Mutant Chinese Hamster Ovary Cells Pleiotropically Defective in Receptor-mediated Endocytosis

APRIL R. ROBBINS, SANDRA S. PENG, and JEAN L. MARSHALL
Genetics and Biochemistry Branch, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, Bethesda, Maryland 20205

ABSTRACT Populations of Chinese hamster ovary cells selected for resistance to diphtheria toxin were found to be highly enriched for mutants deficient in the uptake of lysosomal hydrolases via the mannose 6-phosphate receptor. One doubly defective mutant, DTF 1-5-1, exhibited increased resistance to Sindbis virus, although it was able to bind and internalize virus normally. Normal production of virus was obtained when, subsequent to virus binding, the mutant was exposed for 2 min to acidic pH. Similarly, a shift to acidic pH increased the sensitivity of DTF 1-5-1 to diphtheria toxin 12-fold. Decreased uptake of lysosomal hydrolases by the mutant correlated with decreased mannose 6-phosphate receptor activity at the cell surface; results of lactoperoxidase-catalyzed iodination indicated that the surface-associated receptor was present but inactive on DTF 1-5-1. Total mannose 6-phosphate receptor activity was also decreased in the mutant and this decrease was reflected by increased secretion of lysosomal hydrolases. The phenotype of DTF 1-5-1 resembles in many ways that of cells treated with ammonia. We suggest that the defect in DTF 1-5-1 stems from an inability to deliver virus, diphtheria toxin, and lysosomal hydrolases to an acidic compartment. Other ligands may be endocytosed through a different pathway since the defect of DTF 1-5-1 did not decrease the endocytosis of ricin, modeccin, or *Pseudomonas* toxin and had minimal effects on uptake and degradation of low density lipoprotein.

A variety of macromolecular ligands bind to specific receptors on the cell surface and are subsequently internalized via endocytosis (for reviews see references 1–4). Results of morphological studies suggest that there is a common pathway followed by receptor-bound ligands en route to lysosomes (5–9). To dissect the complex sequence of events culminating in appearance of ligand in lysosomes, it would be helpful to have mutants defective in various steps of this pathway. Mutants deficient in the uptake of lysosomal hydrolases (10), low density lipoprotein (LDL) (11), insulin (12), diphtheria toxin (13, 14), ricin (15), and other ligands have been isolated, but in general these mutants were found to be defective in the initial step of receptor-mediated endocytosis, i.e., ligand binding.

One approach for obtaining mutants altered in steps subsequent to ligand binding is to isolate mutants defective in the uptake of two ligands, entry of which is dependent on unrelated receptors (16). In this paper we present the isolation and characterization of Chinese hamster ovary (CHO) cell mutants resistant to diphtheria toxin and deficient in uptake of lysosomal hydrolases through the mannose 6-phosphate receptor. A preliminary report of this work has been presented (17).

MATERIALS AND METHODS

Materials: Ethyl methanesulfonate was purchased from Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co., (Rochester, NY). Diphtheria toxin was provided by Dr. William H. Habig (Bureau of Biologics, Bethesda, MD), *Pseudomonas* exotoxin by Dr. Stephen Leppla (U.S. Army Medical Research Institute of Infectious Diseases, Frederick MD), and ricin by Dr. Richard J. Youle (National Institutes of Health, Bethesda, MD); modeccin was purchased from Pierce Chemical Co. (Rockford, IL). Iodinated bovine testicular β-galactosidase and rabbit antiserum directed against bovine mannose 6-phosphate receptor were provided by Dr. G. Gary Sahagian (National Institutes of Health). Immuno-precipitin (formalin-fixed *Staphylococcus* A cells, 10% (wt/vol) was purchased from Bethesda Research Laboratories (Rockville, MD), [^{35}S]methionine (1,000–1,500 Ci/mmole) was from Amersham Corp. (Arlington Heights, IL), proteinase K from Boehringer Mannheim Biochemicals (Indianapolis, IN), SDS (L-5750) from Sigma Chemical Co. (St. Louis, MO), bovine serum albumin (A grade) and lactoperoxidase (B grade, 15 U/mg) were from Calbiochem-Behring Corp., American Hoechst Corp., (San Diego, CA). 4-Methylumbelliferyl α-L-iduronide was supplied by Dr. Bernard Weissmann (University of Illinois, Chicago, IL), other 4-methylumbelliferyl substrates were purchased from Research Products International Corp. (Mt Prospect, IL); Percoll was from Pharmacia Inc. (Piscataway, NJ). Human lipoprotein-deficient serum, LDL, and ^{125}I-LDL were provided by Dr. Jeffrey Hoeg (National Institutes of Health). [^{3}H]-Putrescine (39 Ci/mmole) and I^{125} (carrier-free, ~17 Ci/mg) were from New England Nuclear (Boston, MA), N-N'-dimethylated casein was from Calbi-

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 APRIL 1983 1064-1071

ochem-Behring Corp. and methylamine was from Aldrich Chemical Co. (Milwaukee, WI).

Cells and Cell Culture: The isolation of the parental CHO cell line has been previously described (18). All media for cell culture were prepared in the Media Supply Unit of the National Institutes of Health. Growth media (19) and labeling media (20) contained 5% fetal bovine serum or 5% dialyzed fetal bovine serum (both from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), respectively. Procedures of cell culture have been previously described (21). Unless otherwise specified, cells were kept at 34°C.

Preparation of Labeled Secretions: Medium conditioned by CHO cells grown in the presence of ammonia is enriched in newly synthesized lysosomal enzymes that are ligands of the mannose 6-phosphate receptor. Thus for an easily prepared, albeit crude, source of radioactive ligand, WTB cells grown to about 1×10^7 cells/T-150 flasks were washed three times with medium lacking methionine, then labeled for 12–16 h with 10 ml of medium containing 1 mCi [^{35}S]methionine, 10 μg of nonradioactive methionine and NH_4Cl, 10 mM. The secretions were concentrated, dialyzed, and clarified by centrifugation as previously described (20). One flask of cells yielded 1 ml of final material containing 3×10^7 cpm; 5–8% of this radioactivity could be taken up by recipient CHO cells and of that 95% appeared to be dependent on the man 6-P receptor (22). Secretions from human diploid fibroblasts were unsatisfactory as only 2–3% of the radioactive protein was internalized by CHO cells and more than half of this uptake appeared to be independent of the man 6-P receptor.

Isolation of Mutants: A stock of WTB was distributed into three T-75 flasks, "E", "F", and "G." These cultures were grown to densities of ~2×10^6 cells/flask, then treated with the mutagen ethyl methanesulfonate (200 μg/ml). After 18 h "E", "F", and "G" were harvested and subcultured. 5 d after mutagenesis two T-150 flasks each of "E", "F," and "G" (cell density 1.0×10^7 cells/flask) were transferred to 39°C; after 5 h diphtheria toxin was added to each flask at 100 ng/ml. After 24 h toxin was removed, cells were harvested, pooling pairs of flasks, cells were replated on 100-mm dishes in aliquots corresponding to 0.25, 0.5, and 1 of the original flasks per dish and incubated at 34°C; 1 d later the medium was changed to growth medium containing 10% fetal bovine serum and 20 μg/ml insulin (23); Whatman no. 50 filters, sterilized as previously described (21) were added for replica plating (23).

After 10 d the filter paper replicas were separated from the master dishes; the latter were stored in growth medium at room temperature in an atmosphere of 5% CO_2. The replicas were transferred to 39°C; after 5 h growth medium (4 ml) containing 7×10^5 cpm/ml of [^{35}S] secretions from WTB cells was filtered onto each replica through a 0.22 μ Millex filter (Millipore Corp., Bedford, MA). After 12 h medium was removed and the replicas were gently washed once with growth medium, twice with Dulbecco's phosphate buffered saline, pH 7.4, without Ca^{++} or Mg^{++} (PBS) and once with 0.9% NaCl. The replicas were stained in a mixture of 50% methanol, 10% acetic acid, 10% trichloroacetic acid containing 0.5 mg/ml Coomassie Brilliant blue for 1 h, then destained by rinsing four times with 40% methanol, 10% acetic acid. After the stained replicas had been rinsed in water for 15 min, they were immersed in Autofluor (National Diagnostics, Inc., Advanced Applications Institute Inc., Somerville, NJ) for 2 h, dried and exposed to pre-exposed (24) XR-2 film for 20 h at −70°C. Comparison of the stained replicas with the film revealed putative mutants; these were picked from the master dishes and cloned as previously described (21). The letters "E", "F," or "G" in the designation of the mutants indicates the mutagenized culture from which a mutant was obtained.

Protein Synthesis Assay: The assay used was that previously described (25) except that cells were washed three times in methionine-free medium then labeled for 1 h in medium containing 1 μg/ml of methionine and 2 μCi/ml [^{35}S]methionine. Cells were then washed and solubilized in 0.1 N NaOH for 30 min, the lysate was neutralized with 0.1 N HCl, and aliquots were spotted on paper, protein was precipitated and radioactivity in the precipitates was counted as described (25).

Virus: Sindbis virus and vesicular stomatitis virus (VSV) were provided by Dr. Sharon S. Krag (Johns Hopkins University, Baltimore, MD). Stocks of virus were prepared by infecting WTB cells with 0.1 plaque forming unit (pfu)/cell. Viral titers were determined on confluent monolayers of WTB (2.5×10^6 cells/60-mm dish) under solidified growth medium containing 2% fetal bovine serum and 0.75% agarose (Bethesda Research Laboratories). After 24 h (VSV) or 40 h (Sindbis) the solidified medium was removed and the monolayers were stained with 0.5% crystal violet in 50% ethanol.

Sindbis labeled with [^{35}S]methionine was prepared by infecting cells as above with 0.1 pfu/cell. After 5 h the cells were rinsed three times in methionine-free medium then incubated for 12 h in medium containing 0.4 mCi/ml [^{35}S]methionine plus 1.5 μg/ml nonradioactive methionine. The medium was harvested, clarified by centrifugation at 1,000 *g* for 10 min, then viruses were pelleted by centrifugation at 100,000 *g* for 90 min. Viruses were resuspended in 10 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4 and again pelleted at 100,000 *g* for 90 min. Radioactivity of the virus was 1.9 cpm/1,000 pfu. Labeled virions banded as a single sharp peak after centrifugation on a gradient of potassium tartrate (26); analysis of the labeled material by electrophoresis on SDS-polyacrylamide gels (reference 27, with the modification described in reference 28) showed that all of the radioactivity was in viral proteins.

Binding to the Mannose 6-Phosphate Receptor: Binding to intact cells was conducted as previously described (10) except that labeled ammonia-induced secretions were used instead of bovine testicular β-galactosidase and displacement of radioactivity rather than of enzyme activity by mannose 6-phosphate was measured. Briefly, cells were chilled to 4°C, incubated with 5 mM man 6-P to remove endogenous ligand, washed, then incubated for 60 min in 1.5 ml containing 1.3×10^6 cpm of secretions. Following binding cells were washed nine times (the ninth wash was saved), before incubation with man 6-P (5 mM) for 10 min. Radioactivity in the ninth and tenth washes was determined and the difference was taken. Typical values obtained with WTB cells were ninth wash: 300 cpm, tenth wash: 3,300 cpm.

Binding to membranes was also measured by displacement. Membranes were prepared as previously described (29) with the single exception that membranes were collected by centrifugation at 100,000 *g* for 60 min. Membranes were treated on ice for 30 min in buffer (25 mM Tris, 0.25 M sucrose, 5 mM EDTA) pH 7.0, with 0.5% saponin and 5 mM mannose 6-phosphate added (29), then pelleted, resuspended and washed by centrifugation at 100,000 *g* for 60 min; 1 mg total cell protein yielded ~200 μg washed membranes. Samples containing 30 μg of membrane protein were incubated with 2.0×10^5 cpm of ammonia-induced secretions in the above buffer in a final volume of 80 μl for 60 min on ice; 75 μl of the assay mixture was layered onto buffer containing 100 mg/ml bovine serum albumin (BSA), then centrifuged at 100,000 *g* for 20 min at 4°C in a Beckman Airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) to separate bound from free ligand (30). The pellet was resuspended and washed twice in 175 μl buffer plus BSA (1 mg/ml); the supernatant from the second wash was saved. The pellet was then resuspended in buffer plus BSA (1 mg/ml) containing man 6-P (5 mM), incubated on ice for 15 min and re-centrifuged. The difference in radioactivity in washes before and after incubation with man 6-P was taken.

Radiolabeling of the Mannose 6-Phosphate Receptor: For metabolic labeling with [^{35}S]methionine cells grown in 100-mm dishes were washed three times with medium lacking methionine then incubated for 1 h in 4 ml of medium containing 0.4 mCi [^{35}S]methionine and 4 μg of nonradioactive methionine. Radioactivity was chased by changing the medium to normal growth medium.

For iodination of surface receptor, cells in 100-mm dishes were chilled to 4°C for 1 h, then rinsed three times with cold PBS; cold PBS (1.5 ml) was added to each dish, followed by 0.6 mCi of ^{125}I and 0.32 mg lactoperoxidase. The reaction was initiated by addition of 2 μl of H_2O_2 (0.03%) with subsequent additions at 2, 4, 6, and 8 min. At 10 min 1.5 ml of cold PBS containing BSA (10 mg/ml) was added, and the cells were rinsed twice with PBS-BSA.

Solubilization of the receptor and immunoprecipitation with rabbit antiserum directed against bovine man 6-P receptor were accomplished using the procedures described by Sahagian, G. G. and E. F. Neufeld (manuscript submitted for publication).

Enzyme Assays: Acid hydrolase activity from cell extracts, secretions, and gradient fractions was assayed with 4-methylumbelliferyl substrates by methods previously described (20, 21). Transglutaminase activity was assayed as the Ca^{++}-dependent incorporation of [^{3}H]putrescine into casein as described (31). Protein was determined by the standard procedure (32).

RESULTS

Mutant Isolation

Three independently mutagenized populations of wild type CHO cells (WTB) were treated with diphtheria toxin (100 ng/ml) for 24 h, survivors were replicated, and the replicate colonies were screened for the ability to take up lysosomal hydrolases via the mannose 6-phosphate receptor (see Materials and Methods). From a total of 6.3×10^7 cells treated with toxin, 2,100 colonies were obtained. Of 1,800 colonies tested for hydrolase uptake, 130 appeared to be uptake-deficient. In contrast, no uptake-deficient colonies were observed among 3,200 colonies of mutagenized cells not previously treated with toxin. Putative mutants were picked and cloned.

Two independent mutants were tested for their response to a variety of agents that enter the cell via receptor mediated-endocytosis. As expected, both mutants exhibited increased resistance to diphtheria toxin (Table I). No increase was ob-

served in the resistance of the mutants to modeccin, Pseudomonas toxin, or ricin; instead, both mutants showed an increase sensitivity to ricin and mutant DTE 1-6-4 exhibited increased sensitivity to Pseudomonas toxin.

Uptake mediated by the mannose 6-phosphate receptor was reduced in DTE 1-6-4 and DTF 1-5-1 (Table II). Using as ligand either purified bovine testicular β-galactosidase, labeled with ^{125}I, or crude [^{35}S]secretions we found that uptake into DTF 1-5-1 was 4% of that measured with parental cells (Table II).

Increased resistance to diphtheria toxin and decreased endocytosis through the mannose 6-phosphate receptor would be expected in the mutants since these were the characteristics employed in their isolation. In addition, the mutants exhibited increased resistance to Sindbis virus and vesicular stomatitis virus. Resistance was manifested in two ways: first, a two- to six-fold reduction in the number of plaques formed on monolayers of mutant versus parental cells; second, a uniform decrease in the size of plaques formed on the mutants (Table III). That plaques were obtained at fairly high frequency on the mutants does not appear to reflect the presence of revertants; identical results were obtained using freshly cloned mutants; moreover, in a test of 10^4 colonies of DTF 1-5-1 no revertants were detected.

TABLE I

Response of WTB and Mutants to Toxins and Toxic Lectins

Agent	WTB	DTE 1-6-4	DTF 1-5-1
		EC_{50} (ng/ml)	
Diphtheria toxin	30	400	2,000
Ricin	100	30	33
Modeccin	2	2	2
Pseudomonas toxin	550	250	530

Cells grown in 24-well dishes to a density of ~1.5 × 10^5 cells/well were incubated with diphtheria toxin (2 h, growth medium), ricin (3 h, medium + 2 mg/ml BSA without serum), modeccin (2 h, medium + 2 mg/ml BSA without serum) or *Pseudomonas* toxin (3 h, growth medium). Protein synthesis was assayed in duplicate samples as described in Materials and Methods; EC_{50} is the dose required to inhibit protein synthesis to 50% of that measured in parallel samples of untreated cells.

Mechanism

The sensitivity of DTF 1-5-1 to diphtheria toxin was increased if, following incubation with toxin, the mutant was shifted to medium of pH 4.5 (Fig. 1). This procedure has

TABLE II

Mannose 6-Phosphate Receptor-mediated Uptake into WTB and Mutants

Cell	Uptake	
	[^{35}S]Secretions	^{125}I-β-Galactosidase
	cpm/mg protein	
WTB	19,000	35,000
DTE 1-6-4	3,400	Not tested
DTF 1-5-1	800	1,300

Cells grown in 60-mm dishes to ~1.5 × 10^6 cells/dish were incubated with 2 ml of growth medium containing 1.7 × 10^6 cpm of ammonia-induced secretions (see Materials and Methods) or 1 × 10^6 cpm of ^{125}I-β-galactosidase for 4 h. Uptake into WTB cells proceeded linearly during this period. Following uptake the recipient cells were washed and solubilzied in 0.1 M NaOH; lysates were neutralized with 0.1 M HCl, and radioactivity and protein content were determined. Results presented have been corrected for uptake in the presence of 2 mM man 6-P; values for nonspecific uptake were similar in WTB and mutant cells ([^{35}S]secretions, 800 cpm/mg; ^{125}I-β-galactosidase, 3,500 cpm/mg).

TABLE III

Response of WTB and Mutants to Lytic RNA Viruses

	Fraction of WTB Response	
	DTE 1-6-4	DTF 1-5-1
Sindbis virus		
Size of plaques	0.38	0.25
Number of plaques	0.46	0.17
Vesicular stomatitis virus		
Size of plaques	0.40	0.33
Number of plaques	0.50	0.18

More than 40 plaques were measured for each virus on each cell type. It should be noted that the size of the plaques increased with increasing time (e.g. Sindbis plaques on monolayers of DTF 1-5-1 increased from 1 to 3 mm from 40 to 72 h), but the number of plaques did not change.

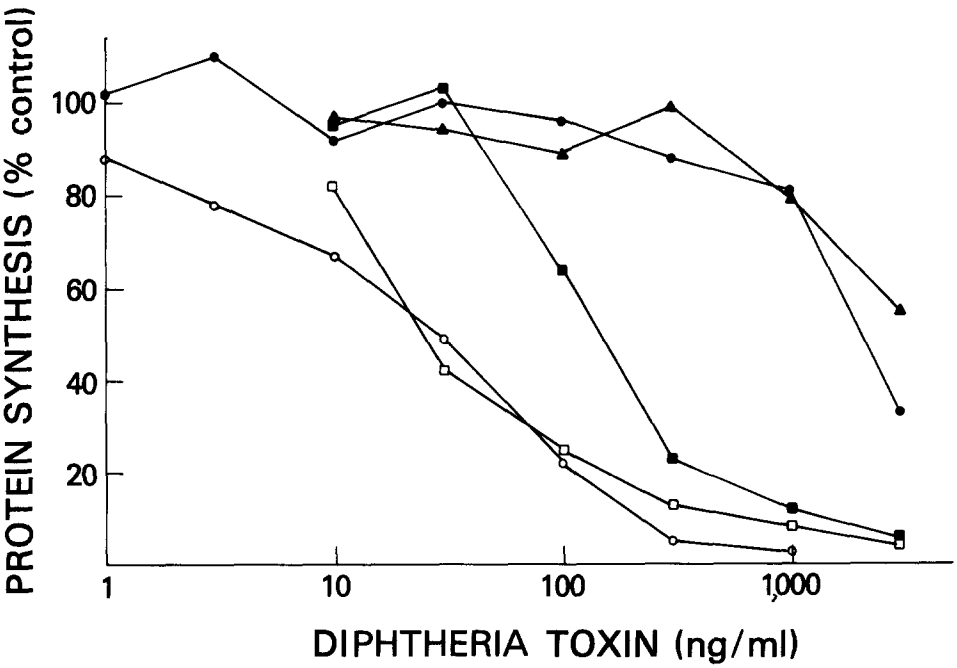

FIGURE 1 Effect of pH on diphtheria toxin sensitivity of WTB and DTF 1-5-1. Cells (grown in 24-well dishes to ~1.8 × 10^5 cells/well) were incubated with the indicated amounts of toxin for 2 h at 34°C, then washed three times and incubated for 30 min at 34°C in growth medium plus 20 mM HEPES, pH 4.5 or pH 7.2. The medium was replaced with standard growth medium and after 1 h protein synthesis was measured in duplicate samples (see Materials and Methods); controls were parallel samples taken through the incubations without added toxin. (○) WTB, pH 7.2; (□) WTB, pH 4.5; (●) DTF 1-5-1, pH 7.2; (■) DTF 1-5-1, pH 4.5; (▲) DTF 1-5-1 incubated with toxin plus 5 mM ATP, pH 4.5.

previously been shown to overcome the block in toxin sensitivity observed in the presence of ammonia (25, 33). The pH-induced sensitivity of DTF 1-5-1 to toxin appeared dependent on binding of toxin to receptors, since the presence of ATP, an inhibitor of toxin binding (34), during incubation with toxin, abolished the subsequent effect of the shift to acidic pH (Fig. 1). These results suggest that resistance of DTF 1-5-1 to toxin reflects a block occurring subsequent to toxin binding, but before the appearance of toxin in the cytoplasm.

In similar fashion, a brief shift (2 min) to pH 5.0 following incubation of DTF 1-5-1 with Sindbis virus increased subsequent production of virus sixfold (Table IV). Thus, the mutant appeared able to bind virus and to support viral replication. Resistance to virus, like resistance to diphtheria toxin, seems to result from either a failure to internalize bound virions or to move virus from within the endocytic apparatus to the cytoplasm.

To distinguish between these possibilities, DTF 1-5-1 and WTB cells were infected with Sindbis labeled with [^{35}S]methionine. Internalization of viral protein was determined by treating the cells with proteinase K to remove surface-bound virions (35), then measuring the remaining cell-associated radioactivity. Mutant and parent cells appeared similar with respect to both the rates of virus binding and internalization (Fig. 2). After 90 min degradation of virus, determined by measuring TCA-soluble radioactivity, respresented only 15% and 25% of the levels of virus internalized by DTF 1-5-1 and WTB, respectively. Binding of virus to mutant and parent at 4°C was similar; 30% of the radioactive virus added was bound to the cells in 1 h. No differences were observed between DTF 1-5-1 and WTB with respect to the rate of internalization of bound virus following a shift of the cells from 4°C to 34°C. Thus, DTF 1-5-1 appeared to be blocked in the translocation of internalized virus to the cytoplasm.

While binding of both diphtheria toxin and Sindbis virus was normal in DTF 1-5-1, binding of ligand to surface-associated mannose 6-phosphate receptors was only 5% of that measured with parental cells (Table V). Lack of surface binding appears to reflect mainly decreased receptor activity, rather than decreased receptor number. Lactoperoxidase-catalyzed iodination followed by immunoprecipitation revealed that the level of surface receptor in DTF 1-5-1 was much greater than that predicted from binding studies (Fig. 3). We observed the same phenomenon in WTB cells that had been preincubated with NH_4Cl; no binding of ligand to man 6-P receptors at the cell surface was detected (Table V), yet on iodination signif-

TABLE IV

Effect of pH on Sindbis Virus Production

Cell	Virus produced (pfu × 10^{-5})	
	pH 7.2	pH 5.5
WTB	6.0	9.0
DTF 1-5-1	0.9	5.8
WTB + NH_4	0.8	4.6

Cells in 24-well dishes (1.0 × 10^5 cells/well) were chilled to 4°C, then incubated for 1 h at 4°C with Sindbis virus at 10 pfu/cell. Cells were washed three times (at 4°C) then incubated for 2 min at 34°C in medium containing 2-[morpholino] ethane sulfonic acid, 10 mM, and BSA, 2 mg/ml, at pH 7.2 or pH 5.5 (35). This medium was replaced with growth medium and incubation was continued at 34°C. (In samples treated with NH_4Cl, 10 mM, the agent was present during the entire course of the experiment). After 5 h, aliquots of the medium were diluted and plated on monolayers of WTB for determination of plaque-forming units (see Materials and Methods).

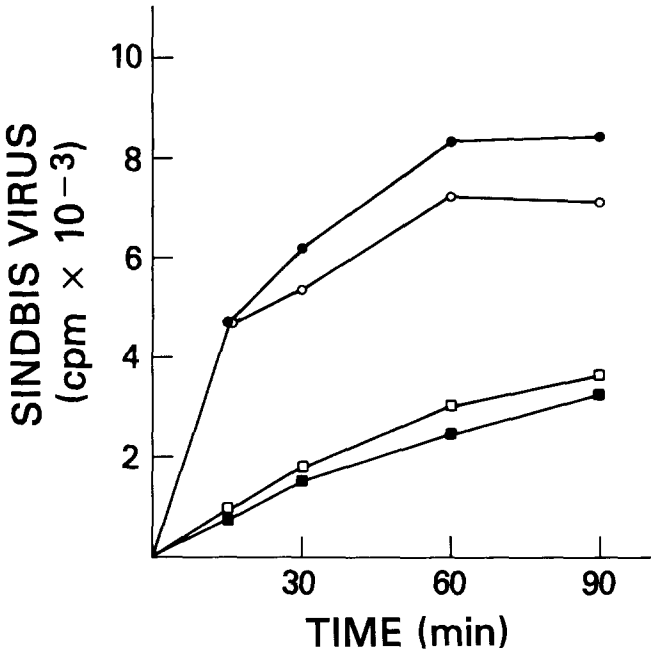

FIGURE 2 Binding and internalization of Sindbis virus by WTB and DTF 1-5-1. Cells (grown to a density of 1 × 10^6 cells/dish in 60-mm dishes) were incubated with [^{35}S]Sindbis, 2.9 × 10^4 cpm, 10 pfu/cell, at 34°C for the times indicated. Infected cells were washed twice with cold PBS, then incubated at 4°C with 1 ml of PBS containing 0.5 mg/ml bovine serum albumin, or 0.5 mg/ml proteinase K, for determination of total cell-associated virions or internalized virions, respectively (35). After 45 min 1 ml PBS containing 30 mg bovine serum albumin and 1 mM phenylmethylsulfonylfluoride was added to each sample, cells were harvested and washed as described (35) then solubilized in 0.1 M NaOH. All assays were done in triplicate; in control samples incubated with virus at 4°C, subsequent treatment with proteinase K removed >95% of cell-associated radioactivity. Values for surface-associated virus were calculated as total cell-associated virus minus internalized (proteinase K-resistant) virus; (○) WTB, surface-associated virus; (□) WTB, internalized virus; (●) DTF 1-5-1, surface-associated virus; (■) DTF 1-5-1, internalized virus.

TABLE V

Mannose 6-Phosphate Receptor Activity in DTF 1-5-1 and WTB

	Binding
	cpm/ mg cell protein
WTB − Cells	5,400
DTF 1-5-1 − Cells	200
WTB + NH_4 − Cells*	20
WTB − Membranes	41,000
DTF 1-5-1 − Membranes	8,300

Binding to the receptor was determined by displacement of radioactivity bound to intact cells or membranes with 5 mM man 6-P (see Materials and Methods). Values given have been corrected for radioactivity displaced from cells or membranes when binding was conducted in the presence of 5 mM man 6-P (intact cells 250, membranes 300 cpm/mg cell protein). No inhibition of binding to intact cells or membranes was observed when binding was conducted in the presence of 5 mM man 1-P.

* WTB cells were grown with 10 mM NH_4Cl for 12 h before the experiment; NH_4Cl was also present during the course of the binding assay.

icant levels of receptor were found on amine-treated cells (Fig. 3). In three experiments the level of iodinated man 6-P receptor in DTF 1-5-1 and amine-treated cells, quantitated by densitometry, ranged from 30–60% of that measured in WTB cells.

Measurements of binding to total cellular mannose 6-phos-

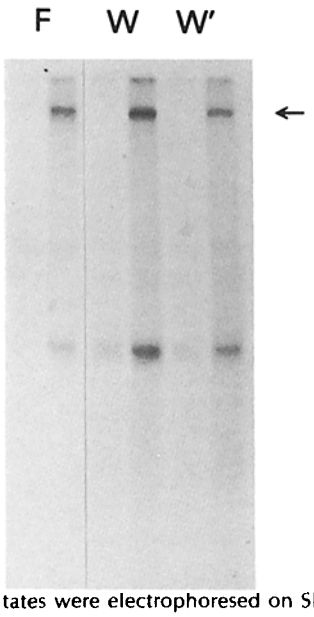

FIGURE 3 Iodination of the mannose 6-phosphate receptor on WTB and DTF 1-5-1. Cells (grown to 4 × 10^6 cells/dish in 100-mm dishes) were chilled to 4°C and iodinated for 10 min at 4°C as described in Materials and Methods. Receptor was solubilized (Sahagian, G. G., and E. F. Neufeld, manuscript submitted for publication) from pairs of dishes; one-half of each sample was immunoprecipitated (Sahagian, G. G., and E. F. Neufeld, manuscript submitted for publication) and the other half was taken through the immunoprecipitation procedure without antireceptor antiserum as a control for nonspecific precipitation. Precipitates were electrophoresed on SDS-polyacrylamide gels (reference 27, as modified in reference 28), and the radioactivity was detected on pre-exposed film (24). The arrow indicates the position of the man 6-P receptor. The left lane of each pair is from the nonspecific precipitation, the right lane is from the immunoprecipitation. *F*, DTF 1-5-1; *W*, WTB; *W'*, WTB cells grown for 12 h in 10 mM NH_4Cl, chilled and iodinated in the presence of NH_4Cl. The level of radioactivity in the second band (M_r ~46,000) visible in both controls and immunoprecipitates varies unpredictably among different preparations.

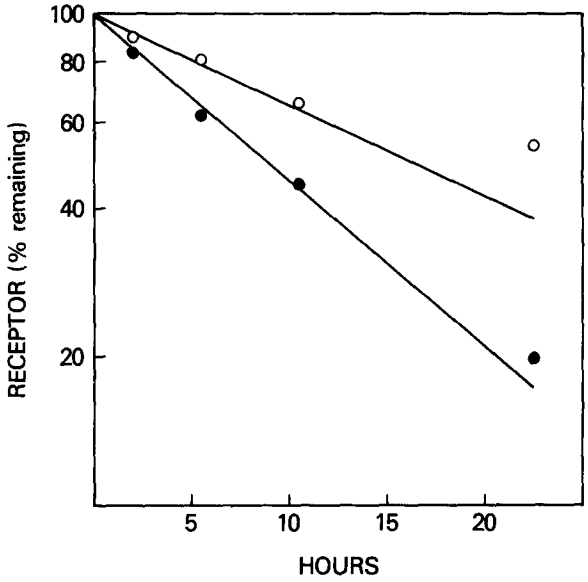

FIGURE 4 Turnover of the mannose 6-phosphate receptor in WTB and DTF 1-5-1. Cells (grown to 3 × 10^6 cells/dish in 100-mm dishes) were incubated with [^{35}S]methionine for 1 h, then label was chased for the times indicated. Precipitation and electrophoresis of the precipitates was performed as described in Fig. 3. The gels were subjected to fluorography (36) radioactivity was detected on pre-exposed film (24) and quantitated by densitometric scanning. Values are expressed as a percentage of the radioactivity measured in the respective cell-type following the 1-h pulse; at this point the level of radioactive receptor measured in WTB cells was 90% of that measured in DTF 1-5-1. (○) WTB; (●) DTF 1-5-1.

phate receptors indicated that the mutant had 20% of the receptor activity of parental cells (Table V). Examination of biosynthesis and degradation of receptor by metabolic labeling indicated equal rates of receptor biosynthesis in DTF 1-5-1 and WTB; the half-life of the receptor was found to be 9.5 h in the mutant and 18 h in the parent (Fig. 4). Addition of NH_4Cl to WTB cells after a radioactive pulse also resulted in increased turnover of the receptor (data not shown).

Consistent with its decreased level of mannose 6-phosphate receptor activity (20), DTF 1-5-1 exhibited reduced intracellular levels of many acid hydrolases and secreted those enzymes in correspondingly elevated amounts (Table VI). Similar results were obtained by metabolically labeling the cells and immunoprecipitating α-L-iduronidase and β-hexosaminidase from cell extracts and media (data not shown). When cell-free extracts of mutant and parent were fractionated on 27% Percoll gradients (37), two peaks of acid hydrolase activity were obtained, consistent with previous reports (21, 38). Extracts of DTF 1-5-1 showed a diminution of enzyme activity only in the denser of the two peaks, i.e., in the region corresponding to lysosomes (Fig. 5).

Fig. 6 shows the uptake and degradation of ^{125}I-low density lipoprotein by mutant and wild type cells. At 5 μg/ml of LDL the rate of uptake into DTF 1-5-1 was 75% of that measured for WTB; degradation was 50% that of WTB. This reduction in the rate of degradation of LDL may reflect the mutant's deficiency in acid hydrolases. The kinetic parameters of LDL uptake differ slightly between DTF 1-5-1 and WTB at concentrations of LDL < 50 μg/ml; at concentrations of ligand ≥50 μg/ml the kinetic parameters were identical for the two cell types (Fig. 7).

The phenotype of DTF 1-5-1 is similar in many ways to that

TABLE VI

Secretion of Lysosomal Hydrolases by WTB and DTF 1-5-1

Enzyme	Enzyme secreted (% total)	
	WTB	DTF 1-5-1
β-Hexosaminidase	15	78
α-L-Fucosidase	15	74
α-Mannosidase	33	67
α-L-Iduronidase	9	60
β-Glucuronidase	5	40

Secreted enzymes were collected for 14 h in serum-free medium from cells grown to densities of 6–8 × 10^6/100-mm dish; preparation of the secretions and assays of enzymatic activity were conducted as previously described (20). Total hydrolase activities from DTF 1-5-1 in comparison to those from WTB were β-hexosaminidase, 116%; α-L-fucosidase, 116%; α-mannosidase, 97%; α-L-iduronidase, 44%; β-glucuronidase, 130%. The decreased level of α-L-iduronidase may reflect the instability of that enzyme in medium (22).

of amine-treated cells. One of the proposed targets in inhibition of endocytosis by the amines is transglutaminase (39), but measurements of the activity of this enzyme in crude extracts of mutant and parent cells revealed no significant differences either in the K_m or rate of transfer of putrescine, or in the inhibition of enzyme activity by methylamine.

DISCUSSION

The initial goal of this study was the isolation of mutants pleiotropically defective in receptor-mediated endocytosis. The mutants described here, DTE 1-6-4 and DTF 1-5-1, were found to be altered in the uptake of diphtheria toxin, lysosomal enzymes, and two lytic RNA viruses, Sindbis virus and VSV. Preliminary studies indicate that DTF 1-5-1 also is unable to accumulate iron from complexes of transferrin-iron (Klausner,

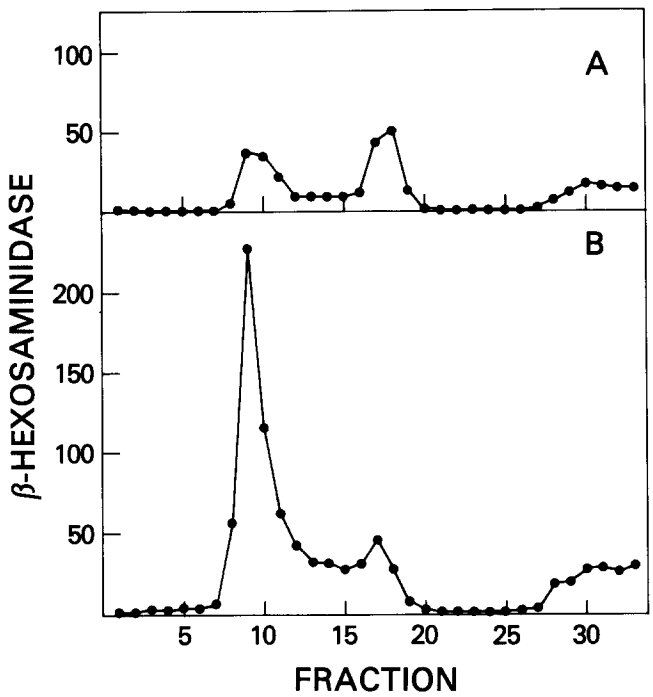

FIGURE 5 Profile of β-hexosaminidase activity from WTB and DTF 1-5-1 on Percoll gradients. Cells (grown to 1.5×10^7 cells/flask in T-150 flasks) were harvested and disrupted as previously described (21, 38). Cell extracts were layered on 27% Percoll suspensions (37) over cushions of 60% sucrose (wt/vol) and centrifuged for 1 h at 18,500 rpm in a Sorvall SV 288 rotor. Fractions (one ml) are numbered in the order of decreasing density. Enzyme activity is expressed as nmol substrate hydrolized per h. (*A*) DTF 1-5-1; (*B*) WTB.

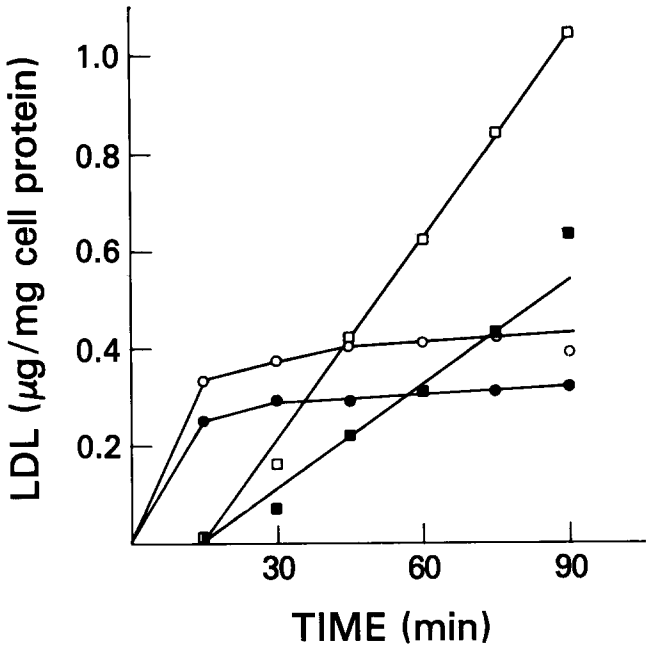

FIGURE 6 Uptake of ^{125}I-LDL into WTB and DTF 1-5-1. Cells (grown to 8×10^5 cells/dish on 60-mm dishes) were incubated for 24 h in medium plus 5% human lipoprotein-deficient serum. Medium (1.5 ml) containing 7.5 μg ^{125}I-LDL, (3.3×10^5 cpm) was added. At the times indicated medium was removed and chilled, the cells were washed four times with cold medium (without serum) and twice with PBS. Cells were solubilized in 0.1 N NaOH for determination of cell-associated radioactivity and protein (32). Aliquots of the medium were precipitated with trichloroacetic acid (15%) for determination of degraded LDL; <2% of the starting material was soluble in trichloracetic acid. Values presented are from duplicate dishes and have been corrected for nonspecific uptake and degradation (measured in the presence of 300 μg/ml LDL); (○) WTB, cell-associated LDL; (□) WTB, degraded LDL; (●) DTF 1-5-1, cell-associated LDL; (■) DTF 1-5-1, degraded LDL.

R. D., and A. R. Robbins, unpublished data). To ensure that these multiple defects result from a single pleiotropic mutation, rather than multiple mutations, it will be necessary to isolate revertants of the mutants. The high frequency at which mutants deficient in lysosomal enzyme uptake were found among the diphtheria-toxin-resistant cells, especially in the absence of selective pressure, argues against the possibility of multiple mutations. Interestingly, cross-resistance to lytic RNA viruses was found in a number of KB cell mutants that had been selected only for resistance to diphtheria toxin (40).

Although temperature-shifts were employed in both selection and screening, neither resistance to diphtheria toxin nor the deficiency in lysosomal enzyme uptake were found to be be temperature-sensitive in any of seven mutants tested to date (data not shown). Using our procedure it may not be possible to isolate mutants with temperature-sensitive defects in steps subsequent to internalization. Toxin accumulated within the cell at the nonpermissive temperature may result in cell death on return to the permissive temperature.

The defect in DTE 1-6-4 and DTF 1-5-1 impairs endocytosis of some ligands but not of others. Resistance to ricin, modeccin, and *Pseudomonas* toxin was not increased in the mutants, and only marginal effects were observed on uptake of low density lipoprotein in DTF 1-5-1. Thus, while morphologic studies indicate that diphtheria toxin (9), Pseudomonas toxin (41), LDL (5), and lysosomal enzymes (8) share a common uptake pathway, endocytosis of these ligands must differ at some stage subsequent to ligand binding. CHO cell mutants defective in uptake, but not in binding, of both *Pseudomonas* toxin and ricin have recently been reported (16). No change was found in sensitivity of these cells to diphtheria toxin. These results, like our own, suggest different structural or functional requirements for endocytosis of different ligands.

Our results indicate that the block in Sindbis infectivity in DTF 1-5-1 occurs at penetration into the cytosol. Our findings with diphtheria toxin are consistent with this localization of the defect. Entry of Sindbis into the cytoplasm, like that of Semliki Forest virus (35, 42), appears to be induced by acidic pH; this is also the case for diphtheria toxin (25, 33). One explanation for the phenotype of DTF 1-5-1 is that internalized virus and toxin never encounter an acidic environment, due either to failure of the endocytic vesicle to fuse with an acidic compartment or to a defect in acidification of the endosome (43) and/or lysosome (44). Alteration in lysosomal pH seems least likely since DTF 1-5-1 was able to degrade LDL.

Is this postulated inability to deliver ligand to an acidic compartment consistent with our observations regarding mannose 6-phosphate receptor mediated uptake in DTF 1-5-1? Dissociation of ligand from the mannose 6-phosphate receptor has been proposed to require an acidic milieu (45); thus lack of surface-binding activity in DTF 1-5-1 and ammonia-treated normal cells (reference 45 and this paper) could result from intracellular accumulation of ligand-receptor complexes. However, iodination at 4°C revealed near-normal numbers of surface-associated receptor molecules in both the mutant and ammonia-treated cells. It is unlikely that these inactive recep-

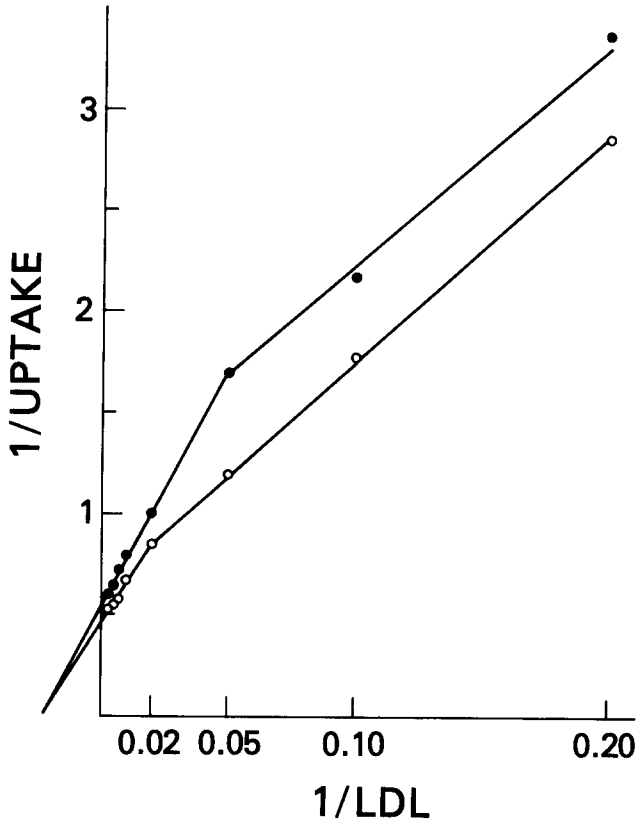

FIGURE 7 Uptake of ^{125}I-LDL into WTB and DTF 1-5-1 as a function of LDL concentration. ^{125}I-LDL was mixed with varying concentrations of unlabeled LDL and cell-associated radioactivity was determined after 60 min of uptake (assayed as described in Fig. 6). Uptake is expressed as μg LDL/mg cell protein, and the concentration of LDL is given as μg/ml (○) WTB; (●) DTF 1-5-1.

tors represent receptors bound to endogenous ligand, since pretreatment with mannose 6-phosphate did not unmask binding activity. Monensin treatment of hepatocytes has been reported to cause a similar phenomenon, i.e. loss of surface asialoglycoprotein receptor activity without loss of surface receptor molecules (46). Total mannose 6-phosphate receptor activity was also decreased in DTF 1-5-1 albeit to a lesser degree than surface receptor. We have at present no explanation for these observations.

The similarities between mutant DTF 1-5-1 and amine-treated parental cells have been emphasized, but the resemblance is not without exception. Amines have been shown to inhibit toxicity of modeccin (47) and *Pseudomonas* toxin (41); DTF 1-5-1 showed no increase in resistance to these agents. The amines act at several sites and the effects of amines on uptake of different ligands may reflect inhibition at different sites. Presumably only one of these sites is defective in DTF 1-5-1.

We thank Drs. William H. Habig, Stephen Leppla, and Richard J. Youle for toxins, Dr. Sharon S. Krag for Sindbis virus and vesicular stomatitis virus, Dr. Jeffrey Hoeg for ^{125}I-LDL, LDL, and lipoprotein-deficient serum, and Dr. G. Gary Sahagian for ^{125}I-β-galactosidase and antiserum directed against the mannose 6-phosphate receptor as well as for making available methods before their publication. We also thank Dr. Elizabeth F. Neufeld for her careful consideration of this manuscript.

Received for publication 18 October 1982, and in revised form 7 January 1983.

REFERENCES

1. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles and receptor-mediated endocytosis. *Nature (Lond.)*. 279:679–685.
2. Pastan, I. H., and M. C. Willingham. 1981. Journey to the center of the cell: role of the receptosome. *Science (Wash. DC)*. 214:504–509.
3. Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* 51:531–554.
4. Sly, W. S. 1982. The uptake and transport of lysosomal enzymes. *In* The Glycoconjugates, Vol. IV, Part B: Glycoproteins, Glycolipids and Proteoglycans. M. I. Horowitz, editor. Academic Press, New York 3–25.
5. Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1976. Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc. Natl. Acad. Sci. USA* 73:2434–2438.
6. Haigler, H., J. F. Ash, S. J. Singer, and S. Cohen. 1978. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA*. 75:3317–3321.
7. Willingham, M. C., and I. Pastan. 1980. The receptosome: an intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. *Cell*. 21:67–77.
8. 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*. 11:6967–6971.
9. Keen, J. H., F. R. Maxfield, M. C. Hardegree, and W. H. Habig. 1982. Receptor-mediated endocytosis of diphtheria toxin by cells in culture. *Proc. Natl. Acad. Sci. USA*. 79:2912–2916.
10. Robbins, A. R., R. Myerowitz, R. J. Youle, G. J. Murray, and D. M. Neville, Jr. 1981. The mannose 6-phosphate receptor of Chinese hamster ovary cells. Isolation of mutants with altered receptors. *J. Biol. Chem.* 256:10618–10622.
11. Krieger, M., M. S. Brown, and J. L. Goldstein. 1981. Isolation of Chinese hamster cell mutants defective in the receptor-mediated endocytosis of low density lipoprotein. *J. Mol. Biol.* 150:167–184.
12. Miskimins, W. K., and N. Shimizu. 1981. Genetics of cell surface receptors for bioactive polypeptides: variants of Swiss/3T3 fibroblasts resistant to a cytotoxic chimeric insulin. *Proc. Natl. Acad. Sci. USA*. 78:445–449.
13. Moehring, T. J., and J. M. Moehring. 1972. Response of cultured mammalian cells to diphtheria toxin. IV. Isolation of KB cells resistant to diphtheria toxin. *Infect. Immun.* 6:487–492.
14. Moehring, T. J., and J. M. Moehring. 1977. Selection and characterization of cells resistant to diphtheria toxin and *Pseudomonas* exotoxin A: presumptive translation mutants. *Cell*. 11:447–454.
15. Stanley, P., V. Caillibot, and L. Siminovitch. 1975. Selection and characterization of eight phenotypically distinct lines of lectin-resistant Chinese hamster ovary cells. *Cell*. 6:121–128.
16. Ray, B., and H. C. Wu. 1982. Chinese hamster ovary cell mutants defective in the internalization of ricin. *Mol. Cell. Biol.* 2:535–544.
17. Robbins, A. R. 1982. Preliminary characterization of a mutant defective in receptor-mediated endocytosis. *J. Cell. Biochem.* 6:120 Supplement.
18. Thompson, L. H., and R. M. Baker. 1973. Isolation of mutants of mammalian cells. *In* Methods in Cell Biology. Vol. 6. D. M. Prescott, editor. Academic Press, New York. 209–281.
19. Cantz, M., H. Kresse, R. Barton, and E. F. Neufeld. 1972. Corrective factors for inborn errors of mucopolysaccharide metabolism. *Methods Enzymol.* 28:884–897.
20. Robbins, A. R., and R. Myerowitz. 1981. The mannose 6-phosphate receptor of Chinese hamster ovary cells. Compartmentalization of acid hydrolases in mutants with altered receptors. *J. Biol. Chem.* 256:10623–10627.
21. Robbins, A. R. 1979. Isolation of lysosomal α-mannosidase mutants of Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA*. 76:1911–1915.
22. Stoll, J., A. R. Robbins, and S. S. Krag. 1982. Mutant of Chinese hamster ovary cells with altered mannose 6-phosphate receptor activity is unable to synthesize mannosylphosphoryldolichol. *Proc. Natl. Acad. Sci. USA*. 79:2296–2300.
23. Esko, J. D., and C. R. H. Raetz. 1978. Replica plating and *in situ* enzymatic assay of animal cell colonies established on filter paper. *Proc. Natl. Acad. Sci. USA*. 75:1190–1193.
24. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
25. Draper, R. K., and M. I. Simon. 1980. The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. *J. Cell Biol.* 87:849–854.
26. Erwin, C., and D. T. Brown. 1980. Intracellular distribution of Sindbis virus membrane proteins in BHK-21 cells infected with wild-type virus and maturation-defective mutants. *J. Virol.* 36:775–786.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
28. Hasilik, A., and E. F. Neufeld. 1980. Biosynthesis of lysosomal enzymes. Synthesis as precursors of higher molecular weight. *J. Biol. Chem.* 255:4937–4945.
29. Fischer, H. D., A. Gonzales-Noriega, and W. S. Sly. 1980. β-Glucuronidase binding to human fibroblast membrane receptors. *J. Biol. Chem.* 255:5069–5074.
30. Rome, L. H., and J. Miller. 1980. Butanedione treatment reduces receptor binding of a lysosomal enzyme to cells and membranes. *Biochem. Biophys. Res. Commun.* 92:986–993.
31. Scott, K. F. F., and D. H. Russell. 1982. Activation of transglutaminase during cell cycle in CHO cells. *J. Cell Physiol.* 111:111–116.
32. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
33. Sandvig, K., and S. Olsnes. 1980. Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell Biol.* 87:828–832.
34. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with Vero cells. Demonstration of a receptor. *J. Biol. Chem.* 253:7325–7330.
35. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* 84:404–420.
36. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
37. Merion, M., and R. D. Poretz. 1981. The resolution of two populations of lysosomal organelles containing endocytosed Wistaria floribunda agglutinin from murine fibroblasts. *J. Supramol. Struct.* 17:337–346.
38. Rome, L. H., A. J. Garvin, M. M. Allietta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts. *Cell*. 17:143–153.
39. Davies, P. J. A., D. R. Davies, A. Levitzki, F. R. Maxfield, P. Milhaud, M. C. Willingham, and I. H. Pastan. 1980. Transglutaminase is essential in receptor-mediated endocytosis of

α_2-macroglobulin and polypeptide hormones. *Nature (Lond.).* 283:162–167.
40. Moehring, T. J., and J. M. Moehring. 1972. Response of cultured mammalian cells to diphtheria toxin V. Concurrent resistance to ribonucleic acid viruses in diphtheria toxin resistant KB cell strains. *Infect. Immun.* 6:493–500.
41. FitzGerald, D., R. E. Morris, and C. B. Saelinger. 1980. Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. *Cell.* 21:867–873.
42. White, J., J. Kartenbeck, and A. Helenius. 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. *J. Cell Biol.* 87:264–272.
43. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α_2-macroglobulin. *Cell.* 28:643–651.
44. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.
45. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell. Biol.* 85:839–852.
46. Fiete, D., M. D. Brownell, and J. U. Baenziger. 1983. Evidence for transmembrane modulation of the ligand binding site of the hepatocyte galactose/N-Acetyl galactosamine specific receptor. *J. Biol. Chem.* Vol. 258:817–823.
47. Sandvig, K., S. Olsnes, and A. Pihl. 1979. Inhibitory effect of ammonium chloride and chloroquine on the entry of the toxic lectin modeccin into HeLa cells. *Biochem. Biophys. Res. Commun.* 90:648–655.