h then returned to 34°C for 5.5 h, all in the presence or absence of 10 mM NH4Cl. Immediately before incubation with 35S-labeled secretions for 1 h, monolayers were washed four times with 3 ml of medium to remove NH4Cl. During the last wash, cells were incubated at 34°C for 5 min.

* Results are averages of duplicate assays, normalized to cell protein, corrected for nonspecific uptake (1,310 cpm/100 μg), and are expressed as percent uptake in untreated cells always kept at 34°C (WTB, 12,160 cpm/100 μg; M311, 8,950 cpm/100 μg; and B3853, 9,130 cpm/100 μg).

**Table 1.** Effect of NH4Cl on Recovery of M6P Uptake

- **Surface Binding Activity**
  One component involved in M6P uptake but not in the other endocytic activities is the CI-M6PR (42, 46). A modified version (see Materials and Methods) of our previously described procedure was used to measure surface binding activity. After 4 h at 41°C, parental cells increased surface binding activity to 1.4 times that at 34°C; M311 and B3853 lost 70-90% of their activity (Fig. 4). Loss in B3853 oc-
Figure 5. Recovery of surface M6P binding activity. After 4 h at 41°C parallel cultures of M311 (A) and B3853 (B) were returned to 34°C. At the indicated times, cells were shifted to 0°C for assays of surface M6P binding activity (open circles; 0 h indicates cells shifted to 0°C after the 4-h incubation at 41°C) or incubated for 1 h with 35S-labeled secretions for assays of M6P uptake (solid circles; 41°C indicates cells kept at 41°C during incubation with ligand). Results are averages of duplicate samples, normalized to cell protein, corrected for nonspecific binding (150 cpm/100 μg) or uptake (1,680 cpm/100 μg), and are expressed as percent binding and uptake in M311 (600 cpm/100 μg and 5,640 cpm/100 μg, respectively) and in B3853 (690 cpm/100 μg and 5,950 cpm/100 μg, respectively) always kept at 34°C.

Figure 6. CI-M6PR at the cell surface: WTB and B3853, grown in 6-well trays, were either kept at 34°C (34°) or incubated for 4 h at 41°C and then returned to 34°C for 8 h (8 h). Cells were chilled to 0°C and surface proteins were labeled with 125I as previously described (29). CI-M6PR was solubilized in RIPA containing 10 mg/ml BSA and 5 mM M6P, immunoprecipitated, electrophoresed on 5% gels, detected by autoradiography, and quantitated by densitometry. Receptor from duplicate wells of WTB and B3853 at each time are shown.

Recovery of binding activity was measured in parallel with recovery of uptake. Recovery of binding in M311 began by 4 h and reached 45-50% of normal by 12 h at which time uptake was 68% of normal (Fig. 5 A). In B3853, binding activity continued to decrease during the first 4 h at 34°C, and then increased to ~20% of normal at 12 h; at 12 h, uptake was ~40% of normal (Fig. 5 B). Thus, recovery of binding and uptake were rapid in M311 and slow in B3853. This suggests that during recovery the level of functional CI-M6PR at the cell surface was rate limiting for uptake.

CI-M6PR at the Cell Surface

Surface CI-M6PR was determined by immunoprecipitating receptor from cells that were labeled at 0°C with 125I by the surface labeling procedure previously described (29). When always kept at 34°C B3853 had 80% of the parental level of surface CI-M6PR (Fig. 6). After a 4-h incubation at 41°C followed by an 8-h return to 34°C, surface receptor in WTB was 76% of normal; however, in B3853 the level of receptor was only 34% of that measured in B3853 always grown at 34°C. Thus, decreased surface M6P binding activity in B3853 at 8-h recovery correlates with decreased numbers of receptors at the cell surface, as measured by iodination.

Segregation of Newly Synthesized Lysosomal Enzymes Out of the Secretory Pathway

M6PRs transport lysosomal enzymes during both endocyto-
with [35S]methionine, radioactivity was chased for 2–3 h at 34°C, and then cells were shifted to and from 41°C. CI-M6PR was solubilized, immunoprecipitated, electrophoresed, and then quantitated by densitometry and/or scintillation counting of the excised bands. WTB and M311 shifted to and from 41°C degraded labeled receptor slowly (Fig. 8, A and B); the time required for 50% degradation ($t_{1/2}$) was 12–18 h. This is similar to the $t_{1/2}$ previously reported for WTB always kept at 34°C (34, 41). In B3853 CI-M6PR was lost much more rapidly (Fig. 8 C); 80% of labeled receptor disappeared during the first 6 h of return to 34°C; the $t_{1/2}$ of labeled receptor was $\sim 5$ h. In some experiments, loss of labeled receptor began during the incubation at 41°C. B3853 always kept at 34°C also degraded receptor more rapidly, $t_{1/2} = 8–9$ h (results not shown).

In contrast to the loss of CI-M6PR synthesized before the shift to 41°C, levels of total CI-M6PR, i.e., receptor synthesized before, during, and after incubation at 41°C, remained high in B3853. Total CI-M6PR was determined by Western blots of cells solubilized in 3% SDS (Fig. 8 C). When always grown at 34°C, B3853 had 90–120% of the parental level of receptor. After 4 h at 41°C receptor was 70–120% of 34°C controls; after 2–4 h of recovery at 34°C, receptor was 70–90% and after 6–8 h, 81–98%. WTB and M311 had amounts of total receptor similar to or slightly more than that in B3853 during recovery except that in some experiments

siv of exogenous and intracellular targeting of newly synthesized acid hydrolases. The intracellular pathway involves segregation of newly synthesized enzymes out of the secretory pathway (9, 22, 32, 52). Recovery of this process was monitored by measuring the amount of newly synthesized enzymes that were secreted. Cells were pulsed with [35S]methionine for 15 min, and then major excreted protein (cathepsin L), and beta-glucuronidase were immunoprecipitated from the medium and electrophoresed on 7% polyacrylamide gels. Radioactive enzyme was excised and counted. Note that cells at the −2 h time were pulsed at 41°C after a 4-h preincubation at that temperature, and radioactivity was chased for 2 h at 41°C and then for 3 h at 34°C. B, Radioactivity was chased for 1 h and MEP was immunoprecipitated, electrophoresed on 10% gels, excised and counted.

**Figure 7.** Secretion of major excreted protein (cathepsin L) and beta-glucuronidase. WTB (solid squares), M311 (open circles), and B3853 (solid circles) were either kept at 34°C (34°) or incubated for 4 h at 41°C (41°) and then returned to 34°C for the indicated times, at which times cells were pulsed for 15 min with [35S]methionine. A, After a 5-h chase, beta-glucuronidase was immunoprecipitated from the medium and electrophoresed on 7% polyacrylamide gels. Radioactive enzyme was excised and counted. Note that cells at the −2 h time were pulsed at 41°C after a 4-h preincubation at that temperature, and radioactivity was chased for 2 h at 41°C and then for 3 h at 34°C. B, Radioactivity was chased for 1 h and MEP was immunoprecipitated, electrophoresed on 10% gels, excised and counted.

**Figure 8.** Turnover of CI-M6PR. After incubation at 41°C for 4 h (41°C) parallel cultures of WTB (A), M311 (B), and B3853 (C) were returned to 34°C for the indicated times before measuring CI-M6PR levels. Total CI-M6PR (solid circles) was determined by immunoblots of extracts from cells solubilized with 3% SDS. Results are from five experiments each for WTB and B3853 and three for M311. To assess degradation of old receptor (open triangles), cells were labeled for 13 h with [35S]methionine, chased for 2–3 h at 34°C, and then shifted to 41°C. Cells were solubilized in RIPA at the indicated times of recovery; receptor was immunoprecipitated, electrophoresed, and detected by fluorography. Results are averages of duplicates of each cell type and are from three experiments. To simultaneously determine levels of total and old CI-M6PR (solid and open squares, respectively) the above procedure for determining degradation was used except that after electrophoresis immunoprecipitates were transferred and blotted. After immunoblottting to determine total CI-M6PR, the nitrocellulose paper was subjected to autoradiography to measure old CI-M6PR. Results in A and C are from two experiments, each of which included WTB and B3853 at two time points. All results are expressed as percent of receptor in the respective cell type always kept at 34°C.
these cells showed increased levels of receptor after 6–8 h of recovery (Fig. 8, A and B).

We were concerned that the apparent differences in old versus total CI-M6PR in B3853 reflected procedural differences in these experiments. In measuring the fate of old receptor, CI-M6PR was immunoprecipitated from cells extracted in RIPA; in measurements of total CI-M6PR, extracts prepared in 3% SDS were electrophoresed directly. To investigate this further, both old and total CI-M6PRs were measured in the same immunoprecipitates. Receptor was immunoprecipitated from RIPA extracts of cells radiolabeled before any temperature shifts, as had been done in measurements of old receptor. Immunoprecipitates were electrophoresed then transferred to nitrocellulose. Total receptor was assayed by the colorometric Western blotting procedure used above and old receptor by subsequent autoradiography of the nitrocellulose. In B3853 total immunoprecipitable CI-M6PR was decreased by 35–50% at 3 h of recovery, but was restored to normal levels by 7–8 h (Fig. 8 C); in contrast, old (radiolabeled) receptor was decreased by 65–78% and 72–78% at 3 and 7–8 h of recovery, respectively.

From these results we conclude that at 3 h of recovery some receptor in B3853 may be insoluble in RIPA. By 7–8 h of recovery, total immunoprecipitable receptor is normal in amount, yet old is markedly (80%) diminished in the immunoprecipitates. Therefore, B3853 (a) mishandles CI-M6PR during recovery, as evidenced by altered solubility and/or accelerated degradation; (b) always mishandles some CI-M6PR as evidenced by the lower t1/2 in B3853 always grown at 34°C; and (c) has normal levels of receptor after an 8-h return to 34°C, and yet does not endocytose lysosomal enzymes because little of this receptor is at the cell surface.

**Recovery of Sialylation**

At 41°C the mutants failed to completely sialylate secreted glycoproteins (36). Sialyltransferase has been localized in TGN (2, 37, 38), an acidic compartment (1, 18, 19). We hypothesize that it is the compartment or the acidification thereof, rather than the sialyltransferase itself, that is affected at the nonpermissive temperature. Recovery of TGN function was monitored by following restoration of complete sialylation of secreted glycoproteins. Secreted polypeptides with pls between 4.0 and 6.5 were analyzed by two-dimensional gel electrophoresis.

Three polypeptides secreted by the mutants at 41°C were found to be more basic than the corresponding polypeptides secreted at 34°C. No differences were seen in polypeptides secreted by WTB at 34°C, 41°C, or after return to 34°C. When returned to 34°C for 6 h, B3853 secreted these three polypeptides with pls identical to those from B3853 always kept at 34°C. After a 6-h return from 41°C, M311 secreted more forms with acidic pls than at 41°C, but basic forms were also present. Therefore, by 6 h, B3853 had restored sialylation and M311 had recovered most but not all of its ability to sialylate (data not shown).

**Recovery in Both Mutants Is Dependent on Protein Synthesis**

The temperature sensitivity of endocytic activity in the mutants likely results from amino acid substitutions that cause conformational instability in the affected protein at elevated temperature. Restoration of activity on return to 34°C could result from either renaturation of old protein or synthesis of new protein. To differentiate between these two possibilities, cells were returned to 34°C in the presence of 5 μM cycloheximide, a concentration that inhibits protein synthesis in CHO cells by >95%.

When cycloheximide was present during 8 h of recovery, B3853 and M311 remained resistant to modeccin (Table II); protein synthesis after treatment with 10 ng/ml modeccin was >95% of control. In parallel cultures without cycloheximide, both mutants became sensitive to modeccin; protein synthesis after toxin treatment was ~20% of control. Cycloheximide had no effect on modeccin sensitivity in WTB or

---

**Table II. Effect of Cycloheximide on Recovery of Modeccin Sensitivity and M6P Uptake**

| Modeccin sensitivity† (Protein synthesis, percent of control) | M6P Uptake§ (percent of 34°C) |
|---------------------------------------------------------------|-------------------------------|
|                  | WTB  | M311 | B3853 | WTB  | M311 |
| Cycloheximide* |      |      |      |      |      |
| 34°C            |      |      |      |      |      |
| –               | 2    | 7    | 2    | 100  | 100  |
| +               | 1    | 39   | 3    | 68   | 106  |
| 41°C            |      |      |      |      |      |
| –               | 1    | 92   | 105  | 98   | 18   |
| +               | 1    | 107  | 99   | n.d. | n.d. |
| Recovery 8 h    |      |      |      |      |      |
| –               | 1    | 18   | 19   | n.d. | n.d. |
| +               | 1    | 96   | 107  | n.d. | n.d. |
| Recovery 4 h    |      |      |      |      |      |
| –               | n.d. | n.d. | n.d. | 80   | 44   |
| +               | n.d. | n.d. | n.d. | 61   | 15   |

* Cycloheximide (5 μM) was added 8.5 h before treatment with modeccin; to remove cycloheximide before the pulse with [35S]methionine, cells were rinsed three times with methionine-free, and cycloheximide-free medium. Before assay of M6P-dependent uptake cells were incubated for 4.5 h with cycloheximide; cycloheximide was present during uptake assays.
† Cells were always kept at 34°C (34°), incubated for 4 h at 41°C (41°), or returned to 34°C for 8 h after a 4-h incubation at 41°C (recovery, 8 h), and then incubated with modeccin (10 ng/ml) for 2 h, pulsed with [35S]methionine for 1 h, and labeled protein was quantitated. Results are expressed as percent of protein synthesis in nontoxin-treated, cycloheximide-treated, or nontreated, cells. At 34°C protein synthesis in control cultures treated with cycloheximide before pulse was 75–85% of that in cells not incubated with cycloheximide; at 41°C synthesis was 56–64%.
§ Cells were returned to 34°C for 4 h and then incubated with [35S]-labeled secretions for 1 h. Results are averages of duplicate samples, normalized to cell protein, corrected for nonspecific uptake (1,070 cpm/100 μg), and are expressed as percent uptake of untreated cells always kept at 34°C (WTB, 3,770 cpm/100 μg and M311, 4,210 cpm/100 μg). n.d., not determined.

Roff et al. *Temperature-sensitive Endocytosis in CHO Mutants*
proximately equal to that measured at 41°C.

The effect of cycloheximide on recovery of M6P uptake in M311 was also tested. The presence of cycloheximide during a 4-h return to 34°C prevented M311 from regaining endocytosis of lysosomal enzymes (Table II); uptake remained approximately equal to that measured at 41°C.

These results suggest that elevated temperature caused an irreversible effect on the polypeptides encoded for by the End1 and End2 loci in B3853 and M311, respectively. To replace lost activity, both mutants needed to synthesize new protein. This eliminates the possibility that M311 and B3853 differed in rates of recovery of M6P uptake because the former could renature a heat-inactivated protein while the latter had to synthesize new protein.

**Discussion**

At the nonpermissive temperature (41°C) B3853 (End1) and M311 (End2) are pleiotropically defective in endocytosis because of decreased acidification of endosomes (36, 44), lysosomes (Robbins, A. R., and C. F. Roff, manuscript in preparation), and possibly TGN (36). In this paper, we have demonstrated that, with one exception, all endocytotic activities examined are restored when mutants are returned to the permissive temperature (34°C) for 10–12 h. Based on results with transferrin and diphtheria toxin, acidification of endosomes is restored rapidly in both B3853 and M311. With both ligands, recovery appeared to begin slightly (1–2 h) earlier in M311 than in B3853: based on transferrin, restoration is complete in both mutants by 6–8 h; based on diphtheria toxin, restoration in M311 is complete by 3 h and in B3853 by 10 h. The somewhat longer time required by both B3853 and M311 for recovery of modeccin sensitivity may indicate that recovery of function in a later endocytotic compartment lags behind that of endosomes. Alternatively, modeccin may require a lower pH than any of the other model ligands we employed.

TGN function, based on sialylation and sorting of newly synthesized lysosomal enzymes out of the secretory pathway, is restored in B3853 by 6 h. In M311, segregation of newly synthesized lysosomal enzymes out of the secretory pathway was restored by 6 h; restoration of sialylation required longer. The latter activity in M311 is never normal.

The CI-M6PR, which appears to have sole responsibility for M6P-dependent surface binding and uptake, has been shown to reside in all of these compartments (4, 5, 6, 15, 16, 17, 20). Recovery of M6P-dependent binding and uptake in M311 occurred at a rate consistent with the recovery of function in these compartments. Recovery of binding and uptake in B3853 was much slower than would be predicted. The different times of recovery in these mutants does not appear to result from residual acidification of organelles: after treatment with NH4Cl during the shift to 41°C and recovery at 34°C, M311 still recovered more uptake than did B3853.

Our results indicate that the slow recovery of M6P-dependent uptake in B3853 reflects decreased movement of CI-M6PR to the cell surface. After 8 h of recovery, B3853 has normal amounts of CI-M6PR but only 15–30% of normal M6P-dependent uptake and 20% of normal M6P-dependent surface binding; throughout recovery surface M6P-specific binding activity recovers in parallel with M6P-dependent uptake (i.e., very slowly). Decreased surface M6P-dependent binding reflects fewer surface CI-M6PR; after 8 h of recovery, B3853 had 34% of its normal level of surface receptor, WTb had 76% of normal. We do not know whether the CI-M6PR participates in any of its normal functions during early stages of recovery; i.e., if more than its movement to the cell surface is affected. Segregation of newly synthesized lysosomal enzymes was restored to normal in both mutants after a 2-h return to 34°C, but whether this reflects activity of the CD-M6PR, CI-M6PR, or both is unknown.

We propose that the slow restoration of M6P-dependent endocytosis in B3853 reflects accumulation of CI-M6PR in a "doomed" compartment during both incubation at 41°C and early (3–4 h) stages of recovery. We suggest that this compartment is the postendosomal, post-TGN, prelysosomal compartment identified by Griffiths et al. (20) as an acidic CI-M6PR+/lysosomal membrane glycoprotein+ compartment (hereafter referred to as MPR+/lgp+). Sequestration of CI-M6PR in MPR+/lgp+ in B3853 results from decreased acidification of that compartment, similar to the accumulation observed by Brown et al. (6) in hepatocytes during a several hour treatment with NH4Cl. Trapping of CI-M6PR in non-acidifying MPR+/lgp+ may lag 4–6 h behind synthesis of receptor. For the first 2–3 h of its lifetime, CI-M6PR resides in biosynthetic compartments (ER, Golgi, and TGN); then, based on results with NH4Cl (6), it may require 2–3 h for mature receptors to accumulate within MPR+/lgp+. In B3853 CI-M6PR in MPR+/lgp+ is doomed because, in contrast to reacidification upon removal of NH4Cl (6), return of B3853 to 34°C does not restore acidification of preexisting MPR+/lgp+. Only as MPR+/lgp+ containing End1 molecules (proton pump subunits?) synthesized after return to 34°C are formed does trapping of CI-M6PR cease. Trapped receptor is degraded in nonacidic MPR+/lgp+ and/or is transported to lysosomes and is degraded there. Thus, the slow recovery of M6P-dependent endocytosis in B3853 reflects the time required to replace the cell's entire complement of CI-M6PR, beginning several hours after return to 34°C.

Although the level of total CI-M6PR remained constant throughout shifts to and from 41°C, we observed that at late stages at 41°C and early times of recovery, previously synthesized CI-M6PR in B3853 was decreased markedly in immunoprecipitates of receptor. At 3 h of recovery, this may reflect both increased degradation and decreased detergent solubility/immunoprecipitability of the receptor. Western blots of receptor extracted with 3% SDS indicated a higher level of total receptor than did blots of immunoprecipitated receptor. Others have shown that addition of anti-CI-M6PR antibodies to intact cells precipitates or decreases solubility of receptor, presumably because of cross-linking (14). If at 3 h of recovery receptor in B3853 is cross-linked by multivalent ligands or some other component, then it also may be less soluble and go undetected in assays involving immunoprecipitation. In contrast, at 8 h of recovery, previously synthesized receptor was 80% depleted, whereas both total and total immunoprecipitable receptors were present at normal levels. To maintain normal steady state levels of total CI-M6PR, B3853 must compensate for increased degradation by increased synthesis. We have found that all cells synthesize more receptor at 41°C (Roff, C. F., and A. R. Robbins, un-
published results); whether this is augmented in recovering B3853 by some regulation of CI-M6PR transcription/translation that balances degradation and synthesis is uncertain at present.

Why, when both B3853 and M311 have decreased acidification of endosomes and lysosomes, do they handle CI-M6PR differently? These mutants differ in three possibly relevant aspects of their phenotype: (a) B3853 is more affected than is M311 with respect to lysosomal acidification (Robbins, A. R., and C. F. Roff, manuscript in preparation); (b) M311 is more defective in sialylation (36) than is B3853, implying a greater effect on the TGN in M311; and (c), even at 34°C M311 routes more receptor to the cell surface than does B3853 or WTB (Robbins, A. R., and C. F. Roff, unpublished results). Any or all of these differences may be involved in preventing the loss of CI-M6PR in M311 and/or increasing the loss in B3853. It is tempting to correlate our observations with the CI-M6PR to the reported difference between Endl and End2 mutants regarding sensitivity to (and evidence demonstrating that loss of organelle acidification is the primary defect in either Endl or End2 mutants. And, it is an unlikely albeit theoretical possibility that B3853 contains, in addition to a ts defect in the Endl locus, a second ts defect in the CI-M6PR.

References

1. Anderson, R. G. W., and R. K. Pathak. 1985. Vesicles and cisternae in the trans Golgi of human fibroblasts are acidic compartments. Cell. 40:635-643.
2. Bennet, G., and D. O’Shaughnessy. 1981. The site of incorporation of sialic acid residues into glycoproteins and the subsequent fates of these molecules in various rat and mouse cell types is shown by radioautography after injection of [3H]-acetil-D-mannosamine. J. Observations in hepatocytes. J. Cell Biol. 88:1-15.
3. Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline phosphatase conjugated anti-antibody on Western blots. Anal. Biochem. 136:175-179.
4. Brown, W. J., and M. G. Farquhar. 1984. The mannose 6-phosphate receptor for lysosomal enzymes is concentrated in cis Golgi cisternae. J. Cell Biol. 100:429-437.
5. Brown, W. J., and M. G. Farquhar. 1984. Accumulation of coated vesicles bearing mannose 6-phosphate receptors for lysosomal enzymes in the Golgi region of I-cell fibroblasts. Proc. Natl. Acad. Sci. USA. 81:5135-5139.
6. Brown, W. J., J. Goodhouse, and M. G. Farquhar. 1986. Mannose 6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. J. Cell Biol. 103:1235-1247.
7. Colbath, P. A., C.-Y. Koo, S.-P. Shia, M. Stookey, and R. K. Draper. 1988. Three new complementation groups of temperature-sensitive Chinese hamster ovary cell mutants defective in endocytosis. J. Cell Biol. 104:499-507.
8. Colbath, P. A., M. Stookey, and R. K. Draper. 1989. Impaired lysosomes in a temperature-sensitive mutant of Chinese hamster ovary cells. J. Cell Biol. 108:2211-2219.
9. Dahms, N. M., P. Lobel, and S. Kornfeld. 1989. Mannose 6-phosphate receptors and lysosomal enzyme targeting. J. Biol. Chem. 264:12115-12118.
10. DeDuve, C., T. DeBarsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof. 1974. Commentary: lysosomotropic agents. Biochem. Pharmacol. 23:2495-2531.
11. Draper, R. K., D. O. O’Keefe, M. Stookey, and J. Graves. 1984. Identification of a cold-sensitive step in the mechanism of membrane action. J. Biol. Chem. 259:4083-4088.
12. Fitzgerald, D. R. E. Morris, and C. B. Saelinger. 1980. Receptor-mediated internalization of Pseudomonas toxin by mouse fibroblasts. Cell. 21:867-873.
13. Gabel, C. A., D. E. Goldberg, and S. Kornfeld. 1983. Identification and characterization of cells deficient in the mannose 6-phosphate receptor: evidence for an alternate pathway for lysosomal enzyme targeting. Proc. Natl. Acad. Sci. USA. 80:775-779.
42. Sahagian, G. G., J. J. Distler, and G. W. Jourdian. 1981. Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular beta-galactosidase. *Proc. Natl. Acad. Sci. USA.* 78:4289–4293.

43. Sandvig, K., A. Sudan, and S. Olins. 1984. Evidence that diphtheria toxin and modeccin enter the cytosol from different vesicular compartments. *J. Cell Biol.* 98:963–970.

44. Schmid, S. R., F. A. Kielian, A. Helenius, and I. Mellman. 1989. Acidification of endosome subpopulations in wild-type Chinese hamster ovary cells and temperature-sensitive acidification-defective mutants. *J. Cell Biol.* 108:1292–1300.

45. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell Biochem.* 18:67–85.

46. Steiner, A. W., and L. H. Rome. 1982. Assay and purification of a solubilized membrane receptor that binds the lysosomal enzyme alpha-l-iduronidase. *Arch. Biochem. Biophys.* 214:681–687.

47. Stone, D. K., M. Marnell, Y. Yang, and R. K. Draper. 1987. Thermolabile proton translocating ATPase and pump activities in a clathrin-coated vesicle fraction from an acidification defective Chinese hamster cell line. *J. Biol. Chem.* 262:9883–9886.

48. Thompson, L. H., and R. M. Baker. 1973. Isolation of mutants in mammalian cells. *Methods Cell Biol.* 6:209–281.

49. Timchak, L. M., F. Kruse, M. H. Marnell, and R. K. Draper. 1986. A thermosensitive lesion in a Chinese hamster ovary cell mutant causing differential effects on the acidification of endosomes and lysosomes. *J. Biol. Chem.* 261:14154–14159.

50. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

51. van Renswoude, J., K. R. Bridges, J. B. Hartford, and R. D. Klausner. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: identification of a nonlysosomal acidic compartment. *Proc. Natl. Acad. Sci. USA.* 79:6186–6190.

52. von Figura, K., and A. Hasilik. 1986. Lysosomal enzymes and their receptors. *Annu. Rev. Biochem.* 55:167–193.

53. Willingham, M. C., I. H. Pastan, G. G. Sahagian, G. W. Jourdian, and E. F. Neufeld. 1981. Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 78:6967–6971.

54. Yamashiro, D. J., and F. R. Maxfield. 1987. Kinetics of endosome acidification in mutant and wild-type Chinese hamster ovary cells. *J. Cell Biol.* 105:2713–2721.

55. Yamashiro, D. J., and F. R. Maxfield. 1987. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. *J. Cell Biol.* 105:2723–2733.

56. Young, D. A., B. P. Voris, E. V. Maytin, and R. A. Colbert. 1983. Very high-resolution two dimensional electrophoretic separation of proteins on giant gels. *Methods Enzymol.* 91:190–214.