Serum Factors Alter the Extent of Dephosphorylation of Ligands Endocytosed via the Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor

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Abstract. Mouse L-cells that contain the cation-independent (CI) mannose 6-phosphate (Man 6-P)/insulin-like growth factor (IGF) II receptor endocytose acid hydrolases and deliver these enzymes to lysosomes. The postendocytic loss of the Man 6-P recognition marker from the cell-associated acid hydrolases was assessed by CI-Man 6-P receptor affinity chromatography. ^{125}I-labeled acid hydrolases internalized by L-cells grown at high density were delivered to lysosomes but were not dephosphorylated. In contrast, the same ^{125}I-labeled hydrolases internalized by L-cells maintained at low density were delivered to lysosomes and were extensively dephosphorylated. The dephosphorylation at low density required 5 h for completion suggesting that the phosphatase responsible for the dephosphorylation is located within the lysosomal compartment. Transition from the high to low density state was rapid and was not inhibited by cycloheximide. Medium substitution experiments indicated that serum factors were necessary to maintain the L-cells in the dephosphorylation-competent (low density) state, and that serum-free conditions led to a dephosphorylation-incompetent (high density) state. Addition of IGF II to cells in serum-free medium allowed acid hydrolases subsequently introduced by endocytosis to be dephosphorylated. The results indicate that the removal of the Man 6-P recognition marker from endocytosed acid hydrolases is regulated by serum factors in the growth medium, including IGF II.

NEWLY synthesized acid hydrolases are withdrawn from the secretory pathway and delivered to lysosomes as a result of an interaction between a specific recognition marker attached to their high mannose-type oligosaccharides, mannose 6-phosphate (Man 6-P),[1] and Golgi-associated Man 6-P receptors (von Figura and Hasilik, 1986; Kornfeld, 1986). Two separate Man 6-P receptors have been identified and purified; a 215-kD cation independent (CI; Sahagian et al., 1981; Steiner and Rome, 1982) and a 46-kD cation dependent (CD; Hoflack and Kornfeld, 1985) form. Both receptors appear to function in the delivery of newly synthesized acid hydrolases to lysosomes (Stein et al., 1987*a*,*b*; Gartung et al., 1985), and affinity chromatography studies indicate that the receptors recognize the same phosphorylated ligands (Hoflack et al., 1987). Although the purpose of the two distinct Man 6-P receptors is unclear, an interesting correlation exists between the presence of the CI Man 6-P receptor and the ability of cells to dephosphorylate newly synthesized acid hydrolases. Cells that express the CI receptor efficiently dephosphorylate acid hydrolases after they reach the lysosomal compartment and, as a result, the Man 6-P recognition marker is short-lived within these cells. In contrast, cells deficient in the CI receptor are inefficient in the dephosphorylation reaction and accumulate phosphorylated acid hydrolases within lysosomes (Gabel et al., 1983). This differential dephosphorylation led to the hypothesis that the two receptors deliver their ligands to separate populations of lysosomes (Gabel et al., 1983; Kornfeld, 1986).

We previously identified a mouse L-cell line that expresses the CI Man 6-P receptor yet maintains steady-state levels of endogenous phosphorylated acid hydrolases (Gabel and Foster, 1986*a*). In addition, we demonstrated that a large percentage of β-glucuronidase internalized by these cells via Man 6-P receptor-mediated endocytosis remains phosphorylated (Gabel and Foster, 1986*b*). The CI receptor-positive L-cells, therefore, constitute an exception to the correlation described above; these cells possess the CI receptor but do not efficiently dephosphorylate endogenous or endocytosed acid hydrolases.

Recent studies have shown that the CI Man 6-P receptor also binds insulin-like growth factor (IGF) II (Morgan et al., 1987; Tong et al., 1988). This dual activity suggested that transport and/or binding of acid hydrolases may be influenced by IGF II. Although the growth factor appears to bind to a region of the receptor distinct from the Man 6-P binding

1. *Abbreviations used in this paper*: BRL, buffalo rat liver; CD, cation dependent; CI, cation independent; IGF, insulin-like growth factor; Man 6-P, mannose 6-phosphate.

sites (Tong et al., 1988; Kiess et al., 1988), the two ligands may influence each other through an allosteric interaction (Roth et al., 1988). To determine whether dephosphorylation of the acid hydrolases is influenced by extracellular stimuli, we compared the postendocytic processing of Man 6-P–bearing proteins within mouse L-cells maintained under different growth conditions. The results of this analysis indicate that serum factors, including IGF II, are necessary for the L-cells to dephosphorylate efficiently endocytosed ligands. Cells maintained at a high density or in serum-free medium internalized but did not dephosphorylate Man 6-P–bearing proteins. In contrast, cells maintained at a low density or in serum-free medium supplemented with IGF II dephosphorylated the internalized ligands. The differential dephosphorylation indicates that the fate of acid hydrolases endocytosed via the Man 6-P/IGF II receptor is regulated by extracellular factors.

Materials and Methods

Cells

Cells were grown in α-MEM containing 10% newborn calf serum (Gibco Laboratories, Grand Island, NY), penicillin (100 U/ml), streptomycin (100 meq/ml), and 10 mM Hepes, pH 7, unless indicated otherwise. Mouse J774 cells were kindly provided by Dr. Stuart Kornfeld (Washington University) and buffalo rat liver (BRL)-3A cells by Dr. Argiris Efstratiadis (Columbia University). The Man 6-P receptor–positive mouse L-cell line was described previously (Gabel and Foster, 1986*a*,*b*). When plated at a specific density, the concentration of cells in a trypsinized suspension was determined using a hemocytometer. Human recombinant IGF I and IGF II were a generous gift of Dr. Michelle Smith, Eli Lilly Corp. (Indianapolis, IN). BSA (1 mg/ml) was added to α-MEM containing 10 mM Hepes, pH 7, when L-cells were cultured in the absence of serum.

Preparation of Man 6-P–bearing Ligands

J774 cells were grown to maximum density in 600 ml of MEM containing serum in a spinner culture. The cells were collected by centrifugation, transferred to 2 liters of serum-free MEM, and maintained for 2 d; glucose was added after 24 h to a final concentration of 5 mM. The culture was harvested and the medium concentrated to 125 ml using a Minitan concentrator (Millipore Continental Water Systems, Bedford, MA) equipped with a 10,000 D molecular mass cut-off filter. The concentrate was dialyzed against 20 mM Tris, pH 8 (buffer A), and applied to a DE52 cellulose column (2.5 × 12 cm) equilibrated in buffer A. Bound proteins were eluted with 1 M NaCl in buffer A, and the column fractions were assayed for β-glucuronidase. Peak fractions were pooled and frozen in a dry ice/ethanol bath and stored at −70°C. The total β-glucuronidase activity recovered from 2 liters of the cell secretions was 2.8 U. Lysosomal enzymes containing the Man 6-P recognition marker were isolated by applying 0.3 ml (110 mU of β-glucuronidase activity) of the concentrated J774 secretions to a 10 ml affinity column containing 1 mg of the CI Man 6-P receptor. The receptor was purified from bovine liver acetone powder (Sigma Chemical Co., St. Louis, MO) and coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) as previously described (Varki and Kornfeld, 1983). Acid hydrolases that bound to the affinity column were eluted with 5 mM Man 6-P; peak fractions as determined by β-glucuronidase activity, were pooled and concentrated using a Centricon 10 filter (Amicon Corp., Danvers, MA). Of the total β-glucuronidase activity applied to the column, 50% bound and eluted with Man 6-P. An aliquot of the concentrated Man 6-P–bearing ligands (containing 39 mU of β-glucuronidase) was iodinated with $Na^{125}I$ (0.1 C/mmol; Amersham Corp., Arlington Heights, IL) using the chloramine-T method as previously described (Greenwood et al., 1963). Excess ^{125}I was separated from labeled proteins by chromatography on a 10 ml column of Sephadex G-25 equilibrated in 50 mM phosphate, pH 7.5, 1 mg/ml ovalbumin, 2 mg/ml potassium iodide. The excluded iodinated proteins were dialyzed against 50 mM phosphate, pH 7.5, and stored on ice.

Ligands also were isolated from the secretions of J774 cells metabolically labeled with [^{35}S]methionine and $^{32}PO_4$. A confluent monolayer (on a 15-cm dish) was preincubated in 15 ml of serum and phosphate-free MEM for 3 h after which 25 μM 5-fluorouracil was added and the cells were incubated for an additional 60 min. 5 mCi of [^{32}P]orthophosphate (8 mCi/ml; Amersham Corp.) and 0.5 mCi of [^{35}S]methionine (1,500 Ci/mmole; Amersham Corp.) were added in the presence of 25 μM 5-fluorouracil in a total volume of 15 ml of serum and phosphate-free MEM. Fluorouracil was added to inhibit DNA synthesis. The cells were incubated overnight after which the medium was collected and dialyzed against buffer A. The dialysate was applied to a 5 ml DE52 cellulose column equilibrated in buffer A; the column was washed with 15 ml of buffer A and eluted with 1 M NaCl in buffer A. Radiolabeled proteins that eluted with NaCl were dialyzed against 50 mM sodium phosphate, pH 7.5, and those that contained Man 6-P were isolated by application of the labeled preparation to the CI Man 6-P receptor affinity column. Proteins that bound and eluted with Man 6-P were concentrated in a Centricon 10 filter.

Receptor-mediated Endocytosis and CI Man 6-P Receptor Affinity Chromatography

Radiolabeled Man 6-P-bearing acid hydrolases were diluted with MEM and filter sterilized before addition to cells; a typical preparation contained 6 × 10^6 cpm/ml. Mouse L-cells were incubated with the ligands at 37°C in the presence and absence of 5 mM Man 6-P. After a 60-min incubation the postuptake supernatant was removed, and the cells were either chased in fresh medium or harvested. To harvest, the dishes were rinsed with PBS (10 mM phosphate, pH 7.3, 150 mM NaCl) containing 2 mg/ml BSA and with PBS alone. The cells were dislodged from the dish with a rubber policeman and collected by centrifugation in a microfuge (Eppendorf, made by Brinkmann Instruments, Inc., Westbury, NY). For studies involving the analysis of total cell-associated proteins, the cell pellet was dissolved directly in sample buffer (Laemmli, 1970) by bath sonication (three 15-s bursts) and analyzed by PAGE. The gels were dried and ^{125}I-labeled proteins were visualized by autoradiography using lightening plus intensifier screens (Dupont Co. Diagnostic & BioResearch Systems; Wilmington, DE). Alternately, the cell pellets were frozen in a dry ice/ethanol bath and stored at −70°C.

The ability of the cell-associated proteins to bind to the Man 6-P receptor affinity column was determined by suspending the frozen cell pellets in 0.5 ml of 50 mM phosphate, pH 7, 5 mM β-glycerophosphate, 2 mM EDTA, 0.1 mM PMSF and sonicating the suspension for four 15-s bursts. The resulting extract was centrifuged in a microfuge (Eppendorf, made by Brinkmann Instruments) for 15 min and the supernatant, containing >95% of the cell-associated radioactivity, was applied to the CI Man 6-P receptor affinity column equilibrated in 50 mM phosphate, pH 7, 150 mM NaCl, 5 mM β-glycerophosphate, 2 mM EDTA, 0.1 mM PMSF (buffer B). The amount of cell extract applied to the column was adjusted so that the total endogenous β-*N*-acetylglucosaminidase activity applied was ≤40 mU. Under these conditions, we verified that binding of the ^{125}I-labeled ligands to the receptor was quantitative. The column was washed with buffer B until the radioactivity within the effluent returned to background. Bound proteins were eluted with buffer B containing 5 mM Man 6-P. Triton X-100 was excluded from the column buffer as the detergent caused broadening of the peaks that eluted from the column. When cell extracts containing the ^{32}P/^{35}S–double-labeled ligands were chromatographed on the receptor affinity column, the nonbound and bound peaks were pooled separately. Hemoglobin was added to each of the fractions to a final concentration of 0.4 mg/ml, and the proteins were precipitated with 10% TCA. Glycopeptides were prepared from the precipitated proteins and fractionated on concanavalin A (Con A)-Sepharose as described previously (Gabel and Foster, 1987).

Percoll Density Gradient Fractionation

L-cells were incubated with the [^{125}I]Man 6-P–bearing ligands for 60 min and then chased in ligand-free medium for 6 h. The monolayers were washed with PBS and then harvested by dislodging the cells with a rubber policeman. The cells were collected by centrifugation, washed with 0.25 M sucrose, and suspended in 7 ml of lysis buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7); cells from a similar, unlabeled confluent 15-cm dish were added as carrier. The cells were disrupted by 30 strokes of a homogenizer (Dounce, made by Kontes Glass Co., Vineland, NJ) and the resulting extract was centrifuged at 800 *g* for 5 min. The pellet was resuspended in 4 ml of lysis buffer, rehomogenized, and centrifuged. The 800 *g* supernatants were combined and added to a solution containing 10.8 ml of Percoll (Sigma Chemical Co.), 1.2 ml of 2.5 M sucrose, 0.4 ml of 1 M Hepes, pH 7; 0.25 M sucrose was added to adjust the total volume to 39 ml. The mixture was layered on top of 1.5 ml of 2.5 M

sucrose and centrifuged (VTi50 rotor; Beckman Instruments, Inc., Palo Alto, CA) for 45 min at 22,000 rpm. The resulting gradient was divided into 1.5 ml fractions by pumping from the bottom of the tube. β-hexosaminidase was assayed using *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide as substrate (Hall et al., 1978).

Preparation of Conditioned Medium

L-cells were grown at high density (5×10^5 cells/cm^2) on 15-cm dishes in 24 ml of MEM containing 10% newborn calf serum. After 24 h, the medium was collected, clarified by centrifugation, and used as L-cell conditioned medium. BRL-3A cells were grown to confluency on 15-cm dishes in 10% newborn calf serum. The monolayer was rinsed three times with serum-free MEM then incubated for 6 h in 20 ml of serum-free MEM. The medium was discarded and 20 ml of fresh serum-free MEM was added. The cells were incubated for 40 h before collection of the conditioned medium.

Other Methods

β-glucuronidase was assayed using *p*-nitrophenol-β-glucuronide as substrate (Hall et al., 1978). One unit of activity is defined as the amount of enzyme required to produce 1 μmol/min of *p*-NO_2-phenol at 37°C. Ligands to be treated with *Escherichia coli* alkaline phosphatase were dialyzed extensively against 60 mM Tris, pH 8.8. 0.45 ml of the dialyzed preparation was incubated with 0.005 ml of alkaline phosphatase (500 U/ml; Calbiochem-Behring Corp., San Diego, CA) at 37°C overnight. An aliquot of the digest was analyzed by SDS-PAGE to verify that the ligands were not degraded by proteolysis and the remainder was chromatographed on the CI Man 6-P receptor affinity column.

Results

Preparation of Man 6-P-bearing Ligands

As a source of ligands for the Man 6-P receptor, secretions from mouse J774 cells were collected in serum-free medium. These cells secrete the precursor forms of several lysosomal enzymes into their growth medium (Jessup and Dean, 1980), and the secreted enzymes are highly enriched in phosphorylated high mannose-type oligosaccharides (Varki and Kornfeld, 1983; Gabel and Foster, 1987). The J774 secretions were applied to a Man 6-P receptor affinity column (Varki and Kornfeld, 1983; Hoflack et al., 1987) and proteins that bound and eluted with Man 6-P were labeled with ^{125}I. Several distinct ^{125}I-labeled polypeptides were detected in the preparation (Fig. 1). The apparent molecular masses of the four major species corresponded to 72,000, 64,000, 52,000, and 38,000 D (Fig. 1, lane *1*). The 72-kD species was demonstrated to be the precursor of β-glucuronidase by immunoprecipitation (not shown); the identity of the other species is unknown. The iodination procedure did not inhibit recognition by the Man 6-P receptor as 96% of the protein-associated radioactivity bound to the receptor affinity column when the ^{125}I-labeled proteins were rechromatographed (Fig. 1, lanes 2 and *3*). Pretreatment of the ^{125}I-labeled proteins with alkaline phosphatase, however, negated binding to the affinity column; this inhibition indicated that interaction of the radiolabeled proteins with the Man 6-P receptor affinity column required an intact Man 6-P recognition marker.

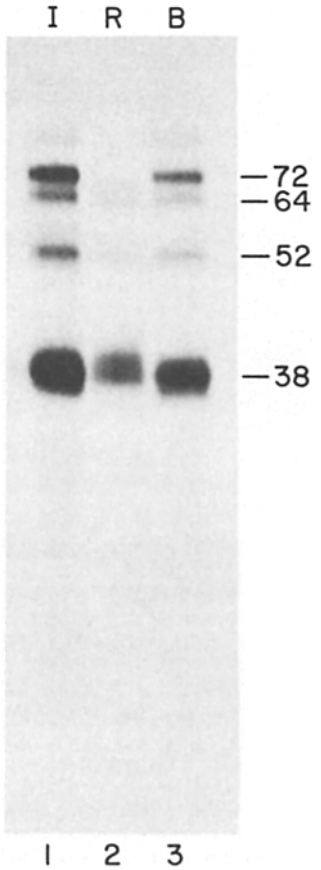

Figure 1. Characterization of the Man 6-P-bearing ligands. ^{125}I-labeled proteins were applied to the CI Man 6-P receptor affinity column. The labeled proteins recovered in the nonbound, and the Man 6-P eluate (bound) fractions were analyzed by SDS-gel electrophoresis. The autoradiogram of the dried gel is shown; the volume of the nonbound sample was 16-fold greater than the volume of the bound sample to load an equal number of cpm in each lane. The autoradiogram shows the ^{125}I-labeled polypeptides detected in the input ligands (lane *1*, *I*), the nonbound fraction (lane *2*, *R*), and the bound fraction (lane *3*, *B*). The numbers on the right indicate apparent molecular masses in kilodaltons.

Internalization of the Man 6-P-bearing Ligands by L-cells

The ^{125}I-labeled Man 6-P-bearing proteins were incubated with mouse L-cells for 60 min, after which the cells were chased in ligand-free medium. The copresence of 5 mM Man 6-P reduced the cellular accumulation of the ^{125}I-ligands by 94%, indicating that cell surface Man 6-P receptors functioned in the internalization process. The bulk of the accumulated radioactivity remained cell-associated during the subsequent chase. 20% of the radioactivity appeared in the medium after 2 h of chase (Fig. 2 *A*); 93% of this was soluble in TCA suggesting that low molecular mass components were released from the cells. Over the next 22 h of chase, however, the cell-associated radioactivity declined only by an additional 10% (Fig. 2 *A*). The stability of individual cell-associated ligands was analyzed by SDS gel electrophoresis and autoradiography (Fig. 2 *B*). The L-cells accumulated each of the input ^{125}I-labeled polypeptides and the cell-associated forms remained intact throughout the 24-h chase. Several of the proteins, however, displayed an increase in their electrophoretic mobility after endocytosis (Fig. 2 *B*). For example, the majority of the cell-associated ^{125}I-β-glucuronidase (68 kD; lanes *3–10*) migrated slightly faster than the input form of the enzyme (72 kD; lane *1*); this shift corresponds to a proteolytic maturation of the precursor form of β-glucuronidase (Gabel and Foster, 1986*b*). Similarly, the 38-kD polypeptide in the input ligand preparation (lane *1*) gave rise to a 35-kD polypeptide during the chase (lanes *3–10*). The cell-associated ligands, therefore, were delivered to intracellular compartments where they were altered but persisted as high molecular mass polypeptides.

To determine if the endocytosed proteins were dephosphorylated, secreted Man 6-P-bearing polypeptides were isolated from J774 cells that were labeled simultaneously with [^{35}S]methionine and $^{32}PO_4$. Proteins that eluted from the CI Man 6-P receptor affinity column with Man 6-P con-

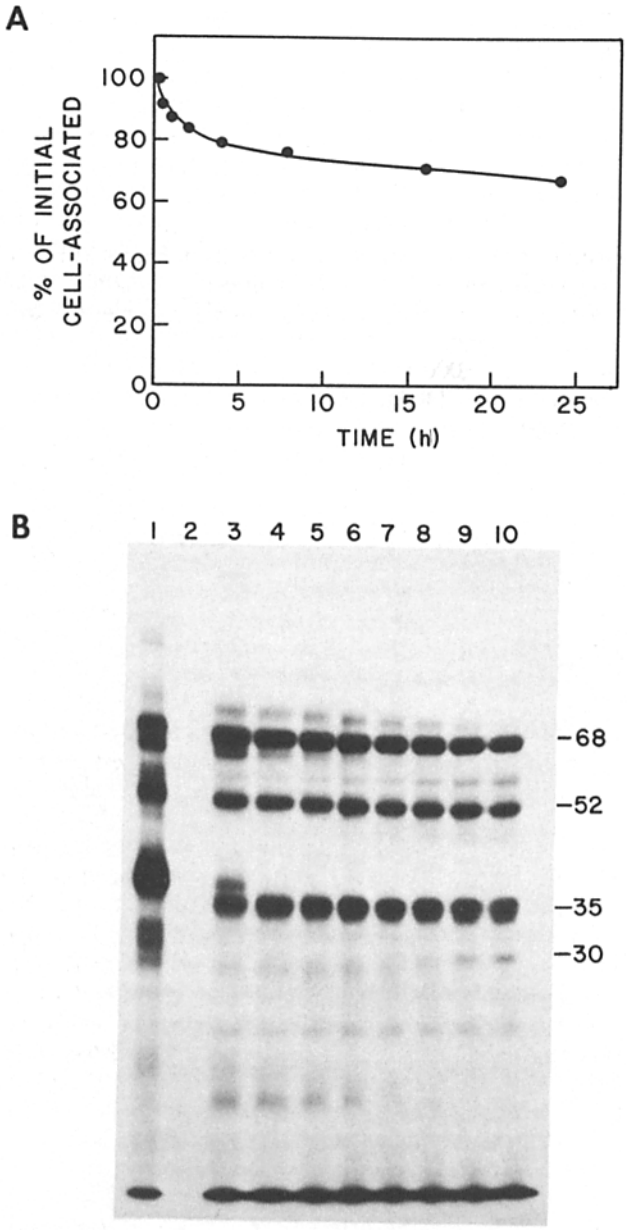

Figure 2. Pulse-chase analysis of cell-associated ^{125}I-Man 6-P-bearing ligands. L-cells (confluent monolayers on 6-cm dishes) were incubated with the ^{125}I-ligands for 60 min. The postuptake supernatant was removed, and the cells were washed and chased in ligand-free medium for the indicated times. The recovery of the total cell-associated radioactivity at each chase time (expressed as the percentage of the cell-associated radioactivity recovered after the 60-min pulse) is shown in *A*. The cell-associated ligands were analyzed by SDS-PAGE; the autoradiogram of the dried gel is shown in *B*. The lanes contain the input ligands (lane *1*) and cell extracts after 0 (lane *3*), 0.5 (lane *4*), 1 (lane *5*), 2 (lane *6*), 4 (lane *7*), 8 (lane *8*), 16 (lane *9*), and 24 (lane *10*) h of chase. The extract in lane 2 was derived from cells that were incubated with the ^{125}I-labeled ligands in the presence of 5 mM Man 6-P. The numbers on the right indicate the apparent molecular masses in kilodaltons.

tained a ^{32}P/^{35}S ratio of 1.1, and were composed of the same polypeptide species as detected in the ^{125}I-ligand preparation (not shown). After a 60-min incubation of the double-labeled ligands with L-cells and a 6-h chase in ligand-free medium, the cell-associated radioactivity partitioned into both the nonbound and bound fractions of the Man 6-P receptor affinity column (Table I). Only 31% of the cell-associated ^{35}S bound to the affinity column and eluted with Man 6-P while 59% of the ^{32}P was recovered in this fraction (Table I). Proteins recovered in the bound and nonbound fractions were precipitated with TCA and digested with pronase. The resulting glycopeptides were applied to Con A-Sepharose to isolate phosphorylated high mannose-type units. Few ^{32}P-labeled high mannose-type oligosaccharides (*peak III*, Table I) were recovered from proteins that did not bind to the receptor affinity column; the majority of the ^{32}P in the nonbound fraction was soluble in TCA suggesting that the phosphate was no longer protein associated. In contrast, proteins that bound to the receptor affinity column yielded a large number of ^{32}P-labeled high mannose oligosaccharides (Table I). The ratio of ^{32}P recovered as high mannose-type glycopeptides divided by the total ^{35}S recovered from Con A-Sepharose was 0.053 and 0.92, respectively, for the nonbound and bound glycoproteins. Proteins that bound to the Man 6-P receptor affinity column, therefore, contained a 17-fold enrichment of ^{32}P-labeled high mannose-type oligosaccharides relative to the nonbound species (Table I). Moreover, 94% of the total ^{32}P-labeled oligosaccharides recovered after the chase were associated with glycoproteins that bound to the receptor affinity column. After endocytosis, therefore, the Man 6-P-bearing ligands were partially dephosphorylated, and the receptor affinity column efficiently separated the phosphorylated and nonphosphorylated species.

Cell Density Influences the Extent of Dephosphorylation

The extent of dephosphorylation of the internalized ligands was compared in L-cells that were plated at densities ranging from 1×10^4 to 60×10^4 cells/cm^2. After an overnight growth period, the cells were incubated with the ^{125}I-labeled Man 6-P-bearing ligands for 60 min and then chased for an additional 24 h. At low cell density (1×10^4 cells/cm^2) the

Table I. Fractionation of Cell-associated Double-labeled Ligands by Man 6-P Receptor Affinity Chromatography

Fraction	Total cpm ^{35}S	Total cpm ^{32}P	Con A peak	cpm ^{35}S	cpm ^{32}P	Ratio
Nonbound	6022	2214	I	3019	198	
			II	26	20	0.053
			III	15	163	
Bound	2753	3174	I	1523	21	
			II	7	121	0.92
			III	0	1401	

L-cells were incubated with ^{32}P/^{35}S double-labeled ligands for 1 h after which the cells were washed and chased in the absence of ligand for 6 h. Cell-associated proteins were fractionated on the CI Man 6-P receptor affinity column to yield a nonbound and bound fraction; the distribution of radioactivity between these two fractions is indicated as the total cpm. Peak fractions were pooled and the proteins were precipitated with TCA, and the precipitated proteins were digested with pronase. The resulting glycopeptides were fractionated on Con A-Sepharose columns to yield components that did not bind to the Con A-Sepharose (peak I), biantennary-type units that bound and eluted with 10 mM α-methylglucoside (peak II), and high mannose-type units that bound and eluted with 100 mM α-methylmannoside (peak III). The ratio of ^{32}P cpm recovered as high mannose-type oligosaccharides divided by the total ^{35}S cpm recovered from Con A-Sepharose is indicated.

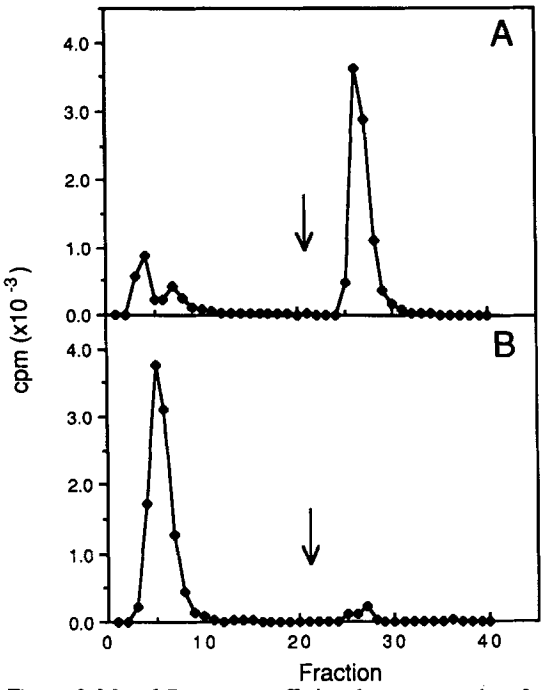

Figure 3. Man 6-P receptor affinity chromatography of cell-associated ^{125}I-ligands at low cell density. L-cells were plated at 10^4 cells/cm^2 on 6-cm dishes. After an overnight growth period, the cells were incubated with the ^{125}I-ligands for 60 min and either collected immediately (*A*) or chased for an additional 24 h (*B*) in the absence of ligand. Cell extracts were fractionated on the CI Man 6-P receptor affinity column; the elution profiles of ^{125}I from the affinity column are shown. The arrows indicate the fractions at which 5 mM Man 6-P was applied to elute bound glycoproteins.

endocytosed ligands underwent a time-dependent dephosphorylation (Fig. 3). 74% of the cell-associated radioactivity bound to the Man 6-P receptor affinity column and eluted with Man 6-P after the initial 60-min incubation (Fig. 3 *A*), but only 4% of the cell-associated ligand bound to the affinity column after the 24-h chase (Fig. 3 *B*). The percentage of the cell-associated radioactivity that bound to the affinity column after the initial 60-min incubation was equivalent at all cell densities (Fig. 4). After 24 h of chase, however, the extent of dephosphorylation showed a marked density-dependence (Fig. 4). At low cell densities the ligands were completely dephosphorylated, but the extent of this dephosphorylation decreased as the cell density increased. At 6×10^5 cell/cm^2, no significant dephosphorylation occurred during the 24-h chase (Fig. 4).

One explanation for the decreased dephosphorylation was that the L-cells internalized more ligand at the high cell density such that the intracellular Man 6-P-ase was limiting. The amount of ^{125}I-ligand internalized per cell, however, actually was less when the L-cells were maintained at high density. As the cell density increased, the amount of ligand internalized relative to the amount of endogenous β-hexosaminidase decreased from 27 to 2.5 cpm/mU at 1×10^4 and 60×10^4 cells/cm^2, respectively. This decrease most likely occurred as a result of the limiting amounts of ligand that were added to the cultures. Cells at high density, therefore, did not internalize more ligand than cells at low density, and the inability of high density cells to dephosphorylate the cell-associated proteins did not result from saturation of the Man 6-P-ase.

A second explanation for the failure of the high density L-cells to dephosphorylate cell-associated ligands was that the endocytosed proteins were not delivered to lysosomes under these growth conditions. The internalized radioactivity recovered from L-cells maintained at high and low density, however, fractionated comparably when cell extracts were separated by Percoll density gradient centrifugation (Fig. 5). In both cases, the cell-associated radioactivity paralleled the distribution of the endogenous lysosomal enzyme β-hexosaminidase (Fig. 5).

Transition between the High and Low Density States

The above results defined two separate states based on the extent of dephosphorylation of the internalized Man 6-P-bearing ligands; cells grown at low density efficiently dephosphorylated endocytosed Man 6-P-bearing ligands while cells grown at high cell density did not dephosphorylate these same molecules. To understand the basis for this differential processing, we examined the transition between the two states. First, the kinetics at which the endocytosed ligands were dephosphorylated was determined in cells grown at low density. After a 60 min incubation with the ^{125}I-ligands, 68% of the cell-associated radioactivity bound to the receptor affinity column (Fig. 6). The percentage of the endocytosed ligands that remained phosphorylated subsequently declined such that by 4 h of chase only 23% of the molecules bound to the receptor affinity column (Fig. 6). With longer chase times the extent of binding continued to decline and achieved a minimum value (12%) after 14 h (Fig. 6).

Next, the rate at which the cells switched from the high to the low density state was analyzed. L-cells established at high density were harvested by trypsinization and plated to low density. At various times after plating the cells were exposed to the ^{125}I-ligands for 60 min and subsequently

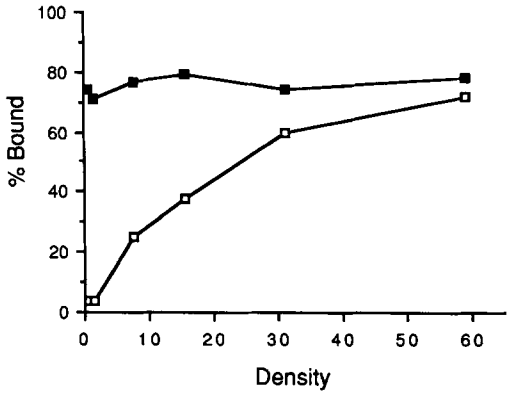

Figure 4. Cell density affects the extent of dephosphorylation of the cell-associated ^{125}I-ligands. L-cells were plated on 6-cm dishes at the indicated densities and maintained overnight in MEM containing 10% newborn calf serum. The cells subsequently were incubated with the ^{125}I-ligands for 60 min and either collected immediately (■) or chased in the absence of ligand for 24 h (□). Cell extracts were fractionated on the CI Man 6-P receptor affinity column. The percentage of the total cell-associated ^{125}I that bound to the affinity column and eluted with Man 6-P is indicated as a function of the density ($\times 10^4$ cells/cm^2) at which the cells initially were plated.

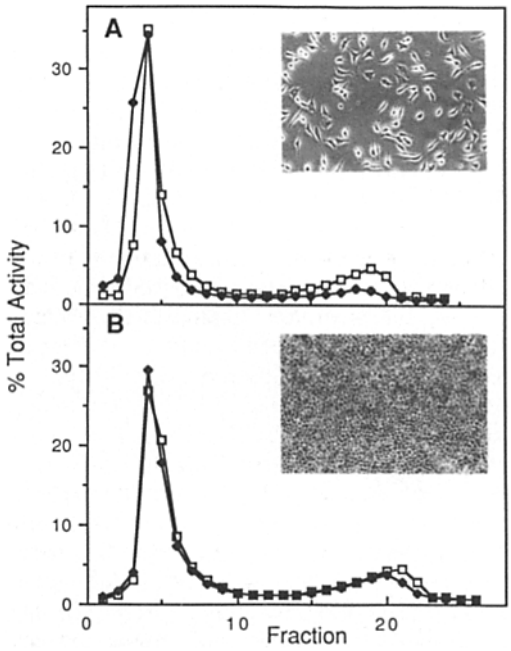

Figure 5. Endocytosed ^{125}I-labeled hydrolases accumulate within lysosomes. L-cells at either low (1×10^4/cm^2; *A*) or high (50×10^4/cm^2; *B*) density were incubated for 60 min with the ^{125}I-ligands followed by a 6-h chase in ligand-free medium. Cell extracts subsequently were separated by Percoll density gradient centrifugation. The gradients were pumped from the bottom of the tube (fraction 1) and the distribution of radioactivity (●) and endogenous β-hexosaminidase (□) is shown. Phase-contrast micrographs of low (*A*) and high (*B*) density cells are shown for comparison.

chased for 6 h; this time period was sufficient for low density cells to achieve maximum dephosphorylation (Fig. 6). The L-cells rapidly attained the low density state (i.e., the ability to dephosphorylate endocytosed ligands). After just 2 h at low density 68% of the internalized ^{125}I-labeled ligands

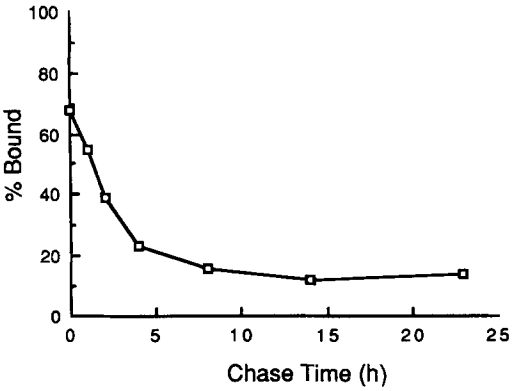

Figure 6. Kinetics of the dephosphorylation reaction at low cell density. L-cells were plated at low density (10^4/cm^2) on 10-cm dishes. After an overnight growth period, the cells were incubated with the ^{125}I-ligands for 60 min and subsequently chased in ligand-free medium. Cells were harvested at the indicated times and the resulting cell extracts were fractionated on the CI Man 6-P receptor affinity column. The percentage of the cell-associated ^{125}I that bound to the affinity column is indicated as a function of the time of chase.

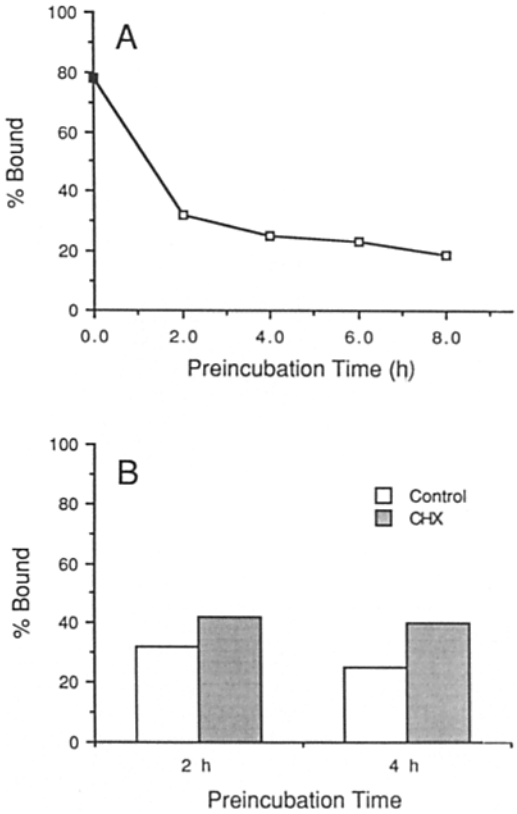

Figure 7. Transition from the high to low cell density state. (*A*) High density L-cells were harvested by trypsinization and replated at 10^4 cells/cm^2 on 10-cm dishes; t_o is the time cells were added to the dishes. After the indicated time the cells were exposed to the ^{125}I-ligands for 60 min and then chased in ligand-free medium for 6 h. The 0-min point refers to cells that were trypsinized and replated at high density (50×10^4 cells/cm^2) for 4 h before the pulse chase. Cell extracts subsequently were analyzed by Man 6-P receptor affinity chromatography. (*B*) High density cells were trypsinized and replated at low density in the presence or absence of 50 μM cycloheximide. After a 2- or 4-h preincubation, the cells were incubated with ^{125}I-ligands for 60 min followed by a 6-h chase. The amount of cell-associated radioactivity that bound to the Man 6-P receptor affinity column is indicated.

were dephosphorylated (Fig. 7 *A*). L-cells that were trypsinized but replated at a high density remained in the high density state; only 22% of the ^{125}I-ligand internalized during a 60 min incubation was dephosphorylated after 6 h of chase.

Finally, the requirement for protein synthesis in the transition from the high to low density state was determined by plating trypsinized high density cells to a low density in the presence and absence of 50 μM cycloheximide. Under these conditions, cycloheximide inhibited the incorporation of [^{35}S]methionine into TCA precipitable components by 95%. The protein synthesis inhibitor, however, did not prevent the L-cells from attaining the low density state. After 2 h at low density, noncycloheximide-treated cells dephosphorylated 70% of the internalized ^{125}I-labeled ligands and the cycloheximide-treated cells dephosphorylated 60% of these molecules (Fig. 7 *B*). After 4 h in the absence of cycloheximide, the low density cells increased the extent of dephosphorylation to 75% whereas the cycloheximide-treated cells remained

Table II. Serum Factors Are Required for the Dephosphorylation of Cell-associated Acid Hydrolases

Experiment	Cell type	Medium	cpm / mU	Percentage bound
1	L-cells	Naive	18,550	30%
		Conditioned	17,030	86%
	CHO	Naive	12,759	0.5%
		Conditioned	8,668	4%
2	L-cells	Naive	34,992	3%
		Serum free	30,304	86%
		IGF-II (130 nM)	11,687	24%
3	L-cells	Naive	11,754	3%
		BRL-3A	14,315	2%
4	L-cells	Naive	16,594	21%
		Serum free	12,653	86%
		IGF-I (1.3 nM)	19,978	71%
		IGF-I (130 nM)	17,657	65%

Cells were plated at low density (10^4/cm^2) and maintained for 16 to 24 h in the indicated medium. The cells subsequently were exposed to the ^{125}I-ligands for 60 min and chased for 6 h. Naive medium contained 10% newborn calf serum. Where indicated, IGF I and II were added to serum-free MEM at the indicated concentrations. Cells were maintained in their respective media throughout all phases of the experiment. The total amount of cell-associated radioactivity (expressed as cpm/mU of endogenous β-*N*-acetylglucosaminidase) and the percentage of this radioactivity that bound to the CI Man 6-P receptor affinity column are indicated. Cells treated with L-cell-conditioned medium consistently internalized less ligand than cells in naive medium. The magnitude of this difference depended upon the preparation of conditioned medium and ranged from 5 to 40%. Since L-cells secrete a small percentage of newly synthesized phosphorylated acid hydrolases into their growth medium (Gabel and Foster, 1986*a*), we believe that this inhibition is because of the competition between the unlabeled phosphorylated acid hydrolases in the conditioned medium and the ^{125}I-labeled ligands for the cell-associated Man 6-P receptor.

at 60%. Protein synthesis, therefore, was not necessary for the initial transition from the high to low cell density state, but in the presence of the inhibitor the level of dephosphorylation appeared to reach a plateau.

Serum Factors Are Necessary for the Low Density State

The rapid conversion of the L-cells from the high to low density state suggested that the medium of high density cells may be responsible for maintenance of the high density state. To examine this possibility, L-cells at low density were treated with naive or conditioned medium; the conditioned medium was isolated from L-cells grown at high density. After a 24-h conditioning period, the L-cells were incubated with ^{125}I-labeled Man 6-P-bearing ligands and then chased for an additional 6 h in the absence of the ligands. Low density cells treated with naive medium containing 10% serum efficiently dephosphorylated endocytosed ligands and only 30% of the cell-associated ^{125}I-labeled protein bound to the receptor affinity column after the chase (Table II). L-cells maintained at low density in the presence of conditioned medium, however, were much less efficient in the dephosphorylation process and 86% of the radiolabeled molecules bound to the receptor affinity column (Table II).

The conditioned medium effect was cell-type dependent (Table II). Chinese hamster ovary (CHO) cells rapidly and efficiently dephosphorylate their newly synthesized acid hydrolases (Gabel et al., 1983). Moreover, we have found that these cells rapidly dephosphorylate endocytosed Man 6-P-bearing polypeptides in a density-independent manner (not shown). CHO cells treated with naive medium efficiently dephosphorylated endocytosed ^{125}I-labeled ligands such that <1% of the cell-associated species bound to the receptor affinity column after the 6 h of chase (Table II). Likewise, CHO cells treated with L-cell conditioned medium efficiently dephosphorylated the endocytosed ligands as only 4% of the ^{125}I-labeled proteins bound to the affinity column (Table II). L-cell-conditioned medium, therefore, altered the fate of the Man 6-P-bearing ligands internalized by L-cells but did not prevent dephosphorylation of the same ligands after entry into CHO cells.

Since conditioned medium would be expected to contain products secreted by cells and to be deficient in serum factors used by the cells, low density L-cells were preconditioned in serum-free medium to determine if serum factors were necessary for maintenance of the low density state. After 10 h of serum-free growth, L-cells returned to the high density state and dephosphorylated only 14% of the proteins subsequently internalized (Table II). Cells maintained in 10% serum dephosphorylated 97% of the endocytosed molecules (Table II). Both serum-free and the L-cell conditioned media, therefore, appeared to lack components required for the L-cells to dephosphorylate endocytosed acid hydrolases.

In view of the ability of the CI Man 6-P receptor to bind both IGF II and Man 6-P-bearing ligands (Kiess et al., 1988; Tong et al., 1988; Waheed et al., 1988), we asked if this growth factor could induce L-cells to attain the dephosphorylation-competent state. IGF II-treated cells differed from cells in serum-free medium in that (*a*) they internalized one-third the amount of ^{125}I-labeled ligand, and (*b*) they dephosphorylated a larger percentage of the endocytosed molecules (Table II). Low density L-cells also were incubated with serum-free conditioned medium isolated from BRL cells; conditioned medium from these cells contains high levels of IGF II (Moses et al., 1980). Unlike L-cell-conditioned medium, the BRL cell-conditioned medium maintained low density L-cells in the dephosphorylation-competent state (Table II). Low density L-cells treated with IGF I, in contrast, did not efficiently dephosphorylate endocytosed acid hydrolases. After an overnight incubation in the presence of 130 nM IGF I, the L-cells dephosphorylated 35% of the internalized ^{125}I-labeled ligands (Table II). Within the same experiment, cells treated with naive medium dephosphorylated 80% of the endocytosed proteins. Lowering the IGF I concentration 100-fold resulted in a similar effect on dephosphorylation, but neither concentration of IGF I inhibited the accumulation of the ^{125}I-labeled ligands (Table II). At the same concentration, therefore, IGF II caused a more extensive induction of the dephosphorylation-competent state and a larger inhibition of ligand accumulation than did IGF I.

Discussion

The majority of the CI Man 6-P receptor is located within Golgi and endosomal compartments, but the intracellular molecules are in equilibrium with a smaller pool of cell surface receptors (Fischer et al., 1980; Duncan and Kornfeld, 1988). Surface CI receptors mediate endocytosis of Man

6-P-bearing acid hydrolases and the delivery of the hydrolases to lysosomes (Willingham et al., 1981; von Figura et al., 1984). 10% of the CD Man 6-P receptor also is located at the cell surface (Stein et al., 1987*a*; Duncan and Kornfeld, 1988), but these surface molecules do not mediate endocytosis (Stein et al., 1987*b*). Internalization of ^{125}I-Man 6-P ligands by the mouse L-cells is assumed, therefore, to reflect the activity of the CI receptor. Several of the ^{125}I-labeled polypeptides were modified after endocytosis such that they migrated faster during SDS gel electrophoresis. The secretions of the J774 mouse macrophage-like cells contain the precursor forms of several acid hydrolases including β-glucuronidase and β-galactosidase (Gabel and Foster, 1987), and the altered mobility of the cell-associated ligands is most likely the result of the proteolytic maturation of the precursor molecules and/or alterations to the proteins' oligosaccharides. Despite residence within lysosomes, the cell-associated ^{125}I-ligands were stable throughout 24 h of chase. Since nonresident proteins are rapidly degraded within lysosomes (Murry and Neville, 1980), the prolonged stability of the ^{125}I-polypeptides indicates that these proteins are lysosomal constituents even though they initially were secreted from the J774 cells.

At low cell densities, the internalized ^{125}I-labeled acid hydrolases were dephosphorylated and, as a result, they failed to bind to the Man 6-P receptor affinity column. Man 6-P-bearing ligands isolated from J774 cells labeled with $^{32}PO_4$ and [^{35}S]methionine contained a ^{32}P/^{35}S ratio of 1.1. After endocytosis, proteins that failed to bind to the receptor column possessed a low ratio of 0.053 while proteins that continued to bind to the column retained a high value of 0.92. Relative to the [^{35}S]methionine-labeled polypeptide chains, therefore, endocytosed ligands that failed to bind to the affinity column contained 17-fold less ^{32}P than those species that bound to the column. The slightly lower ^{32}P/^{35}S ratio of the bound ligands relative to the input polypeptides is expected, as we have shown previously that endocytosed acid hydrolases undergo a limited dephosphorylation within a prelysosomal compartment resulting in the conversion of diphosphorylated oligosaccharides to monophosphorylated species (Gabel and Foster, 1986*b*). This two to one conversion would lead to a lower ^{32}P/^{35}S ratio. The rate at which the internalized proteins were dephosphorylated indicates that loss of the Man 6-P recognition marker occurs, for the most part, within the lysosomal compartment. Macromolecules internalized via receptor-mediated endocytosis are delivered to lysosomes with half-times of 15 to 30 min (Geuze et al., 1983; Willingham et al., 1981). The dephosphorylation reaction, however, occurred over a period of hours and was complete only after 5 h of chase.

In contrast to cells at low density, L-cells grown at high density did not efficiently dephosphorylate endocytosed acid hydrolases. Even after 24 h of chase, 80% of the cell-associated ^{125}I-Man 6-P-bearing polypeptides bound to the CI receptor affinity column. The limited dephosphorylation observed at high cell density was complete after the initial 1-h incubation of the cells with the ligands, suggesting that the prelysosomal phosphatase may be responsible for the bulk of this processing. Despite retention of the Man 6-P recognition marker, the internalized ^{125}I-ligands were associated with dense granules characteristic of lysosomes. Two extreme states were defined, therefore, based on the postendocytic processing. At high cell density the ^{125}I-ligands were delivered to lysosomes but were not dephosphorylated, and at low cell density the same ligands were delivered to lysosomes and were dephosphorylated.

This differential dephosphorylation did not result from the accumulation of excessive amounts of the acid hydrolases at high density that saturated the lysosomal Man 6-Pase. In fact, high density cells accumulated less ^{125}I-ligand than low density cells. Under our experimental conditions only tracer quantities of ligand were added to the cells. The reduced accumulation at high density was due in part to the limiting amount of ligand, but may also reflect alterations in the number of surface Man 6-P receptors. Moreover, the enhanced dephosphorylation observed at low density did not result from the induction of a Man 6-Pase. When cells at high density were trypsinized and replated to low density they rapidly attained the low density state; that is, ligands subsequently introduced by endocytosis were efficiently dephosphorylated. On average, newly synthesized acid hydrolases migrate from the ER to the lysosomal compartment with a half time of 2 h (Brown and Swank, 1983). Since only 2 h was required after replating to achieve a significant level of dephosphorylation, it is unlikely that a newly synthesized phosphatase could accumulate in sufficient quantities within lysosomes to account for the increased dephosphorylation. The transition from the high to low density state also was unaffected by cycloheximide, indicating that protein synthesis was not required.

The rapid transition from the dephosphorylation-incompetent (high density) to the dephosphorylation-competent (low density) state suggested that factors in the medium influenced the postendocytic processing of the endocytosed ^{125}I-ligands. Although L-cells at low density in the presence of serum were efficient in the dephosphorylation reaction, the same cells did not dephosphorylate cell-associated proteins when they were preconditioned with serum-free or L-cell conditioned media. The L-cell conditioned medium did not contain an inhibitor of the lysosomal phosphatase as this preparation did not affect the dephosphorylation of acid hydrolases internalized by CHO cells. The similarity in the effects of serum-free and L-cell conditioned media suggested that the density effect actually was caused by the depletion and/or inactivation of serum factors by L-cells at high density that were necessary for maintenance of the dephosphorylation-competent state.

Two observations suggest that IGF II fulfills part, if not all, of the serum requirement. First, preaddition of IGF II to low density cells in serum-free medium resulted in a dephosphorylation-competent state. Man 6-P-bearing ligands internalized by the IGF II–treated cells were efficiently dephosphorylated. In addition, the IGF II–treated cells consistently internalized 30% of the ^{125}I-ligand accumulated by cells in serum-free medium. This reduction in endocytic activity may result from an allosteric effect of the polypeptide growth factor on the subsequent binding of acid hydrolases to the CI Man 6-P receptor, or from a down regulation of the surface CI Man 6-P receptor in response to IGF II. The number of surface IGF II receptors, for example, are known to change in response to insulin and other growth factors (Wardzala et al., 1984; Braulke et al., 1989).

The second reason for believing that IGF II is the active serum component stems from the ability of conditioned me-

dium derived from BRL-3A cells to confer the dephosphorylation-competent state. IGF II is endocytosed by some cells in culture and degraded within lysosomes (Polychronakos and Piscina, 1988). It is likely, therefore, that L-cell-conditioned medium lacks the growth factor as a result of its internalization and degradation by high density cells. The liver cell serum-free conditioned medium, however, was as effective as fresh serum in maintaining the dephosphorylation-competent state. BRL-3A cells produce large quantities of IGF II, and conditioned medium from these cells is highly enriched in the active factor (Moses et al., 1980).

IGF II is known to bind with a similar affinity to both the IGF I and IGF II receptors (Steele-Perkins et al., 1988). In contrast, IGF I binds to the type II receptor with a 100-fold lower affinity than to its own receptor (Tong et al., 1988). The dephosphorylation-competent state was achieved more effectively by IGF II than by a similar concentration of IGF I. Based on the relative binding affinities, therefore, the effects of IGF II appear to occur through an interaction with the type II receptor. Concentrations of IGF I (1.3 nM) that would not be expected to interact with the type II receptor, however, caused a small stimulation in the extent of dephosphorylation. Although IGF II was more effective than IGF I, in three separate experiments the level of dephosphorylation of ^{125}I-ligands internalized by the IGF II-treated cells was not as extensive as by cells treated with 10% serum. This difference may indicate that a suboptimal IGF II concentration was employed or that multiple factors act in concert to regulate the dephosphorylation competence of the cells; serum contains many growth factors including IGF II (Pfeffer, 1988). Alternatively, serum and BRL-3A cell conditioned medium contain IGF binding proteins (Romanus et al., 1986) that may potentiate the effectiveness of the purified IGFs (Elgin et al., 1987).

The mechanism by which IGF II alters the postendocytic processing of the Man 6-P-bearing acid hydrolases is unknown. The growth factor may cause the activation of a latent lysosomal Man 6-Pase. Alternatively, if the L-cells contain separate populations of lysosomes that differ in their content of an acid Man 6-Pase, then IGF II may act to alter the mechanism by which ligands are allocated between these compartments or may stimulate exchange between the various populations. Under some circumstances, lysosomes are known to exchange soluble constituents (Ferris et al., 1987). The compartmentation model is supported by previous studies showing that mitogens and growth factors alter the intracellular transport of newly synthesized glycoproteins. Secretion of mitogen-regulated protein and cathepsin L, for example, are stimulated by growth factors (Nilsen-Hamilton et al., 1980; Gottesman and Cabral, 1981); interestingly, both of these proteins contain phosphorylated high mannose-type oligosaccharides and, under the appropriate conditions, are targeted to lysosomes (Lee and Nathans, 1988; Sahagian and Gottesman, 1982). Although the mechanism remains to be resolved, results of the present study indicate that the postendocytic maturation of acid hydrolases endocytosed via the L-cell CI Man 6-P receptor is influenced by serum factors including IGF II.

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