Chinese Hamster Ovary Cell Mutants with Temperature-sensitive Defects in Endocytosis. I. Loss of Function on Shifting to the Nonpermissive Temperature

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Abstract. We have isolated three independent Chinese hamster ovary cell mutants (B3853, I223, and M311) with temperature-sensitive, pleiotropic defects in receptor-mediated endocytosis. Activities affected at 41°C include uptake via the D-mannose 6-phosphate receptor, accumulation of Fe from diferric transferrin, uptake of α_2-macroglobulin, compartmentalization of newly synthesized acid hydrolases, resistance to ricin, and sensitivity to diphtheria and *Pseudomonas* toxins and modeccin. The three mutants also displayed decreased sialylation of some secreted glycoproteins at 41°C, reminiscent of the nonconditional mutant DTG1-5-4 that showed both endocytic and Golgi-associated defects (Robbins, A. R., C. Oliver, J. L. Bateman, S. S. Krag, C. J. Galloway, and I. Mellman, 1984, *J. Cell Biol.*, 99:1296–1308). Phenotypic changes were detectable within 30 min after transfer of the mutants to 41°C; maximal alteration of most susceptible functions was obtained 4 h after temperature shift. At 39°C, the mutants exhibited many but not all of the changes manifested at 41°C; resistance to diphtheria and Pseudomonas toxins required the higher temperature.

Analysis of cell hybrids showed that B3853 and DTG1-5-4 are in one complementation group ("End1"); M311 and I223 are in another ("End2"). In the End1 mutants, loss of endocytosis correlated with complete loss of ATP-dependent endosomal acidification in vitro; in the End 2 mutants partial loss of acidification was observed. At the nonpermissive temperature, residual levels of endocytic activity in B3853 and M311 were nearly identical; thus, we conclude that the differences measured in endosomal acidification in vitro reflect the different genetic loci affected, rather than the relative severity of the genetic lesions. The mutations in M311 and I223 appear to have different effects on the same protein; in I223 (but not in M311) the full spectrum of phenotypic changes could be produced at the permissive temperature by inhibition of protein synthesis.

Several laboratories have described mutant Chinese hamster ovary (CHO) cells that are pleiotropically defective in receptor-mediated endocytosis (17, 21, 22, 28, 29). Endosomes isolated from some of these mutants were shown to be defective in ATP-dependent acidification (20, 29). Although decreased endosomal acidification has yet to be demonstrated in vivo, many aspects of the mutants' phenotypes are consistent with such a defect; e.g., increased resistance to diphtheria toxin and enveloped RNA viruses (21, 22, 28, 29) and decreased accumulation of Fe from transferrin (13).

However, other facets of the mutants' phenotypes do not appear to follow directly from loss of endosomal acidification; decreased release of Sindbis virus and decreased galactosylation of Sindbis glycoproteins (29) suggest a defect in Golgi function. Other phenotypic changes, e.g., oversecretion of newly synthesized acid hydrolases (28, 29, 36), are ambiguous in that they could result from either Golgi-associated or endosomal defects.

Through analysis of revertants and cell–cell hybrids we have shown that Golgi-associated and endosomal alterations are genetically related (29). But, with the nonconditional mutants we could not determine whether some of the phenotypic changes represented secondary consequences of the primary defect and/or adaptations by those mutants necessary for survival when continuously in the defective state. To refine our dissection of the mutants' phenotypes we have isolated mutants with temperature-sensitive (*ts*)[1] defects. In this paper, we examine the alteration of various activities in

1. *Abbreviations used in this paper*: Man 6-P, D-mannose 6-phosphate; MEP, Major Excreted Protein; *ts*, temperature sensitive.

these mutants as a function of time at the nonpermissive temperature.

Materials and Methods

Materials

Diphtheria toxin was from List Biological Laboratories Inc. (Campbell, CA), modeccin from Pierce Chemical Co. (Rockford, IL); *Pseudomonas* toxin was provided by Dr. Stephen Leppla (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD), and ricin by Dr. Richard J. Youle (National Institute of Mental Health, Bethesda, MD). Cycloheximide, L-leucine-β-naphthylamide HCl, inositol hexaphosphate, D-mannose 6-phosphate (Man 6-P), FITC-dextran (mol wt 70,000), FITC-celite, ATP, ouabain, sodium lauryl sulfate (L-5750), human α_1-acid glycoprotein, and neuraminidase Type X (*Clostridium perfringens*) were from Sigma Chemical Co. (St. Louis, MO). Ethyl methanesulfonate was purchased from Eastman Kodak Co. (Rochester, NY), polyester-PeCap HD7-17 from TETKO Inc. (Elmsford, NY), and polyethylene glycol-1000 and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside were from Research Products International Corp. (Mount Prospect, IL). [^{35}S]Methionine (~1 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL); ^{125}I (carrier-free, ~17 Ci/mg), [^{3}H] GDP-mannose (10.7 Ci/mmol), and [^{14}C] UDP-galactose (337 mCi/mmol) were from New England Nuclear (Boston, MA). Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ). Lactoperoxidase, neuraminidase (*Vibrio cholerae*), human apotransferrin, and BSA were from Calbiochem-Behring Corp. (La Jolla, CA). ^{59}Fe-Transferrin and ^{125}I-transferrin were provided by Dr. Richard D. Klausner (National Institute of Child Health and Development, Bethesda, MD); ^{125}I-α_2-macroglobulin was provided by Dr. Frederick R. Maxfield (New York University School of Medicine, New York, NY); goat anti-β-glucuronidase antiserum (34) by Dr. Richard T. Swank (Roswell Park Memorial Institute, Buffalo, NY); rabbit anti-Sindbis antiserum and dolichol phosphate were provided by Dr. Sharon S. Krag (Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD); and rabbit anti-Major Excreted Protein (MEP) antiserum and rabbit anti-Man 6-P receptor antiserum by Dr. G. Gary Sahagian (Tufts University School of Medicine, Boston, MA). Immuno-Precipitin (formalin-fixed *Staphylococcus aureus*) was purchased from Bethesda Research Laboratories (Gaithersburg, MD), and ricin-agarose (A-2001) from E-Y Laboratories, Inc. (San Mateo, CA).

Cells

The isolation of parent cells WTB (39), of the endocytosis mutant DTG1-5-4 (28), and of WTB111 and DTG1-5-4-122, derivatives of these cells resistant to both ouabain and thioguanine (29), has been described previously. Detailed procedures for isolation of mutants with *ts* defects in endocytosis are described elsewhere (30). Briefly, the mutants were obtained from populations of WTB or WTB111, independently mutagenized with ethyl methanesulfonate (200 μg/ml), subcultured for 5 d to allow phenotypic expression, then shifted from 34°C to 39°C 3 h before selection. For isolation of B3853 from WTB, cells were incubated with 100 ng/ml diphtheria toxin at 39°C for 24 h; before return to 34°C, cells were rinsed three times, trypsinized, then washed three times by centrifugation and resuspension, all in Dulbecco's phosphate-buffered saline without divalent cations (PBS) supplemented with 10 mg/ml inositol hexaphosphate (6). Cells were plated on 100-mm dishes and replicated on polyester discs (26). Replicate colonies were tested in situ for uptake via the mannose 6-phosphate (Man 6-P) receptor and for protein synthesis in the presence of 100 ng/ml diphtheria toxin, each at 34°C and 39°C. For isolation of I223 from WTB, cells were incubated with 2 ng/ml modeccin for 24 h at 39°C, rinsed, trypsinized, and replated on 100-mm dishes. Dishes were left at 39°C for 5 d, then were returned to 34°C. For isolation of M311 from WTB111, cells were incubated with modeccin at 39°C as above, then replated and grown at 34°C in medium containing 10 mM NH_4Cl (17). After 7 d, medium without NH_4Cl was substituted. Replicate colonies from the latter two selections were screened for uptake via the Man 6-P receptor at 34°C and 39°C.

Putative mutants were picked from the master dishes and cloned as previously described (27). Using the above selection and screening procedures we obtained mutants with *ts* defects in endocytosis at frequencies $\geq 1 \times 10^{-7}$.

Hybrid cells were generated by polyethylene glycol-mediated cell fusion, then selected in hypoxanthine/aminopterin/thymidine (HAT) medium (42) containing ouabain as described previously (29) with the single exception that cells were plated at 4×10^5 of each cell type per well in 6-well trays 24 h before fusion.

Cell Culture

FBS was from Biofluids (Rockville, MD), Hazleton Research Products (Denver, PA), and Armour Pharmaceutical Co. (Kanakee, IL); dialyzed FBS was from Gibco (Grand Island, NY). Growth medium (3) and procedures of cell culture have been previously described (27). Stock cultures were kept at 34°C.

Unless otherwise stated, cells were plated 2–2.5 d before experiments; WTB, B3853, and I223 were seeded at densities of 5×10^4, 1.5×10^5, 3×10^5 and 1.0×10^6 cells in 24-well trays, 6-well trays, 60-mm dishes, and 100-mm dishes, respectively; M311 was plated at 10–20% higher densities to compensate for its slower growth rate. To initiate a temperature shift, cells were transferred to incubators equilibrated to 39°C or 41°C.

Endocytosis Assays

Uptake via the Man 6-P receptor was measured using ammonia-induced secretions from WTB cells grown in the presence of [^{35}S]methionine as previously described (28), except that cells in 6-well trays were incubated for 1 h with 1 ml medium containing 5×10^5 cpm [^{35}S] secretions. Nonspecific uptake was measured in the presence of 10 mM Man 6-P. Accumulation of ^{59}Fe from ^{59}Fe-transferrin was measured with cells in 6-well trays; incubation with diferric ^{59}Fe-transferrin (3.8×10^4 cpm/μg; 10 μg/ml per well) was for 2 h (13). To harvest, cells were washed and solubilized as for Man 6-P uptake, then radioactivity and cell protein (16) were determined. Nonspecific uptake was measured in the presence of a 100-fold excess of nonradioactive diferric transferrin. Uptake of ^{125}I-α_2-macroglobulin was measured in 6-well trays; incubation with ligand (2×10^6 cpm/μg; 2 μg/ml per well) was for 15 min (40); then cells were washed five times with medium and radioactivity was chased for 2 h. To harvest, cells were solubilized; intact and degraded α_2-macroglobulin in the medium were determined as TCA-precipitable and -soluble radioactivity, respectively (40). Nonspecific uptake was measured in the presence of a 100-fold excess of nonradioactive α_2-macroglobulin. For all ligands listed above, nonspecific uptake did not differ significantly when measured with mutants or WTB cells, or when determined at 34° or 41°C.

Sensitivity to toxins and toxic lectins was measured by the ability of those agents to inhibit protein synthesis using methods previously described (28).

Man 6-P-dependent Binding

Man 6-P-dependent binding to intact cells (28) was measured at 0–4°C; unless otherwise stated, washes and incubations were in growth medium. Cells in 6-well dishes were placed on ice for 10 min, incubated for 30 min in 2 ml medium containing 10 mM Man 6-P, washed four times with 2 ml, then incubated for 1 h with 7.5×10^5 cpm [^{35}S] secretions in 1 ml (saturation was obtained at $0.5–1.5 \times 10^6$ cpm/ml depending on the preparation of secretions). After this, cells were washed five times with 2 ml, (the last two washes were left on the cells for 10 min), then incubated for 30 min in 1.5 ml of 10 mM Man 6-P, washed twice with 3 ml PBS, then solubilized. Radioactivity in the solubilized cell fraction (nondissociable ligand) and Man 6-P wash (dissociable ligand) were determined and normalized to cell protein; nonspecific binding was measured in the presence of 10 mM Man 6-P. Increasing the time of either preincubation or displacement with Man 6-P from 30 to 120 min resulted in 30–40% and 5–10% increases in total binding to cells previously maintained at 34° and 41°C, respectively. Results obtained using these longer incubations were in complete accord with conclusions presented below.

Man 6-P-dependent binding to total membranes was measured as previously described (28).

Cell Fractionation

Postnuclear supernates from cells grown in 100-mm dishes (1 or 2 dishes per gradient) were prepared as previously described (31) except that cells were harvested by scraping. Percoll gradients (19) were prepared as follows: 9 vol of Percoll (as supplied by Pharmacia Fine Chemicals) were mixed with 1 vol of 2.5 M sucrose containing 10 mM EDTA, pH 6.8; for 27% and 17% gradients, 9 ml and 5.7 ml, respectively, of this solution were brought to 30 ml by addition of 0.25 M sucrose containing 1 mM EDTA, pH 6.8. 25 ml of 27% or 17% Percoll was layered onto a cushion of 4–5 ml sucrose (2.5 M containing 10 mM EDTA, pH 6.8); 4 ml of sample was layered onto the Percoll. Centrifugation was performed in a Sorvall SV288 vertical rotor

(DuPont Co., Sorvall Biomedical Div., Newton, CT) for 1 h at 34,500 *g* at 4°C. Fractions (1.0 -1.1 ml) were collected using a Beckman Universal Fraction Recovery System (Beckman Instruments, Inc., Palo Alto, CA).

The marker enzymes β-hexosaminidase (lysosomes), leucyl β-naphthylamidase (plasma membrane), and mannosylphosphoryldolichol synthase (endoplasmic reticulum) were assayed as previously described (references 31, 24, and 38, respectively). Galactosyl transferase (Golgi apparatus) was assayed by published procedures (2, 31) with the following modifications: agalactoorosomucoid was used as acceptor; after incubation at 37°C for 60 min, 25 μl of the assay mix was spotted on strips of Whatman 3MM paper; these strips were soaked in 10% TCA containing 1% pyrophosphate (three changes at 15-min intervals, 200 ml per bath). Strips were then washed in ethanol and air dried before determination of the radioactivity in each spot. Greater than 70% of the marker enzyme activities present in whole cell lysates were recovered in the postnuclear supernates.

Isotopic Labeling

For metabolic labeling with [^{35}S]methionine, cells in 6-well trays were rinsed three times with methionine-free labeling medium, then pulsed with 100 μCi [^{35}S]methionine in 1 ml of labeling medium containing 0.25–0.5 μg/ml nonradioactive methionine, for 15 min (β-glucuronidase and MEP) or 2 h (Man 6-P receptor). To chase radioactivity, cells were rinsed twice in 2 ml growth medium, then incubated in 1 ml of growth medium.

For surface labeling, cells in 6-well trays were chilled to 0°C, then iodination was performed as previously described (29) using the method of Morrison (23).

Immunoprecipitation

β-Glucuronidase, MEP, and Man 6-P receptor were immunoprecipitated from medium and/or cells by methods previously described (32). If not electrophoresed immediately, samples were stored at −20°C as Immuno-Precipitin pellets.

PAGE, Fluorography, and Quantitation

Antigen was dissociated from Immuno-Precipitin by heating for 5 min at 98°C in 0.1 ml electrophoresis buffer (14) containing 2% SDS, 10 mM dithiothreitol, and 2% 2-mercaptoethanol; samples were centrifuged for 5 min in the microcentrifuge and 70 μl of the supernate was loaded onto the gel. Man 6-P receptor, MEP, and β-glucuronidase were electrophoresed on 5–7% gradient, 10% and 7% SDS polyacrylamide gels, respectively, as described by Laemmli (14). Gels were subjected to fluorography (1), and exposed to pre-flashed (15) XAR-2 film (Eastman Kodak Co., Rochester, NY); labeled bands were excised from the gels and radioactivity was quantitated as previously described (29).

Labeling and Preparation of Endosomes for Acidification Assays

Human transferrin was rendered diferric and fluoresceinated using 10% FITC-Celite as described (41) except that dialyses were replaced by centrifugation (400 *g*, 5 min) of 1 ml samples through columns packed with 10 ml of Sephadex G-25 (fine) equilibrated and pre-centrifuged in the desired buffer. Before use, the FITC-transferrin conjugate was dialyzed extensively against PBS at 4°C. Eight FITC groups were coupled per molecule of diferric transferrin, as determined spectrophotometrically (12). A standard curve relating FITC fluorescence intensity (excitation 485 nm, emission 515 nm) to pH was found to be similar to that obtained for FITC–dextran (10).

Cells grown to confluence in 15-cm dishes (usually 10 per experiment) were incubated in medium without serum for 30 min at 34°C or 41°C, then re-fed with serum-free medium containing 20 μg/ml FITC-transferrin and incubated for an additional 30 min at the appropriate temperature. Cells were washed with cold PBS, removed from the dishes by scraping, washed three times in cold PBS by centrifugation (1,600 rpm, 5 min) and an additional time in cold TAE buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM NaOAc, 1 mM EDTA, pH 7.4), then disrupted in 4 ml of TAE buffer containing 1 mM fresh phenylmethylsulfonyl fluoride using a stainless steel Dounce homogenizer (Kontes Co., Vineland, NJ). The postnuclear supernate was brought to 1.15 M sucrose, overlaid in a Beckman SW-40 ultracentrifuge tube with 2 ml each of 1, 0.86, and 0.25 M sucrose, all in TAE buffer. Gradients were centrifuged for 1.5 h (40,000 rpm, 4°C). Membranes banding at the 0.86/1.0 M interface were found to contain most of the FITC–transferrin as well as most of the ATP-dependent acidification activity.

No FITC fluorescence was detected in cells labeled in the presence of 250 μg/ml unconjugated diferric transferrin. Removal of surface-bound transferrin (see Method 2 in Table III of reference 13) prior to cell harvest had no qualitative effects on the results obtained. Centrifugation on 17% Percoll gradients showed that all of the FITC fluorescence was in light membranes (fraction I, see below).

Endosomes labeled with FITC–dextran were prepared by Percoll gradient centrifugation as described previously (10, 29).

Acidification Assays

Measurement of ATP-dependent acidification of endosomes from WTB and mutant cells was performed using modifications of previously established methods (9, 10, 29). Aliquots corresponding to 10–50 μg protein were diluted into 150 mM KCl, 5 mM $MgSO_4$, and 20 mM Hepes (adjusted to pH 7.4 with tetramethylammonium hydroxide) and equilibrated for at least 1 h (room temperature) to dissipate pre-existing ion gradients. The rate of acidification was measured at ambient temperature by following the decrease in fluorescence after addition of ATP (1.7–5.0 mM, from a 0.35 M stock solution adjusted to pH 7.4 with KOH), using a Perkin-Elmer LS-5 spectrofluorometer (excitation and emission wavelengths set at 485 nm and 515 nm, respectively; slit widths, 10 nm).

Preparation of Secreted 92-kD Protein

Cells in 6-well trays were pulsed with 100 μCi [^{35}S]methionine for 15 min; cells were rinsed twice and radioactivity was chased for 90 min, all in serum-free medium supplemented with 2 mg/ml BSA. Medium was removed, clarified by centrifugation, and Tris-HCl, pH 7.5, was added to 10 mM. Samples were stored at −20°C.

The following were all performed at 4°C and all washes were by centrifugation. To 0.5 ml of each media sample we added 0.8 ml of a ricin–agarose suspension (prepared by washing ricin-agarose three times with 10 mM Tris-HCl, pH 7.5, then incubating with Tris buffer containing 2 mg/ml BSA for 20 min, followed by three more washes, then resuspension of the resin in a volume of Tris buffer equal to the packed volume). The media–resin mixture was gently agitated for 20 min, then the resin was washed three times with 1 ml Tris buffer, and incubated for 20 min with 0.7 ml of buffer containing 0.2 M lactose. Ricin–agarose was removed by centrifugation.

Two 0.3-ml aliquots of each supernate were diluted with 0.6 ml of 0.11 M Na acetate, pH 5.5; to one of each pair was added 0.1 IU of neuraminidase. After incubation at 37°C for 2 h, samples were brought to a final concentration of 0.1 M Tris, pH 7, then protein was precipitated with TCA (25). Precipitates were solubilized and electrophoresed on 10% SDS polyacrylamide gels. Whereas ricin binding of other secreted glycoproteins was found to be increased by pre-treating with neuraminidase, recovery of the 92-kD protein by the above procedure was near quantitative as compared to TCA precipitation (25).

Results

Isolation of Temperature-sensitive Mutants

The basic strategy was identical to that previously described for isolation of nonconditional mutants; i.e. selection with toxin (diphtheria or modeccin) followed by autoradiographic screening of survivors for those deficient in uptake of radiolabeled ligand via the Man 6-P receptor (28). 3 h before selection, cells were shifted to the nonpermissive temperature (39°C); after 24 h toxin was removed and the cells were replated. To circumvent cytotoxicity of cell-associated toxin upon return to the permissive temperature (34°C), three different methods were used: mutant B3853, cells were treated with trypsin plus inositol hexaphosphate to remove surface-associated diphtheria toxin (6); mutant I223, survivors were grown at 39°C for 5 d before return to 34°C to decrease the level of toxin per cell; mutant M311, survivors were grown in the presence of NH_4Cl at 34°C to prevent penetration of cell-associated toxin (17). Replicas of the colonies from the master dishes were assayed for Man 6-P–de-

Table I. Endocytosis in WTB and Mutants at 34°, 39°, and 41°C

Cell	Temperature	Man 6-P Uptake	Toxins		Toxic lectins	
			Diphtheria	Pseudomonas	Modeccin	Ricin
		% WTB, 34°C	*EC_{50} ng/ml*	*EC_{50} ng/ml*	*EC_{50} ng/ml*	*EC_{50} ng/ml*
WTB	34°C	100	37	335	3.7	145
	39°C	90–100	12	68	0.8	82
	41°C	80–100	8	50	0.4	40
B3853	34°C	90–100	35	160	4.0	160
	39°C	10–15	100*	130	>3,000	2.9
	41°C	<10	780	7,000	>1,000	1.9
I223	34°C	55–65	51	265	2.2	78
	39°C	10–15	35	94	>1,000	18
	41°C	<10	70	800	>1,000	2.8
M311	34°C	75–85	66	150	11	25
	39°C	<10	66	110	>1,000	2.7
	41°C	<10	1,100	6,000	>1,000	3.2

Cells were kept at 34°C or shifted to 39° or 41°C for 4 h before assay. Uptake of [^{35}S]secretions was determined as described in Materials and Methods. Results are expressed as percent specific uptake of WTB at 34°C (≡100%) and are given as ranges compiled from many experiments. Inhibition of protein synthesis by toxins and toxic lectins was determined as previously described (28). EC_{50} is the dose required to inhibit protein synthesis to 50% of that measured in parallel samples of untreated cells. Cells were kept at the indicated temperature during the entire course of the uptake or protein synthesis assay.
* At 39°C, resistance to diphtheria toxin has been variable; we ascribe this to slight fluctuations in incubator temperature. In some experiments, including the one shown, protein synthesis in B3853 at 39°C did not decrease below 30% of normal after incubation with up to 6,000 ng/ml toxin. In B3853 at 34°C, diphtheria toxin always reduced protein synthesis to <10% of untreated cells.

pendent uptake at both 34° and 39°C to identify those colonies defective only at the latter temperature.

Phenotypic Changes as a Function of Temperature

Although the mutants were isolated by selection at 39°C, some of the phenotypic changes previously observed in nonconditional mutants (21, 22, 28, 29) required incubation at 41°C. Incubation at 41°C for up to 8 h did not decrease protein synthesis; indeed incorporation of [^{35}S]methionine into protein was 1.4–1.9 times greater at 41°C than at 34°C for all cell types. Viability, measured as subsequent plating efficiency at 34°C, did not decrease after 24 h at 39°C, nor after 5 h at 41°C, but was reduced in both parent and mutants by 25% after 12.5 h at 41°C.

Toxin sensitivity in parental cells (WTB) increased with temperature from 34° to 41°C; Man 6-P receptor-dependent uptake in WTB varied little over this range of temperatures (Table I). At the permissive temperature (34°C), mutant B3853 was very similar to WTB with respect to endocytic activity. Incubation at 39°C for 4 h was sufficient to increase modeccin-resistance of B3853 >750-fold, ricin-sensitivity 50-fold, and to decrease Man 6-P uptake to 10–15%; however, incubation at 41°C was required to effect significant increases in resistance to *Pseudomonas* toxin and diphtheria toxin (Table I). Increasing the time of preincubation at 39°C

Table II. Effect of Inhibition of Protein Synthesis on Response to Toxins and Toxic Lectins in WTB and Mutant Cells

Cell	Cyclo-heximide	Temperature	Toxins		Toxic lectins	
			Diphtheria	Pseudomonas	Modeccin	Ricin
			EC_{50} ng/ml	*EC_{50} ng/ml*	*EC_{50} ng/ml*	*EC_{50} ng/ml*
WTB	−	34°C	62	150	1.4	120
WTB	+	34°C	125	230	1.7	32
I223	−	34°C	125	80	1.2	70
I223	+	34°C	>6,000	>3,000	>1,000	1.2
B3853	−	34°C	110		1.5	115
B3853	+	34°C	280		1.5	60
M311	−	34°C	130		1.6	42
M311	+	34°C	480		5.5	11
WTB	−	41°C	12		0.6	
WTB	+	41°C	70		0.5	
I223	−	41°C	150		>1,000	
I223	+	41°C	>6,000		>1,000	

Cells were incubated at the indicated temperature in the presence or absence of 5 μM cycloheximide, which concentration reduced protein synthesis to <10% after 30 min. After 4 h toxins or toxic lectins were added, again in the presence or absence of cycloheximide. After toxin treatment cells were rinsed three times to remove inhibitor and toxins, then protein synthesis was assayed. Protein synthesis in control cells preincubated with cycloheximide ranged from 65 to 90% of that measured in untreated controls; appropriate controls were used to calculate EC_{50}'s.

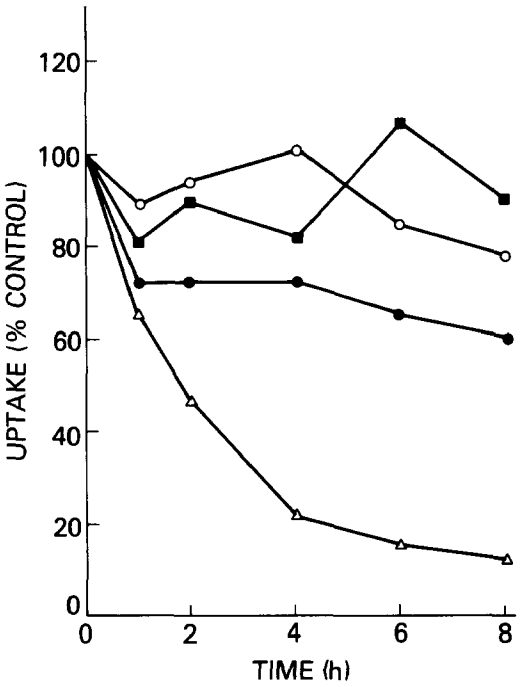

Figure 1. Effect of cycloheximide on Man 6-P–dependent endocytosis. WTB (*solid squares*), B3853 (*solid circles*), I223 (*open triangles*), and M311 (*open circles*) were incubated at 34°C with 5 μM cycloheximide for the indicated times, then assayed for Man 6-P–dependent uptake of [^{35}S] secretions as described in Materials and Methods; cycloheximide was included during the 1-h uptake assay. Results are averages of duplicate assays, normalized to cell protein, corrected for Man 6-P–independent uptake (8,000 cpm/mg cell protein) and expressed as percent uptake of untreated cells. In the absence of cycloheximide Man 6-P–specific uptake was 25,200, 28,700, 18,000, and 29,700 cpm/mg for WTB, B3853, I223, and M311, respectively.

to 48 h did not increase the resistance of B3853 to these toxins. Penetration of diphtheria toxin into the cytoplasm was delayed in B3853 at 39°C; protection from inhibition of protein synthesis could be achieved by addition of 10 mM NH_4Cl to WTB and B3853 up to 7 and 15 min, respectively, following addition of 5 μg/ml toxin (data not shown).

Mutant M311 at 34°C exhibited fivefold higher ricin sensitivity than parental cells; other parameters showed less or no difference. After 4 h at 39°C, M311 resembled mutant B3853; i.e. modeccin resistance, uptake via the Man 6-P receptor and ricin sensitivity all were significantly affected, whereas resistance to diphtheria and *Pseudomonas* toxins was not. In contrast to B3853, prolonged (18 h) incubation of M311 at 39°C did increase resistance to the latter toxins ~30-fold. After 4 h at 41°C, the phenotypes of B3853 and M311 were similar (Table I).

I223 differed from the other mutants in several respects: incubation at 39°C from 4 to 15 h produced only a fourfold increase in ricin sensitivity; 41°C was required to produce maximal sensitivity to this lectin. Even at 41°C, resistance of I223 to diphtheria and *Pseudomonas* toxins was minimal, with most of the difference between parent and mutant reflecting increased toxin sensitivity of WTB, rather than increased resistance of I223 (Table I).

Inhibition of protein synthesis in I223 at 34°C not only produced the phenotypic changes obtained on shifting that mutant to 41°C, but also effected significant increases in resistance to both diphtheria and *Pseudomonas* toxins (Table II and Fig. 1). B3853, M311, and WTB showed little or no changes in either toxin resistance (Table II) or Man 6-P uptake (Fig. 1) after incubation with cycloheximide. It should be noted that inclusion of cycloheximide during the shift of B3853 and M311 to the nonpermissive temperature did not prevent either the loss of Man 6-P uptake or increase in resistance to diphtheria toxin or modeccin (data not shown).

Genetic Complementation Analyses

Hybrids generated by polyethylene glycol–mediated cell fusion were selected for ability to grow in HAT medium containing ouabain, cloned, then tested for modeccin resistance and Man 6-P uptake. As ouabain-resistant, thioguanine-resistant parents in these crosses we used WTB111, a doubly marked variant of WTB; M311, which was isolated from WTB111; and DTG1-5-4-122, a doubly marked variant of DTG1-5-4, a nonconditional mutant whose phenotype at 34°C (29) closely resembles those of B3853 and M311 at 41°C. As shown in Table III, the *ts* defects are recessive in hybrids formed with parental cells. Hybrids comprised of a *ts* mutant and a mutant of the same complementation group should be normal at 34°C and aberrant at 41°C, whereas hybrids from different complementation groups should be normal at both temperatures. B3853 and DTG1-5-4-122 are in one complementation group (designated "End1"), and I223 and M311 are in another ("End2"). Based on its failure to complement M311 in tests of both diphtheria toxin resistance and Man 6-P uptake, the nonconditional mutant DTF1-5-1, previously shown (29) to complement DTG1-5-4, also is a member of End2 (data not shown). I223 × WTB111 and I223 × DTG1-5-4-122 hybrids showed no loss of Man 6-P uptake

Table III. Endocytosis in Cell–Cell Hybrids

Hybrids	Temperature	Modeccin	Man 6-P Uptake
		EC_{50} ng/ml	*% WTB*
B3853 × WTB111	34°C	1.9	97
	41°C	0.9	87
I223 × WTB111	34°C	1.5	80
	41°C	0.5	83
M311 × WTB	34°C	1.3	97
	41°C	0.8	95
B3853 × DTG1-5-4-122	34°C	1.4	94
	41°C	>100	4
B3853 × M311	34°C	1.5	92
	41°C	0.8	94
M311 × DTG1-5-4	34°C	1.1	83
	41°C	0.7	76
I223 × DTG1-5-4-122	34°C	1.6	72
	41°C	0.8	79
I223 × M311	34°C	3.0	85
	41°C	>100	13

Where indicated cells were shifted to 41°C for 4 h before addition of modeccin (0.3 to 100 ng/ml) or [^{35}S] secretions. Inhibition of protein synthesis and Man 6-P uptake were measured as described in Table I. At least three independent cloned hybrids from each fusion were tested; uncloned populations of hybrids gave similar results.

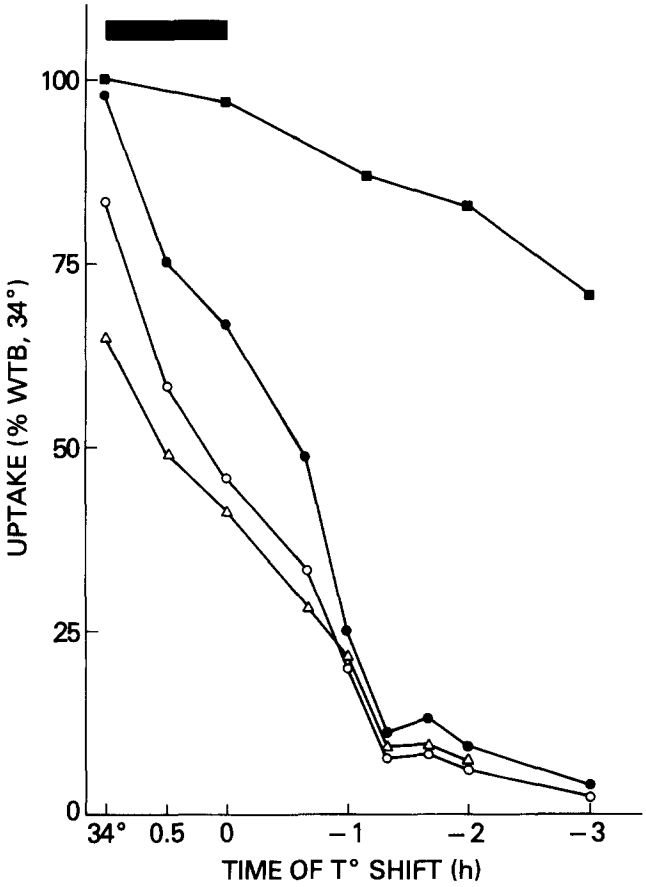

Figure 2. Loss of Man 6-P-dependent endocytosis at 41°C. WTB (*solid squares*), B3853 (*solid circles*), I223 (*open triangles*), and M311 (*open circles*) were kept at 34°C (34° values) or shifted to 41°C before (−h), at (0 h), or after (+h) addition of [^{35}S] secretions; incubation with ligand, indicated by the solid bar, was for 1 h. Results, corrected for nonspecific uptake (12,000–14,000 cpm/mg) and normalized for cell protein, are expressed as percent of Man 6-P-dependent uptake by WTB at 34°C (68,500 cpm/mg).

after incubation with cycloheximide for up to 8 h at 34°C; thus the abnormal dependence of endocytosis on protein synthesis also is recessive.

Sequence of Events after Temperature Shift

Because a wider spectrum of phenotypic changes could be elicited at 41°C, we used that temperature for examination of the kinetics with which activities were lost from the *ts* mutants.

Graphical Presentation. On shifting the mutants from 34° to 41°C some functions were affected at times shorter than were required to assay those functions; in such cases it was necessary to shift temperature during the assays. We express Time of Temperature Shift with respect to initiation of the assay; i.e. 0 h is the time of addition of ligand or radiolabeled metabolite; negative times indicate temperature shifts before addition (e.g., a point at −2 h represents cells shifted to 41°C 2 h before addition of ligand), and positive times indicate shifts after addition. Thus, increasing values for Time of Temperature Shift correspond to decreasing times spent at the nonpermissive temperature.

Man 6-P Receptor-dependent Uptake. After a shift to 41°C, loss of Man 6-P uptake in the three mutants was both early and rapid (Fig. 2). Incubation at 41°C for 0.5 h (i.e., shifting the cells 0.5 h after addition of ligand [+0.5 h]) decreased activity by 25%; shifting the cells 1.5 h before assay (−1.5 h) reduced uptake to <10% of that measured in WTB. Decreased uptake at 41°C did not reflect loss of receptor molecules from the cell surface; lactoperoxidase-catalyzed iodination at 0°C of cells incubated at 41°C for 1.5 and 4 h resulted in 1.6–2.3 and 1.0–1.6 times, respectively, the level of iodinated surface receptors (215 kD) measured in cells kept at 34°C (Fig. 3). At the permissive temperature B3853, M311, and WTB cells had similar amounts of iodinated receptors, while I223 had fewer (Fig. 3).

In WTB cells incubated at 41°C, increased incorporation of ^{125}I into Man 6-P receptors was paralleled by increased surface Man 6-P binding activity (measured after incubating cells with Man 6-P to displace bound endogenous ligands). In contrast, after 4 h at 41°C binding activity on the mutants had decreased to 30–40% of that measured in cells kept at 34°C (Table IV). Loss of surface binding activity was slower than loss of Man 6-P uptake; 1.5 h at 41°C reduced uptake to <10% (Fig. 2), while binding was 70–90% (Table IV).

We observed two types of surface Man 6-P binding sites: addition of Man 6-P displaced ligand from one type of site, but not from the other. Preliminary characterization of nondissociable binding activity indicated that it was inhibited ~50% in the presence of 10 mM glucose 1-phosphate, glucose 6-phosphate or mannose 1-phosphate; neither glucose nor mannose effected inhibition. After incubation at 41°C, B3853 showed decreased activity of both types of binding sites, whereas the End2 mutants I223 and M311 primarily lost nondissociable binding activity (Table IV); none of the mutants showed the increase in dissociable binding activity observed on shifting WTB to 41°C. The relationship between dissociable and nondissociable Man 6-P binding sites, if any, is unknown.

At 41°C, mutant and parent cells synthesized the 215-kD Man 6-P receptor in similar amounts, based on incorporation of [^{35}S]methionine during a 2-h pulse. In the mutants at 41°C, no increased degradation of receptor synthesized at either 41°C or at 34°C was observed (data not shown). In contrast to the cell surface, total membranes from mutant cells shifted to 41°C for 4 h before harvest had 50–100% (variability among membrane preparations, not cell types) of normal Man 6-P binding, measured as divalent cation–independent, Man 6-P–dissociable binding activity (data not shown).

Compartmentalization of Acid Hydrolases. At 41°C, the mutants divert newly synthesized acid hydrolases to a secretory pathway. Cells were pulsed for 15 min with [^{35}S]methionine, radioactivity was chased, and β-glucuronidase and MEP, a thiol-dependent acid protease (8), were immunoprecipitated from media and cell extracts. In WTB at 41°C, secretion and/or lysosomal compartmentalization of MEP was nearly complete after 1 h of chase (data not shown). Shifting the mutants to 41°C at the time of or prior to the pulse caused secretion of essentially all the newly synthesized MEP during a 1-h chase (Fig. 4). Thus, less than 1.25 h at 41°C is required for the mutants to divert MEP to the secretory pathway. In I223, over-secretion of MEP could also be induced at 34°C by pretreatment with cycloheximide for 4 h (data not shown).

Analysis of secretion of β-glucuronidase is complicated by two factors: first, although this enzyme begins to appear in

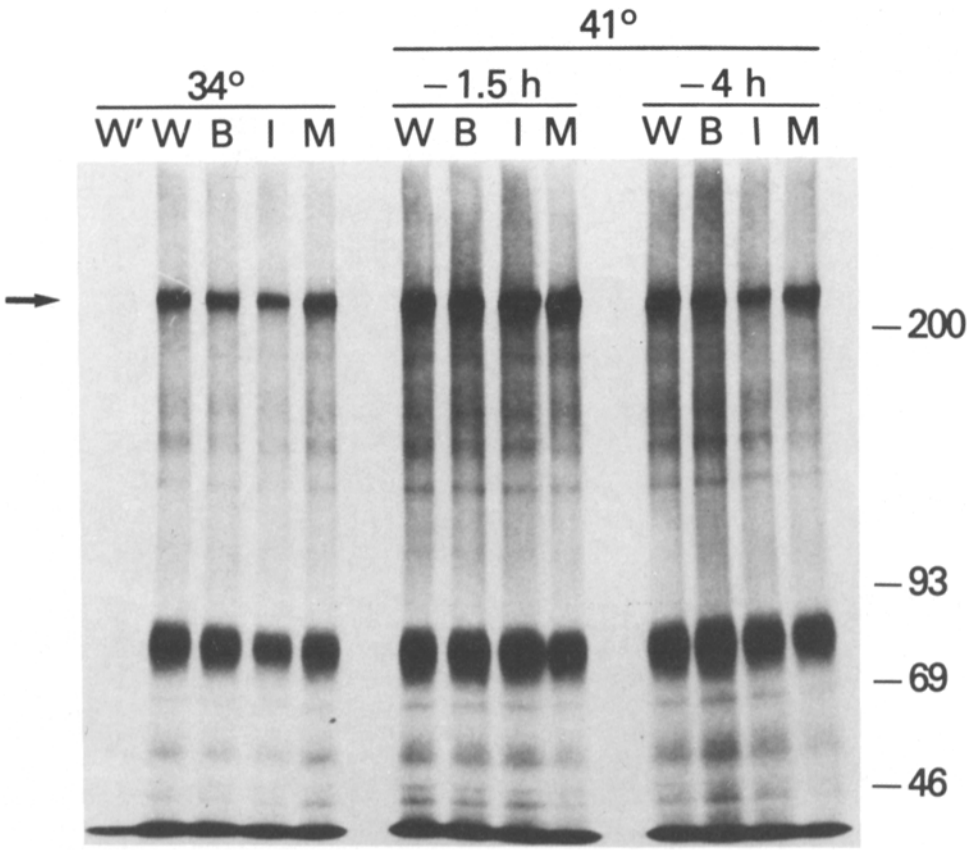

Figure 3. Mannose 6-phosphate receptor (215 kD) at the cell surface at 41°C. WTB, B3853, I223, and M311 (lanes *W, B, I,* and *M*, respectively) were kept at 34°C (*34°* lanes) or shifted to 41°C for 1.5 h (*−1.5 h* lanes) or 4 h (*−4 h* lanes); then chilled to 0°C for lactoperoxidase-catalyzed surface iodination. Man 6-P receptor, indicated by the arrow on the left, was immunoprecipitated and electrophoresed on SDS–5–7% gradient polyacrylamide gels. A parallel extract of WTB cells was carried through the immunoprecipitation procedure without antiserum (lane *W'*). Numbers on the right indicate the positions of standards, molecular masses ($\times 10^{-3}$). The identity of the prominent radioactive band M_r 82,000 is unknown. This polypeptide was not observed when antiserum was either omitted from the precipitation procedure (lane *W'*) or replaced by preimmune serum, or when receptor was immunoprecipitated from extracts of [^{35}S]methionine-labeled cells (data not shown); nor was it previously observed in immunoprecipitates of iodinated cells obtained with a different anti-Man 6-P receptor serum (see reference 28).

the medium (and the lysosomes in WTB) after a 1 h chase, it continues to accumulate in these compartments for >4 h; second, in CHO cells, >50% of the β-glucuronidase remains in a precursor form associated with light membranes (Fraction I, see Fig. 7); this portion of β-glucuronidase was unaffected in the mutants at 41°C. After shifting cells to 41°C before or at the time of the radioactive pulse, then immunoprecipitating β-glucuronidase from medium and cell extracts after a 5-h chase, we observed similar amounts of precursor β-glucuronidase (73 kD) in mutant and WTB cells. But, cell extracts of WTB also contained a mature form (71 kD) of the enzyme while the mutants were devoid of this form; instead they showed compensating amounts of precursor β-glucuronidase in the medium (Fig. 4). β-glucuronidase secreted by the mutants at 41°C was found to be endocytosed and proteolytically processed by recipient WTB cells, with uptake inhibitable by Man 6-P (data not shown). Thus, secretion of the enzyme by the *ts* mutants does not reflect faulty posttranslational modification of acid hydrolases.

Accumulation of Iron from Transferrin. Shifting the mutants to 41°C at the initiation of a 2-h uptake of ^{59}Fe-transferrin resulted in one-half maximal inhibition of ^{59}Fe accumulation; a shift to 41°C 2 h before assay produced maximal inhibition (Fig. 5). In B3853 and M311, the level of cell-associated ^{59}Fe observed at maximal inhibition appeared to reflect the amount of ^{59}Fe-transferrin bound; after 3 h at 41°C, cell-associated radioactivity in those mutants did not increase in assays of uptake ranging from 15 to 120 min. By the same criterion, the higher levels of cell-associated ^{59}Fe measured in I223 at maximal inhibition appeared to result from residual ability to accumulate ^{59}Fe. Comparison of cell-associated ^{125}I-transferrin in WTB and B3853 following a shift to the nonpermissive temperature revealed no differences in the number of transferrin-binding sites (data not shown).

Endocytosis and Degradation of α_2-Macroglobulin. After a brief increase in activity after temperature shift, degradation of internalized α_2-macroglobulin decreased in the mutants at 41°C (Fig. 6). Cells were pulsed with ligand for 15 min, then ligand was chased for 2 h. After 3 h at 41°C, uptake of α_2-macroglobulin, estimated as the sum of TCA-soluble and -precipitable radioactivity from the medium, plus cell-associated radioactivity, was <25% of that observed at 34°C; the significance of the latter two parameters was difficult to assess because values obtained were ≤2 times those observed in the presence of excess ligand.

Transport of Endocytosed Ligand through Intracellular Compartments. At 34°C, ligands endocytosed via the Man 6-P receptor were similarly distributed in B3853 and WTB cells (Fig. 7). After uptake for 1 h, radiolabeled ligand was

Table IV. Cell Surface Man 6-P Binding Activity in WTB and Mutants

Cell	Time at 41°C	Total	Dissociable	Nondissociable
	h	*% 34°C*	*cpm/mg*	*cpm/mg*
WTB	0 (34°C)	100	2,530	4,750
WTB	1.5	140	5,050	5,340
WTB	4	140	6,690	3,490
B3853	0	100	3,190	6,530
B3853	1.5	70	3,350	3,380
B3853	4	30	1,460	1,880
I223	0	100	1,630	3,700
I223	1.5	90	2,340	2,500
I223	4	40	1,840	370
M311	0	100	2,560	5,920
M311	1.5	70	2.660	3,100
M311	4	30	1,700	970

Binding of [^{35}S] secretions was determined at 4°C as described in Materials and Methods. Two types of ligand binding were measured: *Dissociable* indicates ligand that could be displaced from the cells by incubation in 10 mM Man 6-P for 30 min; *Nondissociable* indicates ligand that remained cell-associated after treatment with Man 6-P. Total binding is presented as a ratio of the sum of dissociable and nondissociable binding measured for a given condition to the sum obtained with samples incubated at 34°C. Results are from duplicate determinations, normalized for cell protein and corrected for nonspecific binding measured in the presence of 10 mM Man 6-P (980 and 2,900 cpm/mg for dissociable and nondissociable binding, respectively).

found in three subcellular fractions, separable on sequential Percoll gradients. Characterization of these fractions for marker enzymes (see Materials and Methods) revealed that fraction I (light membranes) contained 90, 80, 70, and 20% of the activities of plasma membrane, endoplasmic reticulum, Golgi and lysosomes, respectively; fraction I also contained >90% of the endosomes, based on the sedimentation of internalized FITC-transferrin (see below); fraction II (light lysosomes [31]) contained <2% of plasma membrane and endoplasmic reticulum, ~5% of Golgi apparatus and 17% of lysosomal enzyme activities; fraction III (dense lysosomes) contained 43% of the lysosomal marker, and <2% of the other three marker enzymes. After a 30-min chase, the level of ligand decreased in fractions I and II and increased in fraction III; some radioactivity was lost on the chase, presumably due to proteolytic processing of endocytosed hydrolases, with consequent loss of [^{35}S]methionine.

Shifting B3853 to 41°C for 15 min before a 45-min incubation with [^{35}S]secretions resulted in radioactivity sedimenting almost exclusively with fraction I; most of the radioactivity remained in this fraction after a 30-min chase (Fig. 7). Because cells were incubated with Man 6-P at 0°C before harvest, we assume that this radioactivity is in endosomes, rather than on the plasma membrane. However, we cannot

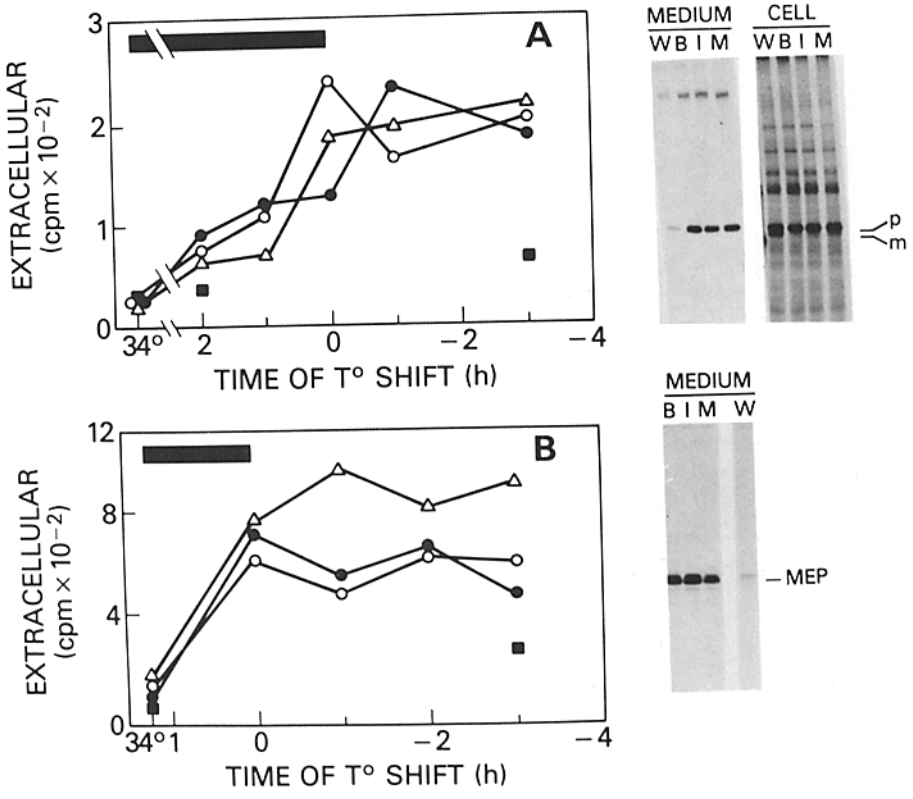

Figure 4. Secretion of β-glucuronidase and MEP at 41°C. WTB (*solid squares*), B3858 (*solid circles*), I223 (*open triangles*), and M311 (*open circles*) were pulsed with [^{35}S]methionine (100 μCi/ml per well) for 15 min. (*A*) After a 5-h chase, β-glucuronidase was immunoprecipitated from the medium and electrophoresed on 7% SDS polyacrylamide gels. Radioactive bands were located by fluorography and precursor β-glucuronidase (*p*, M_r 73,000) was excised from the gel, solubilized and counted. A fluorograph of immunoprecipitates from media and cell extracts after a 3-h shift to 41°C (*−3 h*) are shown on the right; note the absence of labeled mature β-glucuronidase (*m*, M_r 71,000) in cell lysates of B3853, I223, and M311 (*B*, *I*, and *M*, respectively). Molecular masses of precursor and mature β-glucuronidase are similar to those reported for mouse macrophages (35). (*B*) Radioactivity was chased for 1 h and MEP was immunoprecipitated from the medium and electrophoresed on 10% SDS polyacrylamide gels; bands of M_r 37,000 and 39,000 were excised and counted. The gel of immunoprecipitates from the media from B3853, I223, and M311 after a 1-h shift to 41°C is shown on the right. Also shown is the lane for WTB (*W*) after a 3-h shift to 41°C before the pulse. Bars indicate the times of pulse-chase with [^{35}S]methionine.

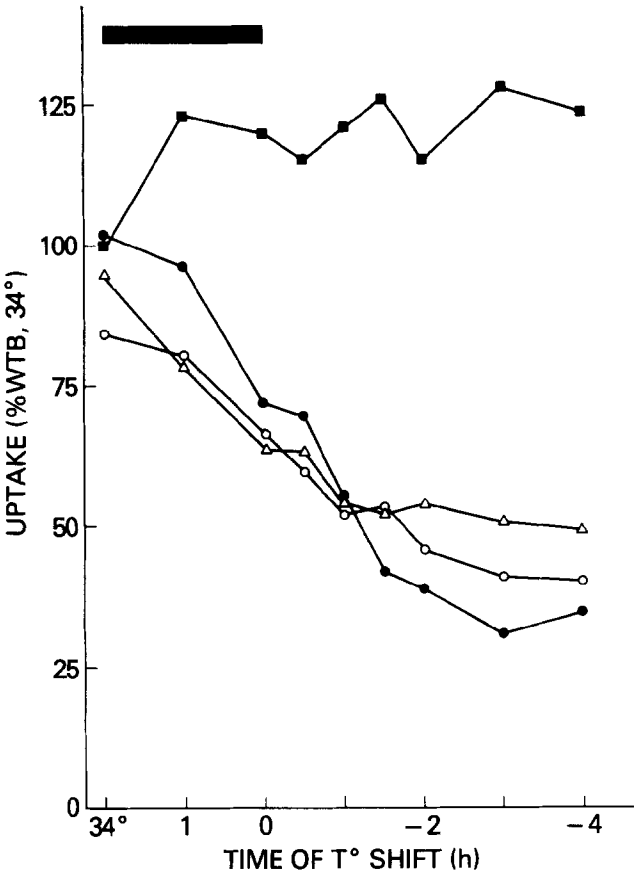

Figure 5. Loss of transferrin-mediated Fe accumulation at 41°C. After incubation at 41°C for the indicated times WTB (*solid squares*), B3853 (*solid circles*), I223 (*open triangles*), and M311 (*open circles*) were incubated with ^{59}Fe-transferrin (10 μg/ml per well, 3.8 × 10^4 cpm/μg) for 2 h, indicated by the bar. Uptake was determined as described in Materials and Methods. Results are averages of duplicate assays, normalized to cell protein, corrected for nonspecific ^{59}Fe accumulation (6,600 cpm/mg cell protein) and expressed as percent specific uptake of WTB at 34°C (79,700 cpm/mg cell protein).

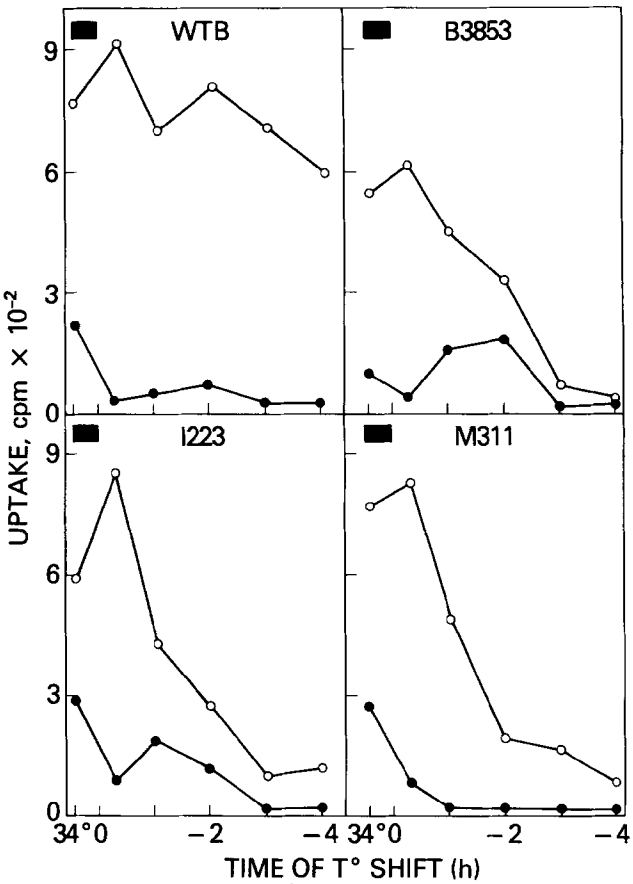

Figure 6. Loss of uptake of α_2-macroglobulin at 41°C. WTB, B3853, and I223 were seeded at 1.7 × 10^5 and M311 at 1.8 × 10^5 cells/well in 6-well dishes and assayed 2.5 d later. Cells were incubated with ^{125}I-labeled α_2-macroglobulin (2 μg/l ml per well, 2 × 10^3 cpm/ng) for 15 min, indicated by the bars, then washed to remove free ligand. Radioactivity was chased for 2 h, the cells and media were harvested, and radioactivity was determined as described in Materials and Methods. Cell-associated α_2-macroglobulin (*solid circles*) is radioactivity from NaOH-solubilized cells; degraded α_2-macroglobulin (*open circles*) is soluble radioactivity obtained after TCA precipitation of the media. All values are averages of duplicate assays, corrected for uptake in the presence of excess (200 μg/ml) unlabeled α_2-macroglobulin (210 and 260 cpm/well for degraded and cell-associated α_2-macroglobulin, respectively), and are expressed as cpm/well.

rule out the contribution of nondissociably bound ligand (see Table IV).

Immunocytochemical studies have shown that endocytosed lysosomal hydrolases and α_2-macroglobulin follow the same pathway to lysosomes (43). In contrast to ligand taken up via the Man 6-P receptor, α_2-macroglobulin endocytosed by B3853 at 41°C continued to be transported from light membranes to both light and dense lysosomes (Fig. 8). Transport was also observed in B3853 shifted to 41°C 1.5 h before the addition of ligand (data not shown).

Response to Toxins and Toxic Lectins. Increased resistance of B3853 and M311 to diphtheria and *Pseudomonas* toxins required incubation for 2 h at 41°C before addition of toxin; maximal resistance was attained after a 4-h shift (Fig. 9). I223 showed little or no change at 41°C (see also Table I). All three mutants exhibited a very abrupt increase in modeccin resistance: after 1 h at 41°C, dose–response curves for mutant and parental cells were identical; after 2 h at 41°C modeccin resistance of the mutants had increased more than 3,000-fold (Fig. 9). Increased sensitivity of B3853, I223, and M311 to ricin occurred after 15, 45, and 75 min at 41°C, respectively (Fig. 9). Maximal sensitivity was reached after 105 min at 41°C.

ATP-dependent Acidification of Endosomes. Mutant and WTB cells maintained at 34°C or at 41°C for 4 h were pulse-labeled with FITC-dextran (a marker of fluid phase endocytosis) for 10–15 min at the respective temperature, then fractionated by Percoll density gradient centrifugation to separate endosomes and lysosomes (see Fig. 7). Endosomes isolated from WTB grown at 34° and 41°C exhibited the same rate and extent of acidification (15% quenching of initial fluorescence); endosomes from all three mutant cell lines were found to have reduced acidification activity after growth for 4 h at 41°C (Table V). B3853 endosomes were the most severely affected, exhibiting virtually no quenching of FITC-dextran fluorescence after ATP addition. With M311 and I223, growth at 41°C resulted in 30 and 50% decreases,

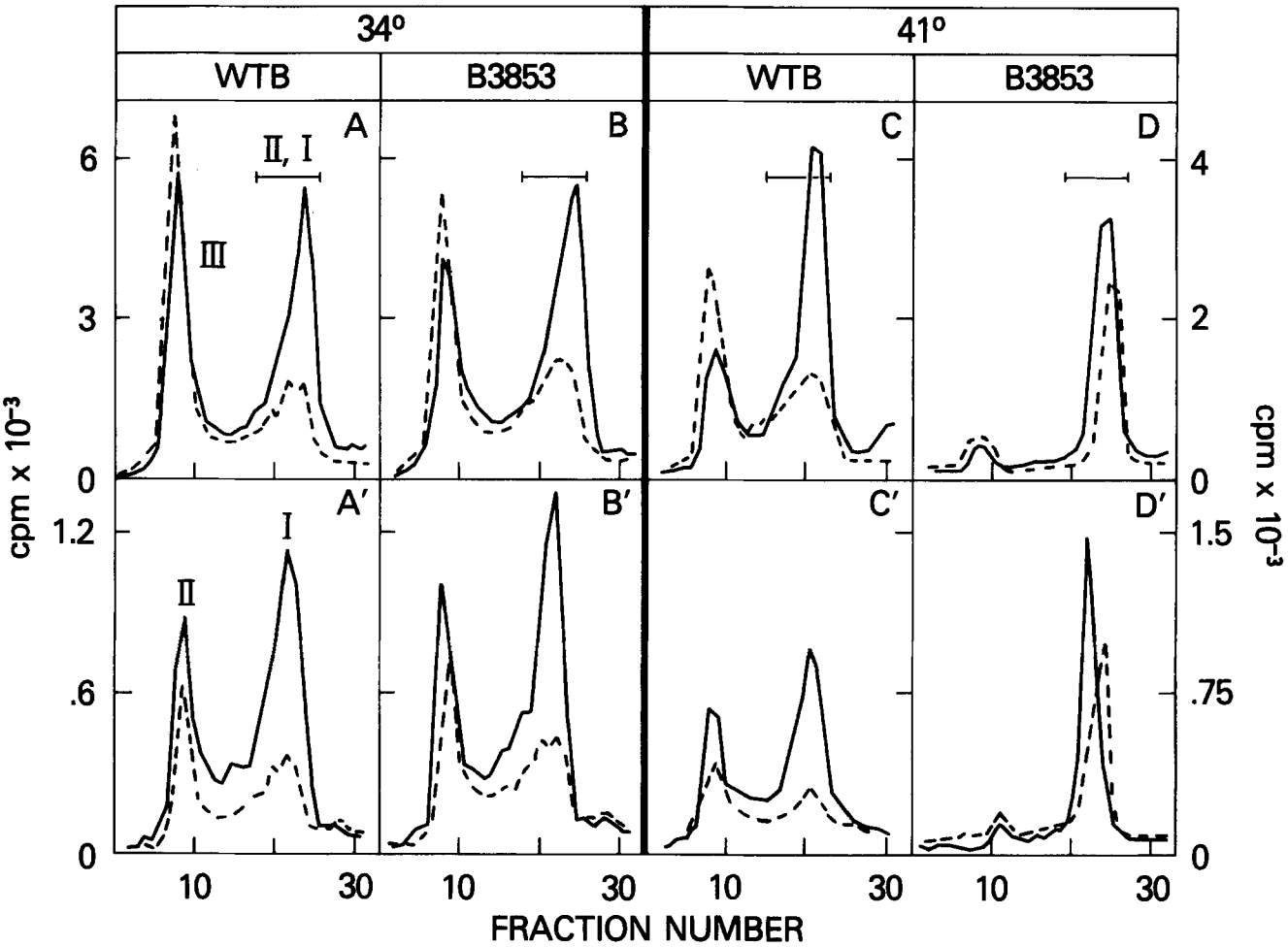

Figure 7. Effect of 41°C on intracellular transport of endocytosed acid hydrolases. Cells in 100-mm dishes were incubated at 34°C with 4 ml [^{35}S] secretions, 5 × 10^5 cpm/ml, for 1 h (*A*, *A'*, *B*, and *B'*), or shifted to 41°C for 15 min, then incubated with [^{35}S] secretions for 45 min at 41°C (*C*, *C'*, *D*, and *D'*). Samples were harvested at the end of incubation with ligand (*solid lines*) or after a 30-min chase (*broken lines*). Before harvest, cells were washed for 20 min with 10 mM Man 6-P at 4°C to remove surface-bound ligand. Postnuclear supernates were fractionated, as described in Materials and Methods, on 27% (*A*, *B*, and *C*) or 29% Percoll gradients (*D*); the peak of lower density (I + II, indicated by bars) was further fractionated on 17% Percoll gradients (*A'*, *B'*, *C'*, and *D'*). The source of peak II (i.e., 27 vs. 29% gradients) did not affect its subsequent sedimentation on 17% gradients. Inclusion of 10 mM Man 6-P during incubation with [^{35}S] secretions reduced radioactivity in the peak fractions by >90%. Results are from three experiments: (No. 1) WTB (*A* and *A'*) and B3853 (*B* and *B'*) at 34°C; (No. 2) WTB at 41°C (*C* and *C'*); (No. 3) B3853 at 41°C (*D* and *D'*). Fractions are numbered from bottom to top of the gradients; total radioactivity for each fraction (1 ml), normalized for number of cells harvested, is shown.

respectively, in ATP-driven acidification as compared to 34°C controls. Especially in the case of I223, the 34°C controls were themselves reduced as compared to WTB. Because endocytosis in I223 at 34°C depends on continued protein synthesis (Fig. 1 and Table II) it is possible that the activity of a labile protein was decreasing during preparation and/or pre-equilibration of endosomes.

Endosomal acidification was also measured using FITC-labeled transferrin, a probe chosen because release of transferrin-bound iron was inhibited in mutant cells at 41°C (Fig. 5), yet normal ligand binding was retained. Also, transferrin is not transported to lysosomes (41). This result was confirmed with CHO cells; homogenates prepared from cells labeled with FITC-transferrin at 34° or 41°C then stripped of surface-associated ligand were centrifuged in 17% Percoll gradients (see Figs. 8 and 9); label was found exclusively in fraction I (data not shown).

FITC-transferrin-labeled endosomes isolated from B3853 and M311 grown at 41°C for 2 or 4 h exhibited reductions in ATP-dependent acidification activity when compared to 34°C controls or endosomes from WTB. As shown in Fig. 10, acidification of B3853 endosomes decreased to ~50 and <10% after 2 and 4 h, respectively, at 41°C (Fig. 10, *A* and *B*). Lesser reductions were observed with M311 endosomes, 60 and 40% of 34°C controls (Fig. 10, *C* and *D*). As was found using FITC-dextran (Table V), acidification of FITC-transferrin-labeled endosomes from M311 grown at 34°C was also reduced relative to controls.

Golgi-associated Functions. The initial observation of a single genetic defect altering both Golgi and endocytic functions was made with the nonconditional Endl mutant DTG1-5-4; that mutant failed to galactosylate E1 and E2 of Sindbis virus (29). No endogenous protein was found to exhibit decreased levels of galactose (Robbins, A. R., unpublished data); however several secreted proteins from this mutant appeared to be less sialylated. These proteins migrate more

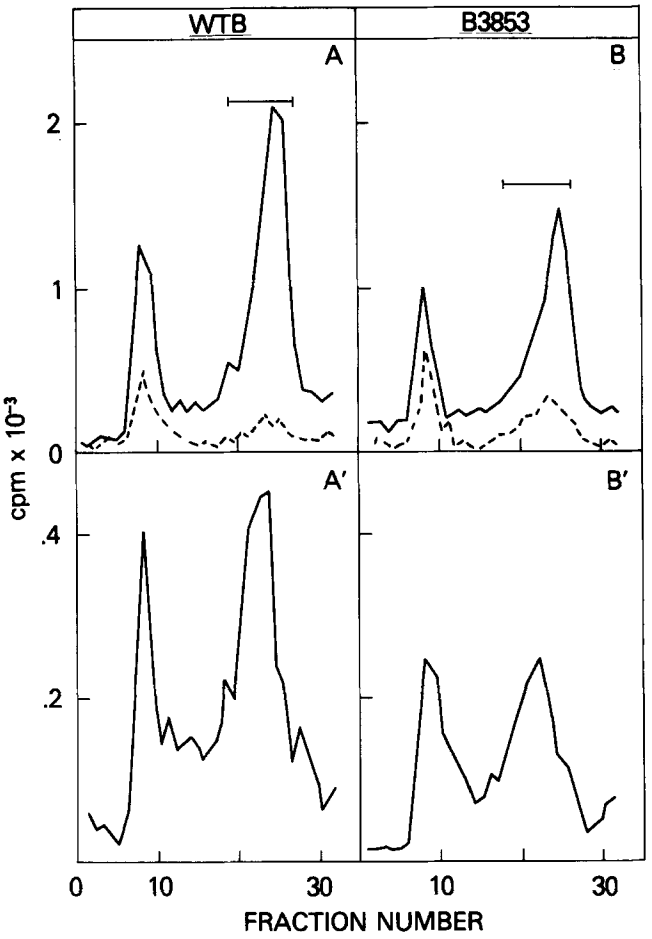

Figure 8. Intracellular transport of endocytosed α_2-macroglobulin at 41°C. WTB (*A* and *A'*) and B3853 (*B* and *B'*) were shifted to 41°C for 15 min, then incubated with 2 μg/ml ^{125}I-α_2-macroglobulin for 45 min. One set of samples was harvested at the end of incubation with ligand (*solid lines*), the other after a 30-min chase (*broken lines*). Postnuclear supernates (from ~8 × 10^6 cells) were fractionated as described in Materials and Methods on 27% Percoll gradients (*A* and *B*); the peak of lower density (I + II; indicated by bars) was further fractionated on 17% Percoll gradients (*A'* and *B'*). Fractions (1 ml) are numbered from bottom to top of gradients and the total radioactivity for each fraction is given.

rapidly on SDS gels than their normal counterparts (Fig. 11 *A*); treatment with neuraminidase abolished the increased electrophoretic mobility of the secreted 92-kD protein from DTG1-5-4 (Fig. 11 *B*). Similar results were obtained with fibronectin (data not shown).

Sialylation appeared to be most affected in M311 (Fig. 12, *A–C*). Shifting to 41°C at 0 time was sufficient to effect a change in the protein from M311; preincubation for 3 h at 41°C produced maximal loss of sialylation. I223 and B3853 required 1 and 2 h, respectively, at 41°C to initiate alteration in the 92-kD protein and 2 and 3 h, respectively, for maximum effect. Note that some sialylation of the 92-kD protein occurred even under conditions of maximal inhibition; i.e., much of the labeled protein isolated from mutant cells incubated at 41°C electrophoresed to a position between that of untreated and neuraminidase-treated 92-kD protein from WTB. Identical results were obtained using neuraminidase Type X from *Clostridium perfringens*.

Decreased sialylation of the 92-kD protein also was observed in M311 and B3853 after 2–4 and 4–8 h at 39°C, respectively; I223 was inconsistent in response to 39°C, sometimes showing an effect, sometimes not. After incubation of I223 with cycloheximide for 4 h at 34°C, 92-kD protein, labeled during a 15-min pulse commenced 15 min after removal of the inhibitor, also exhibited undersialylation. The other *ts* mutants and WTB secreted normal 92-kD protein under these conditions (data not shown).

The decreased levels of sialic acid on the protein secreted by the mutants at 41°C does not result from removal of sialic acid after secretion. As shown in Fig. 12 *D*, labeled protein from parental cells was not altered after incubation with the mutants. The 92-kD protein secreted by M311 at 41°C consistently showed some increase in electrophoretic mobility even after neuraminidase treatment (Fig. 12, *A–C*), irrespective of the presence of pepstatin and phenylmethylsulfonyl fluoride (data not shown). This difference was not observed with the other mutants or with other undersialylated proteins from M311 (see below).

Fibronectin and Sindbis virus glycoproteins E1 and E2, immunoprecipitated from the mutants, also exhibited increased electrophoretic mobilities, abolished by treatment with neuraminidase (data not shown). Sialylation appeared to be most altered in M311 and least in I223; even at 34°C fibronectin and Sindbis glycoproteins from M311 exhibited some decrease in sialic acid content, whereas fibronectin from I223 appeared identical to that from WTB even after preincubation of the mutant for 6 h at 41°C. Decreased sialylation of Sindbis glycoproteins by I223 and B3853 and of fibronectin by the latter mutant was observed after 2–3 h at 41°C.

Although the *ts* mutants responded like DTG1-5-4 with respect to sialylation, after 6 h at 41°C or 24 h at 39°C, no decrease in galactosylation of Sindbis virus glycoproteins was observed. In addition, the reduced release of Sindbis reported for all the nonconditional mutants (29) could be effected in the *ts* mutants only after prolonged (>24 h) preincubation of the cells at 39°C (data not shown).

Discussion

The three CHO mutants, B3853, I223, and M311, described here exhibit temperature-sensitive, pleiotropic defects in

Table V. ATP-dependent Acidification of Endosomes from WTB and Mutants

Cell	Fluorescence quenching		Inhibition
	34°C	41°C	
	%	%	%
WTB	15	15	0
B3853	12	0	100
I223	8	4	50
M311	11	8	30

Cells were maintained at 34°C or shifted to 41°C for 4 h before labeling with FITC-dextran at the indicated temperature. ATP-dependent acidification of endosomal fractions was measured as described in Materials and Methods. The data are expressed as the maximum amount of FITC fluorescence quenching relative to initial fluorescence, (observed 5–7 min after addition of ATP). After acidification, FITC fluorescence could be restored to initial values by addition of 1–2 μM nigericin or carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone.

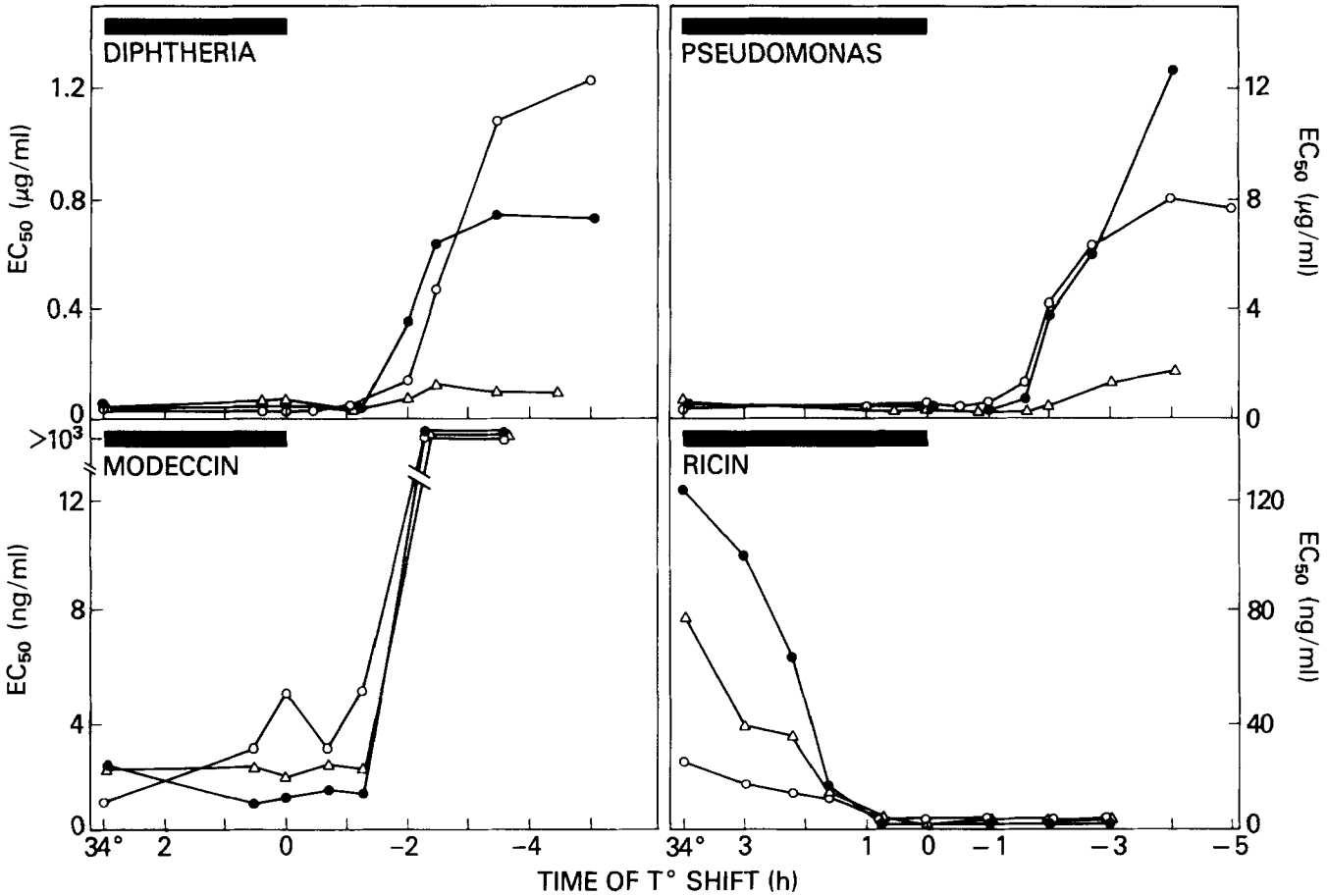

Figure 9. Responses of the mutants to toxins and toxic lectins at 41°C. After temperature shifts for the times indicated, dose-responses of B3853 (*solid circles*), I223 (*open triangles*), and M311 (*open circles*) to modeccin, *Pseudomonas* toxin, diphtheria toxin, and ricin were measured as described in Table I, and the EC_{50} was determined for each agent with each mutant at each time point. The solid bars indicate the times of incubation with the toxic agents and with [^{35}S]methionine.

receptor-mediated endocytosis. These mutants fall into the two genetic complementation groups previously defined (29) by the nonconditional mutants DTG1-5-4 and DTF1-5-1: B3853 and DTG1-5-4 are in one group—End1; I223, M311, and DTF1-5-1 are in the other—End2. No significant differences in endocytosis were observed between the more defective members (B3853, DTG1-5-4, M311) of the two complementation groups with respect to either the spectrum of ligands affected or the levels of residual activity. With both B3853 and M311, ATP-dependent acidification of endosomes, measured in vitro, decreased with increasing time at the nonpermissive temperature. Defective acidification was observed using both FITC-dextran, a fluid-phase tracer destined for lysosomes, and FITC-transferrin, a receptor-bound ligand that recycles from prelysosomal organelles (41). As was observed previously with the End1 mutant DTG1-5-4 (29), in B3853 loss of endocytosis in vivo correlated with essentially complete loss of endosomal acidification in vitro; in contrast, some residual acidification activity remained in endosomes isolated from M311. Because B3853 and M311 are so closely matched with respect to the degree of their endocytic defects, we assume that the disparity in their levels of endosomal acidification in vitro reflects the nature of the affected genetic loci, rather than the relative severity of the genetic lesions in these two mutants. Whether the acidification defect represents the primary lesion in either End1 or End2 mutants is still unknown. We have been unable to measure acidification of endosomes incubated at the nonpermissive temperature in vitro.

The lesion in I223 appears to result in accelerated turnover of the affected component, since the defective phenotype of this mutant could be reproduced at the permissive temperature by inhibition of protein synthesis. In fact, inhibition of protein synthesis produced a wider range of alterations in endocytosis than did incubation at 41°C, suggesting that at 41°C, new synthesis of the defective protein compensates in part for its lability. Endocytosis in M311, also End2, was unaffected by inhibition of protein synthesis.

At the nonpermissive temperature, decreased sialylation of some glycoproteins was observed with both End1 and End2 *ts* mutants. Comparison of membrane versus secreted proteins (labeled with either mannose or methionine) indicated that decreased sialylation occurred far more frequently with secreted glycoproteins (data not shown); with no protein did we observe complete abolition of sialylation. If the rate of sialylation were decreased in the endocytosis mutants, secreted proteins might be expected to be more affected because they are irreversibly removed from the site of sialylation, whereas membrane proteins may recycle through the appropriate cellular compartment (37), thus, increasing their chances of sialylation.

One of the reasons for isolating *ts* mutants was to order,

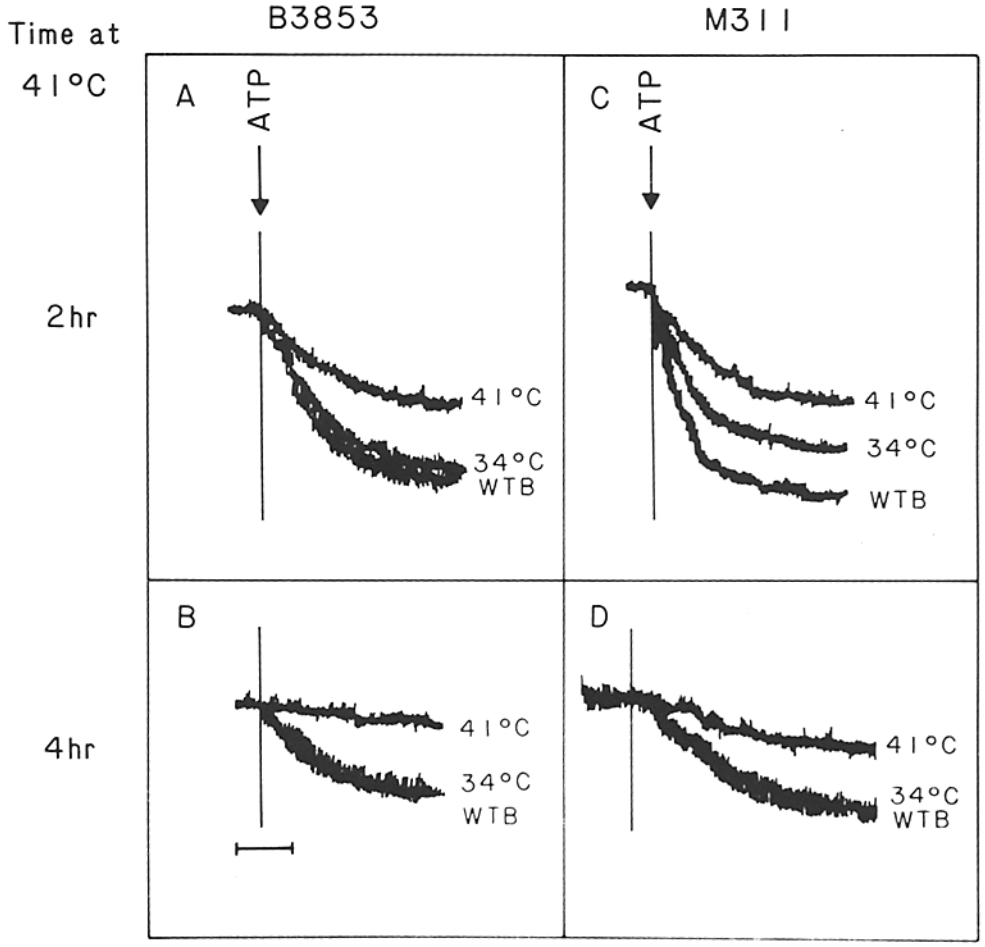

Figure 10. ATP-dependent acidification of FITC-transferrin labeled endosomes from WTB and mutant cells grown at 34° and 41°C. FITC–transferrin-labeled endosomes were isolated from cells which had been maintained at 34°C or shifted to 41°C for 2 or 4 h (including the 30-min labeling period). Fluorescence intensity (at 515 nm) was determined following addition of ATP. Each panel shows the fluorometer traces obtained from parallel cultures of a mutant grown at 34° and 41°C as well as of WTB grown at 34°C (identical results were obtained with WTB grown at 41°C). After 2 and 4 h at 41°C, ATP-dependent acidification of B3853 endosomes decreased to 53 and <10%, respectively, of that exhibited by endosomes obtained from B3853 grown at 34°C (*A* and *B*). Acidification of M311 endosomes decreased to 59 and 40% of the 34°C controls after 2 and 4 h, respectively (*C* and *D*). Bar, 1 min.

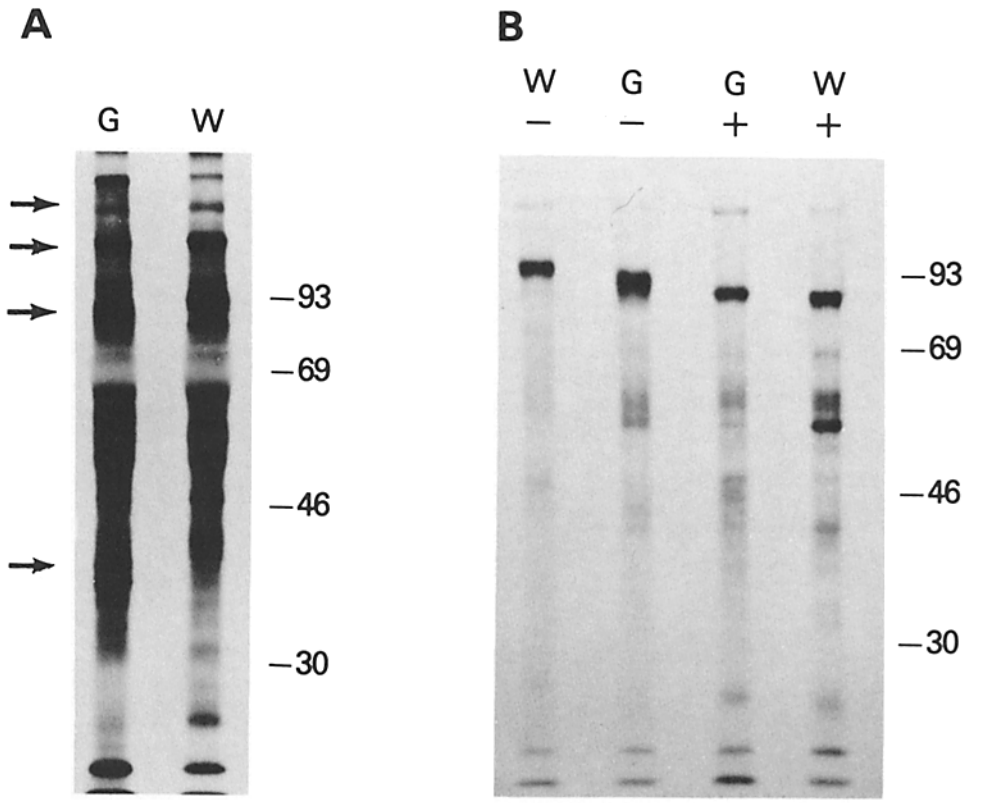

Figure 11. Decreased sialylation of proteins secreted by the nonconditonal Endl mutant DTG1-5-4. Cells in 6-well trays at 34°C were pulsed with 100 μCi [^{35}S]methionine for 15 min and radioactivity was chased for 90 min, all in serum-free medium containing 2 mg/ml BSA. (*A*) Samples of de-albuminated media were TCA precipitated (25) and electrophoresed on 10% SDS polyacrylamide gels. Numbers on the right indicate molecular mass standards (×10^{-3}). Arrows on the left indicate those proteins from DTG1-5-4 (lane *G*) whose electrophoretic mobilities appear increased with respect to the corresponding proteins from WTB (lane *W*). (*B*) 92-kD protein secreted by WTB (lanes *W*) and DTG1-5-4 (lanes *G*) was prepared as described in Materials and Methods. Parallel samples were incubated with (+) or without (−) neuraminidase (*Vibrio cholerae*) before precipitation and electrophoresis. Numbers on the right indicate molecular mass markers (×10^{-3}).

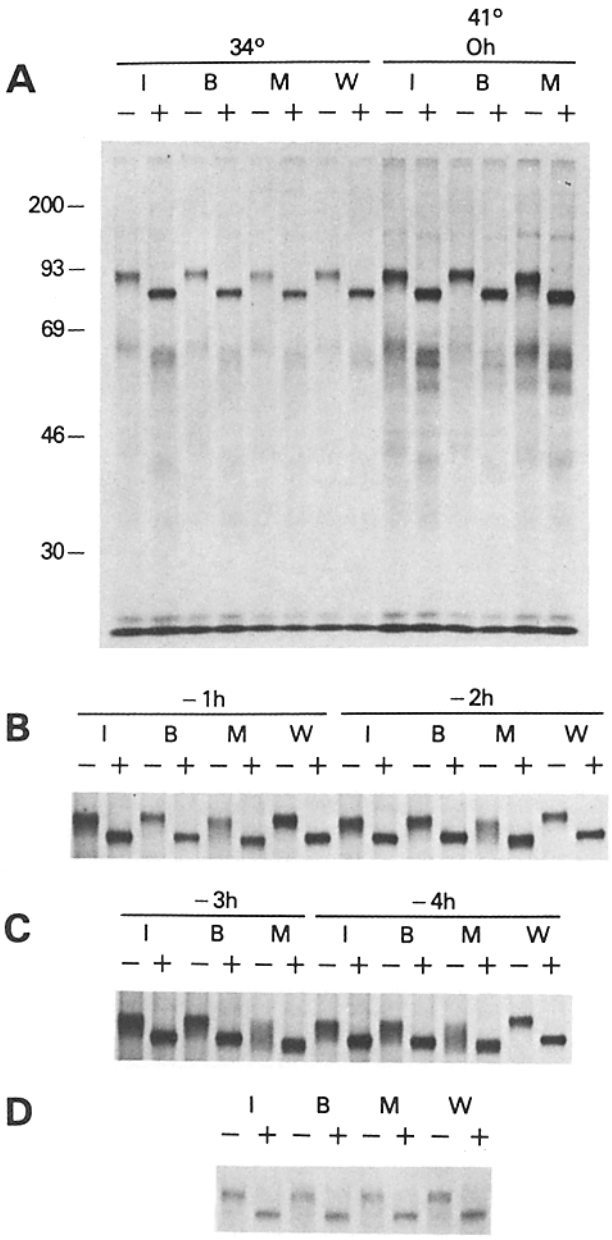

Figure 12. Decreased sialylation of a secreted protein at 41°C. (*A–C*) Cells in 6-well trays were maintained at 34°C or shifted to 41°C at the time of (*0 h*) or before (*−h*) a 15-min pulse with [^{35}S]methionine. Radioactivity was chased for 90 min, then the 92-kD protein was prepared from media samples as described in Materials and Methods. Parallel samples were incubated with (+) or without (−) neuraminidase (*Vibrio cholerae*) before precipitation and electrophoresis. Numbers on the left indicate molecular mass standards ($\times 10^{-3}$). Bands present in the 46–68-kD region of *A* were also present in the fluorographs from which *B* and *C* were taken; changes in electrophoretic mobilities of these proteins paralleled those observed with the 92-kD protein. (*D*) Media were removed from four wells of WTB after a 15-min pulse and 90-min chase at 34°C and transferred to wells containing mutant or WTB cells that had been shifted to 41°C 4 h earlier. After 90-min, media were removed, then 92-kD protein was prepared and incubated with or without neuraminidase as above. (Lanes *I*, *B*, *M*, and *W*) I223, B3853, M311, and WTB, respectively.

based on time of appearance after shift to the nonpermissive temperature, some of the many phenotypic changes previously observed in nonconditional endocytosis mutants (28, 29, 36). Although changes in the mutants occurred rapidly and quite synchronously, several distinctions did emerge. (*a*) Loss of cell surface–associated Man 6-P binding activity was significantly slower than loss of Man 6-P–dependent uptake; thus, loss of binding does not cause loss of uptake. (*b*) In the Endl mutant B3853 the earliest effect on endocytosis was loss of transport of endocytosed acid hydrolases to lysosomes; this occurred within minutes of the temperature shift. Because intracellular transport of α_2-macroglobulin was unaffected, the block observed with acid hydrolases may reflect failure to dissociate hydrolase-receptor complexes due to decreased endosomal acidification. If this is the case, then the acidification defect in B3853 is expressed several hours before any observable alteration in glycosylation. (*c*) Failure to release Sindbis virus, which correlated closely with the degree of the endocytic defect in nonconditional mutants (29), is clearly a secondary phenomenon; to elicit this phenotypic change in the *ts* mutants required incubation for more than a generation time at the nonpermissive temperature.

It was expected that individual endocytic activities in the *ts* mutants would be affected in the order of their pH requirements—the lower the pH needed for dissociation or penetration, the more readily an activity would be lost. Although the thresholds (39° vs. 41°C, length of incubation at 41°C, and, in I223, inhibition of protein synthesis vs. 41°C) observed with respect to inhibition of each endocytic activity were internally consistent, these thresholds did not always correspond to reported pH requirements. For example, although the pH necessary for diphtheria toxin penetration (5, 7, 33) is the same as or lower than that required for dissociation of Fe from transferrin (4) or hydrolases from the Man 6-P receptor (11), at 39°C the three mutants remained sensitive to toxin, but were unable to accumulate either Fe from transferrin or acid hydrolases. In contrast to the slower-than-expected loss of diphtheria toxin sensitivity, the rapid loss of α_2-macroglobulin uptake at 41°C is not in keeping with the observed dissociation of this ligand from its receptor at near neutral pH (18). These inconsistencies are magnified dramatically on examination of recovery of endocytic activity after return to the permissive temperature (Roff, C. F., and A. R. Robbins, manuscript in preparation).

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