Biosynthesis and secretion of the mannose 6-phosphate receptor and its ligands in polarized Caco-2 cells

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Wick, Debra A., Bellur Seetharam, and Nancy M. Dahms. Biosynthesis and secretion of the mannose 6-phosphate receptor and its ligands in polarized Caco-2 cells. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G506–G514, 1999.—We have analyzed the transport of newly synthesized mannose 6-phosphate (Man-6-P)-bearing proteins (i.e., lysosomal enzymes) in the polarized human colon adenocarcinoma cell line, Caco-2, by subjecting filter-grown cells to a pulse-chase labeling protocol using [35S]methionine, and the resulting cell lysate, apical medium, and basolateral medium were immunoprecipitated with insulin-like growth factor II/MPR (IGF-II/MPR)-specific antisera. The results showed that the majority of secreted lysosomal enzymes accumulated in the apical medium at >2 h of chase and that this polarized distribution was facilitated by the IGF-II/MPR selectively endocytosing lysosomal enzymes from the basolateral surface. Treatment with various agents known to affect vesicular transport events demonstrated that incubations at 16°C or incubations with brefeldin A inhibited the secretion of lysosomal enzymes from both the apical and basolateral surface, whereas treatment with nocodazole selectively blocked apical secretion. In contrast, incubation with NH4Cl or nocodazole had a stimulatory effect on basolateral secretion. Taken together, these results demonstrate that the sorting of Man-6-P-containing proteins into the apical and basolateral secretory pathways is regulated by distinct components of the intracellular trafficking machinery.

lysosomal enzymes; intracellular trafficking; insulin-like growth factor II receptor

THE SELECTIVE DELIVERY OF newly synthesized lysosomal enzymes to the lysosome is an essential process for the functional maturation of this organelle that involves a number of specific recognition and segregation events. In higher eukaryotic cells, newly synthesized soluble acid hydrolases acquire mannose 6-phosphate (Man-6-P) residues on their N-linked oligosaccharides by the action of UDP-N-acetylglucosaminyl-lysosomal enzyme N-acetylglucosamine-1-phosphotransferase. The ability of this phosphotransferase to recognize a protein determinant that is common to lysosomal enzymes provides the specificity required for the subsequent segregation of lysosomal enzymes from secretory proteins (5, 27). In the Golgi, phosphomannosyl residues serve as high-affinity ligands for binding to two distinct Man-6-P receptors (MPRs), the 300-kDa insulin-like growth factor II/MPR (IGF-II/MPR) and the 46-kDa cation-dependent MPR. The removal of acid hydrolases from the secretory pathway occurs when the receptor-lysosomal enzyme complex enters into clathrin-coated pits and vesicles for delivery from the trans-Golgi network (TGN) to an acidified late endosomal compartment. The acidic pH of this compartment induces the complex to dissociate. The released lysosomal enzymes are then delivered to lysosomes, whereas the receptors either return to the Golgi to repeat the process or move to the plasma membrane where, at least for the IGF-II/MPR, they function to internalize extracellular ligands via a recapture pathway (6, 26, 35, 48).

Most cells secrete a small percentage of their newly synthesized lysosomal enzymes. However, under certain physiological conditions, some acid hydrolases are overproduced and the majority of these enzymes are secreted instead of being delivered to the lysosome (12, 20). A number of mechanisms have been proposed to explain how acid hydrolases may be selectively targeted to escape transport to the lysosome, resulting in their secretion: 1) decreased binding to MPRs due to an altered Man-6-P content on the lysosomal enzyme (38), 2) decreased binding to MPRs due to noncarbohydrate effects on the acid hydrolase (28), 3) altered availability of the MPRs to bind ligand due to saturation, downregulation, or redistribution of the receptors to the plasma membrane (1, 39), and 4) involvement of MPRs in the delivery of lysosomal enzymes to the cell surface (2). However, the exact mechanisms by which these processes are regulated to mediate the delivery of newly synthesized lysosomal enzymes to the cell surface for secretion, rather than to the lysosome, are not known.

Polarized epithelial cells present a more complicated problem when it comes to the delivery of proteins to the cell surface in that their plasma membrane is divided into two morphologically, functionally, and biochemically distinct cell surface domains: an apical domain that faces the exterior of the organism and a basolateral domain that faces the internal environment. Polarized epithelial cells are able to selectively direct newly synthesized membrane or secretory proteins to either of these domains (44). To begin to evaluate how the secretion of lysosomal enzymes may be regulated in a polarized cell, we have analyzed the biosynthesis and transport of Man-6-P-containing ligands (i.e., lysosomal enzymes) in the polarized human intestinal epithelial cell line, Caco-2. Our results indicate that the majority of newly synthesized lysosomal enzymes accumulate in the apical medium. The enrichment of distinct Man-6-P-containing ligands in the apical and basolateral medium plus the differential effects displayed by various agents known to alter vesicular transport events indicate that entry into and passage through the apical and basolateral pathways are differentially regulated.
MATERIALS AND METHODS

Materials. The following reagents were obtained commercially as indicated: EXPRE\(^{35S}\) protein labeling mix (1,200 Ci/mmol, NEN Life Science Products); fetal bovine serum (FBS; HyClone Laboratories); DMEM and trypsin-EDTA (GIBCO BRL Life Technologies); protein A-Sepharose, brefeldin A (BFA), and Man-6-P (Sigma); nucodazole (Aldrich); and endo-\(\beta\)-N-acetylglucosaminidase (endo H) (Boehringer Mannheim). Caco-2 cells were kindly provided by Dr. Ward Olsen of the Veterans Affairs Hospital (Madison, WI).

Cell culture. Caco-2 cells (passages 76–96) were grown in DMEM (25 mM glucose) supplemented with 20% heat-inactivated FBS, 4 mM glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin in a humidified atmosphere containing 5% CO\(_2\). For polarity experiments, cells were grown as epithelial layers by high-density seeding (1.5 \(\times\) 10\(^6\) cells/filter) onto nitrocellulose membrane filter inserts (Millicell-HA, 30 mm diameter, 0.45 \(\mu\)m pore size, Millipore). The formation and integrity of monolayers were assessed by the development of a significant transepithelial electrical resistance of \(250–300\) Ohm/cm\(^2\) over the resistance of the filter alone. Resistance readings were measured with a Millicell-ERS voltohmeter (Millipore). All polarity studies were performed 14 days after plating.

Metabolic labeling. Cells grown on filter inserts were starved for 15 min in DMEM lacking methionine and cysteine (GIBCO BRL Life Technologies) and containing 10% heat-inactivated FBS (DMEM-FBS). The cells were then incubated in DMEM-FBS containing EXPRE\(^{35S}\) protein labeling mix (0.25–0.5 mCi/ml) (pulse medium) for 1 h followed by incubation for the indicated times with DMEM containing 20% FBS, 1 mM methionine, and 1 mM cysteine (chase medium). In some experiments, Man-6-P was added to the pulse and chase medium to a final concentration of 10 mM. Incubations at 16°C were carried out in chase medium supplemented with 20 mM HEPES, pH 7.2. In some experiments, BFA (10 \(\mu\)g/ml) or NH\(_4\)Cl (10 mM) was added to the chase medium. Treatment with nucodazole was carried out by preincubating the cells for 2 h and 45 min in DMEM-20% FBS containing nucodazole (10 \(\mu\)g/ml) followed by the above labeling protocol with nucodazole (10 \(\mu\)g/ml) present in the starvation, pulse, and chase media. The apical and basolateral media were harvested. Unless otherwise indicated, the cells were solubilized for 1 h in ice in buffer containing 0.1 M Tris, pH 8.0, 0.1 M NaCl, 10 mM EDTA, Triton X-100 (1% vol/vol), sodium deoxycholate (0.1% wt/vol), aprotinin (1% vol/vol), antipain (4 \(\mu\)g/ml), benzamidine (20 \(\mu\)g/ml), and 2 \(\mu\)g/ml each of leupeptin, chymostatin, and pepstatin. The total amount of protein in the resulting cell lysate was determined using the Bradford protein assay as recommended by the manufacturer (Bio-Rad). In some experiments, the cells were labeled under serum-free conditions in DMEM lacking methionine and cysteine and supplemented with human serum albumin (0.05% vol/vol), insulin (5 \(\mu\)g/ml), transferrin (5 \(\mu\)g/ml), and selenium (5 \(\mu\)g/ml).

Immunoprecipitations. Cell lysates or medium were incubated at 4°C for 16–24 h with protein A-Sepharose plus anti-MPR polyclonal antibodies. In some samples, Man-6-P (10 mM) or purified IGF-II/MPR (0.27 nM), which had been isolated from bovine liver by pentamannosyl phosphate-agarose affinity chromatography (7, 18), was added. After recovery by centrifugation, the protein A-Sepharose beads were washed four times with buffer containing 0.1 M Tris (pH 8.0), 0.1 M NaCl, 10 mM EDTA, and 1% Triton X-100 and once in buffer containing 20 mM Tris (pH 8.0) and 20 mM NaCl. Bound proteins were eluted by the addition of Laemmli sample buffer and analyzed on 7.5% or 9% SDS polyacrylamide gels under reducing conditions. The radiolabeled bands were visualized by fluorography and quantified by using an Ambis radioanalytical imaging system or a PhosphorImager (Molecular Dynamics Storm 860) with ImageQuant (version 4.1) software.

Endo H digestion. Immunoprecipitated samples were eluted from the protein A-Sepharose beads by incubation with buffer containing 1% SDS and 10 mM Tris·HCl (pH 7.4) for 5 min at 95°C. The eluates were precipitated with acetone. Endo H digestion was carried out in a buffer containing 0.1 M citrate (pH 6.0), 0.075% SDS, 0.2% 2-mercaptoethanol, and 1 mM endo H for 16 h at 37°C. The samples were analyzed by SDS-PAGE as described above.

IGF-II/MPR-agarose affinity chromatography. A fraction of the dialyzed medium sample was passed over bovine liver IGF-II/MPR affinity columns (21). After washing, the columns were eluted sequentially with 5 mM glucose 6-phosphate in column buffer followed by 5 mM Man-6-P in column buffer. The eluates were precipitated with TCA, subjected to SDS-PAGE, and visualized by fluorography.

RESULTS

Expression of IGF-II/MPR in Caco-2 cells. To analyze the biosynthesis of the IGF-II/MPR in polarized Caco-2 cells, cells grown on filter inserts were metabolically labeled and the cell lysate, apical medium, and basolateral medium were incubated with IGF-II/MPR-specific antisera. The results of the immunoprecipitation show the expected band of \(\sim 250\) kDa, corresponding to the receptor, in the cell lysate plus multiple lower-molecular-weight species (Fig. 1A, lane 1). These lower-molecular-weight species were also detected in both the apical and basolateral media (Fig. 1A, lanes 3 and 5) and in immunoprecipitations using monoclonal or affinity-purified antibodies specific for the IGF-II/MPR (data not shown). To determine whether these low-molecular-weight species represented newly synthesized lysosomal enzymes that were bound to the IGF-II/MPR en route to the prelysosomal compartment, immunoprecipitations were carried out in the presence of Man-6-P. Figure 1A (lanes 2, 4, and 6) shows that the lower-molecular-weight species (i.e., major species of 124, 90, doublet at 65, doublet at 53, and 46 kDa molecular mass) were completely eliminated when the solubilized cell lysate or medium was incubated with IGF-II/MPR-specific antiserum in the presence of 10 mM Man-6-P.

In contrast, the \(\sim 250\) kDa species was efficiently immunoprecipitated from the cell lysate in the presence or absence of Man-6-P. The identity of the \(\sim 250\) kDa species as the IGF-II/MPR was confirmed by pentamannosyl phosphate-agarose affinity chromatography (data not shown). Thus these results demonstrate that the multiple low-molecular-weight species are Man-6-P-containing ligands (i.e., lysosomal enzymes) that remain bound to the receptor and are communoprecipitated along with the receptor. The observed relative molecular weight of these species indicates the size of the Man-6-P-containing proteins rather than the size of the receptor-ligand complex because of the dissociation of the complex during the preparation of the samples for SDS-PAGE. The results also show that no detectable receptor is secreted into the medium (see also Fig.
2). Although a 250-kDa species is detected in the basolateral medium (Fig. 1A, lanes 5 and 6), this band, which is highly abundant in the basolateral medium as determined by precipitating an aliquot of the medium with TCA (data not shown), is nonspecific, since it is precipitated with preimmune serum (Fig. 1B, lanes 1 and 6, and Fig. 2A).

Although our results demonstrate that Caco-2 cells do not release significant amounts of their endogenous IGF-II/MPR into the medium as has been reported in other cell lines (3, 43), Man-6-P-containing ligands were coimmunoprecipitated from both the apical and basolateral media using IGF-II/MPR-specific antisera. This is likely due to the presence of exogenous soluble IGF-II/MPRs in the chase medium that contains 20% FBS (50): using quantitative Western analysis, we have found that typical lots of FBS contain ~1 nM soluble IGF-II/MPR (Y. Zhang and N. M. Dahms, unpublished observations), which would provide the source of the receptor in the medium to interact with the secreted ligands. To confirm this hypothesis, medium from Caco-2 cells labeled in the absence or presence of FBS was immunoprecipitated with IGF-II/MPR-specific antisera. The results show that, in the absence of FBS, no low-molecular-weight species were immunoprecipitated (Fig. 1B, compare lanes 2 and 7). Addition of purified bovine liver IGF-II/MPR to serum-free medium resulted in the immunoprecipitation of the low-molecular-weight species, which were eliminated in the presence of 10 mM Man-6-P (Fig. 1B, lanes 4 and 5), similar to that observed in the presence of FBS (Fig. 1B, lanes 7 and 8). The identical low-molecular-weight species could also be isolated directly from the medium of Caco-2 cells by passage over an IGF-II/MPR affinity column (Fig. 1B, lanes 9 and 10). Glucose 6-phosphate and mannose 6-phosphate eluates were precipitated with TCA. Samples were analyzed on a 9% SDS-polyacrylamide gel under reducing conditions.

Fig. 1. Immunoprecipitation of insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor (MPR) and its ligands. A: Caco-2 cells grown on filter inserts were labeled with 35S protein-labeling mix (250 µCi/ml) for 1 h followed by a 2-h incubation in medium containing 1 mM unlabeled methionine and cysteine. Equal aliquots of the cell lysate (Cells), apical medium (Ap), and basolateral medium (Bl) were immunoprecipitated with polyclonal antiserum specific for the IGF-II/MPR in the absence (−) or presence (+) of 10 mM mannose 6-phosphate (M6P). Samples were analyzed on a 7.5% SDS-polyacrylamide gel under reducing conditions. Size of each of the major lower-molecular-weight species [relative molecular weight (Mr) × 10^3] is indicated on right. B: Caco-2 cells in 60-mm dishes were labeled with 35S protein-labeling mix (50 µCi/ml) for 20 h in the absence (−) or presence (+) of fetal bovine serum (FBS). Medium was harvested and immunoprecipitated with preimmune serum (Pre) or with polyclonal antiserum specific for the IGF-II/MPR (B2.5) in the absence (−) or presence (+) of 10 mM mannose 6-phosphate. To the supernatants from the immunoprecipitations in lanes 2 and 3, purified bovine liver IGF-II/MPR (0.08 µg) was added along with the B2.5 antibody, and the samples were analyzed in lanes 4 and 5, respectively. In a separate experiment, medium from Caco-2 cells labeled with 35S protein-labeling mix (50 µCi/ml) for 20 h in the absence of FBS was chromatographed on an IGF-II/MPR-Affi-Gel-10 affinity column (MPR column) that was eluted with glucose 6-phosphate (G, nonspecific ligand, lane 9) followed by mannose 6-phosphate (M, specific ligand, lane 10). Glucose 6-phosphate and mannose 6-phosphate eluates were precipitated with TCA. Samples were analyzed on a 9% SDS-polyacrylamide gel under reducing conditions.
incubated in chase medium containing nonradioactive methionine and cysteine for various times, and the cell lysates and media were immunoprecipitated with IGF-II/MPR-specific antisera. The newly synthesized, 250-kDa IGF-II/MPR was completely endo H sensitive at 0 h of chase, indicating the presence of high mannose oligosaccharides, and gradually acquired complex-type oligosaccharides that were completely resistant to endo H digestion (half-life 5 h) (Fig. 2A). The half-life of the receptor was 27 h, which is similar to that reported in several other cell lines (4, 17, 23).

Fig. 2. Biosynthesis of the IGF-II/MPR and phosphorylated ligands. A: Caco-2 cells grown as a polarized monolayer on filter inserts were labeled with 35S protein-labeling mix (250 µCi/ml) for 1 h followed by incubation for the indicated times in chase medium containing 1 mM unlabeled methionine and cysteine. Cell lysates, apical medium, and basolateral medium were immunoprecipitated with either polyclonal antiserum specific for the IGF-II/MPR or preimmune serum (Pre). Samples were then incubated in the absence (−) or presence (+) of endo H before SDS-PAGE on 7.5% resolving gels. Closed and open arrows indicate the migration of the endo H-resistant and endo H-sensitive IGF-II/MPR, respectively. *90-kDa species.

Analysis of the Man-6-P-containing ligands revealed a transient appearance of these low-molecular-weight species in the cell, with no detectable intracellular ligands after 8 h of chase (Fig. 2). These results are consistent with the dissociation of newly synthesized lysosomal enzymes from the receptor when they reach the prelysosomal compartment where the acidic environment of this compartment results in dissociation of the receptor from its ligands (26). The detection of Man-6-P-containing ligands in the apical and basolateral media indicates that these proteins have entered the secretory pathway, having escaped interaction with the MPRs and the subsequent delivery to the prelysosomal compartment. The Man-6-P-containing ligands present in the cells and media were sensitive to endo H digestion (Fig. 2A), which is consistent with the presence of Man-6-P on high mannose-type, N-linked oligosaccharides (15, 47). Although each of the major species of lysosomal enzymes was detected in both the apical and basolateral media, the apical medium showed an enrichment of the 90-kDa species, whereas the basolat-
eral medium showed an enrichment of the 65-kDa species at all time points (Fig. 2A), suggesting the existence of a selective mechanism for the entry of individual lysosomal enzymes into the apical or basolateral secretory pathways. Quantitation of the total phosphorylated population of lysosomal enzymes revealed that significantly more Man-6-P-containing ligands were observed in the apical medium than in the basolateral medium at >2 h of chase time, with ~60% and >80% of the total lysosomal enzymes detected in the apical medium at 6 and 36 h of chase, respectively (Fig. 2B). However, the presence of 10 mM Man-6-P in the pulse and chase media resulted in similar amounts of lysosomal enzymes present in the apical and basolateral media, with 42% and 48% of the total Man-6-P-containing ligands detected in the apical medium at 6 and 36 h of chase, respectively (Fig. 2B). Because the addition of Man-6-P to the medium of cells has been shown to inhibit the endocytosis of extracellular ligands by the IGF-II/MPR (22), these results implicate a role for the receptor in establishing the polarized steady-state levels of secreted lysosomal enzymes of Caco-2 cells.

Apical and basolateral sorting pathways. To determine whether the sorting of lysosomal enzymes into the apical and basolateral secretory pathways may be regulated differently, filter-grown Caco-2 cells were treated with several agents known to affect vesicular transport processes. The results are summarized in Table 1. Incubation of the cells at 16°C, which is known to block vesicular transport (31, 42), resulted in the nearly complete inhibition of secretion of lysosomal enzymes from both the apical and basolateral surfaces, indicating a similar temperature dependence for transport to the apical and basolateral cell surface. In addition, the low temperature significantly decreased the amount of newly synthesized lysosomal enzymes that associated with the IGF-II/MPR in the cell fraction (Fig. 3 and Table 1), suggesting that 16°C either blocked the phosphorylation of lysosomal enzymes or prevented the targeting of lysosomal enzymes and/or the IGF-II/MPR to the compartment where the receptor normally binds its ligand. Similar results were obtained with BFA, a fungal metabolite that is known to interfere with the function of a membrane-bound guanine nucleotide-exchange protein to prevent association of 20-kDa GTP-binding ADP ribosylation factors (ARFs) with membranes (11, 16). A significant decrease was seen in the amount of lysosomal enzymes that associated with the IGF-II/MPR in the cell fraction as well as the amount secreted at both surfaces, with a slightly greater impact on apical secretion (Fig. 4 and Table 1). Treatment of Caco-2 cells with nocodazole, which causes depolymerization of microtubules (10), resulted in about a 50% reduction in the amount of lysosomal enzymes secreted apically and a twofold increase both in the amount secreted basolaterally as well as the amount associated with the IGF-II/MPR in the cell fraction at the 6-h chase time (Fig. 5 and Table 1). These results demonstrate the requirement for an intact microtubular network for lysosomal enzymes to efficiently traverse the apical secretory pathway. Treatment of the cells with the weak base, NH₄Cl, which raises the pH of intracellular organelles (9, 33), resulted in a selective threefold increase in the secretion of lysosomal enzymes at the basolateral surface with a slight inhibitory effect on the apical secretory pathway. In contrast to that observed with nocodazole, the amount of lysosomal enzymes associated with the cell fraction was unchanged at the 6-h chase time (Fig. 6 and Table 1). Taken together, these results demonstrate that the secretion of lysosomal enzymes via the apical and basolateral secretory pathways is mediated through distinct machinery.

Table 1. Quantitation of mannose 6-phosphate-containing proteins in Caco-2 cells and media following various treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 h Chase</th>
<th>6 h Chase</th>
<th>2 h Chase</th>
<th>6 h Chase</th>
</tr>
</thead>
<tbody>
<tr>
<td>16°C</td>
<td>17 ± 5</td>
<td>2 ± 2</td>
<td>8 ± 1</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>BFA</td>
<td>10 ± 2</td>
<td>7 ± 1</td>
<td>20 ± 1</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>88 ± 2</td>
<td>39 ± 8</td>
<td>125 ± 1</td>
<td>218 ± 9</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>50 ± 2</td>
<td>86 ± 2</td>
<td>314 ± 58</td>
<td>93 ± 6</td>
</tr>
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</table>

Values are means ± SE, based on 3 experiments performed in duplicate. Percent of control is percent of mannose 6-phosphate-containing proteins in each fraction compared with control. Cells were pulse labeled for 1 h followed by a chase of 2 or 6 h. Representative gels for each type of treatment are shown in Figs. 3–6. Ap, apical medium; Bl, basolateral medium; BFA, brefeldin A.
DISCUSSION

MPRs mediate the lysosomal targeting of Man-6-P-containing soluble acid hydrolases, a heterogeneous population of >40 enzymes that differ in size, oligomeric state, number of N-linked oligosaccharides, and extent of phosphorylation of their oligosaccharide chains. Although much information is available concerning the essential role of this phosphomannosyl recognition system in the biogenesis of lysosomes in nonpolarized cells (36), little is known about the trafficking of the receptor and its ligands in polarized epithelial cells. To analyze the biosynthesis and transport of newly synthesized lysosomal enzymes in polarized cells, we have used IGF-II/MPR-specific antisera to detect by coimmunoprecipitation the total pool of Man-6-P-containing proteins in the human colon adenocarcinoma cell line, Caco-2.

It has been reported that Caco-2 cells release the majority of their secretory glycoproteins and lipoproteins (41, 46) as well as some nonglycoproteins (40) into the basolateral medium, and it was suggested (41, 46) that the basolateral pathway represents a default pathway for exocytosis. Subsequent studies have revealed that the acid hydrolase, α-glucosidase, is secreted predominantly from the apical surface in Caco-2 cells, whereas the other lysosomal enzymes tested, namely, cathepsin D, β-glucuronidase, and β-hexosaminidase, are secreted predominantly via the basolateral pathway (24). Our current studies clearly demonstrate that the majority of secreted phosphorylated lysosomal enzymes accumulate in the apical medium of Caco-2 cells. The discrepancy between our results and those of Klumperman et al. (24) may be due to the difference in the type of assay utilized: Klumperman et al. measured the activity of selected lysosomal enzymes, whereas in the current study IGF-II/MPR-specific antisera was used to detect the total endogenous pool of phosphorylated lysosomal enzymes, which coimmunoprecipitate with the receptor. The observa-
tion that the presence of 10 mM Man-6-P in the medium resulted in an increase in the amount of lysosomal enzymes in the basolateral, but not the apical, medium (Fig. 2B) is consistent with our previous findings that, although present on both plasma membrane domains, the IGF-II/MPR is capable of endocytosing phosphorylated ligands via the recapture pathway exclusively from the basolateral surface (8).

Therefore, the presence of Man-6-P in the medium would be expected to inhibit the uptake of lysosomal enzymes only from the basolateral surface, as observed in the current report. In addition, the observed decrease in the total amount of phosphorylated lysosomal enzymes in the apical medium following incubation with Man-6-P (Fig. 2B) suggests that inhibition of the IGF-II/MPR recapture pathway at the basolateral surface by Man-6-P partially inhibits the delivery of lysosomal enzymes to the apical surface. Thus the IGF-II/MPR plays a role in mediating the delivery of at least a portion of lysosomal enzymes to the apical surface via an indirect secretory pathway (Fig. 7). Taken together, these results indicate that the IGF-II/MPR recapture pathway plays a critical role in establishing the steady-state polarized distribution (i.e., apical enrichment) of phosphorylated lysosomal enzymes in intestinal epithelial cells.

The fungal metabolite BFA has been shown to interfere with various steps of intracellular vesicular transport. With respect to endoplasmic reticulum (ER)-to-Golgi transport, BFA treatment leads to an inhibition of anterograde vesicular transport, whereas the retrograde pathway seems to remain unaffected. At the morphological level, this results in an almost complete disappearance of the Golgi apparatus and relocation of Golgi constituents to the ER. These effects are caused by the inability of cytosolic coat components to bind to organelar surfaces: BFA inhibits GDP-GTP exchange on ARF proteins, which are key components for the recruitment of coat proteins (30, 34, 37). Our results show that BFA, as well as incubation at 16°C, inhibits both apical and basolateral secretion to a similar extent. In addition, little association of the enzymes with the IGF-II/MPR was observed in the current report (see Table 1 and Figs. 3 and 4). These results are consistent with BFA and low temperature blocking anterograde movement through the Golgi, thus either preventing the newly synthesized lysosomal enzymes from entering into compartments containing the phosphotransferase and/or preventing the phosphorylated enzymes from interacting with the MPRs in the TGN (Fig. 7).

The transport of brush-border enzymes to the apical membrane has been shown to occur in Caco-2 cells via either a direct pathway or an indirect pathway in which the proteins are first delivered to the basolateral membrane and then undergo transcytosis for delivery to the apical membrane (29, 32). Studies using nocodazole, a microtubule-depolymerizing agent, demonstrated that the delivery of membrane and secretory proteins to the apical surface was inhibited in the presence of the drug, whereas the delivery of basolateral proteins was unaffected (13). Consistent with these studies are our results that show that treatment with nocodazole resulted in an overall decrease in the amount of lysosomal enzymes secreted apically (see Table 1 and Fig. 5). The overall increase in the amount of lysosomal enzymes secreted at the basolateral surface and contained within the cell fraction in the presence of nocodazole (see Table 1 and Fig. 5) is likely due to an increase in the overall flux of the enzymes through the basolateral secretory pathway caused by an accumulation of the acid hydrolases in the TGN and/or endosomal compartments that results from the blockage of the apical secretory pathway (Fig. 7).

The targeting of newly synthesized acid hydrolases involves both the binding and release of ligand. The receptor-ligand complex forms in the Golgi and is transported to a prelysosomal compartment. The low pH of this late endosomal compartment induces dissociation of the complex by causing a change in the conformation of the receptor (49). A change in the ability to dissociate ligand can have an overall impact in the targeting pathway. This is demonstrated in the treatment of cells with lysosomotropic amines, such as NH₄Cl, which accumulate in acidic intracellular compartments, causing an increase in the intracellular pH and an impairment of receptor-ligand dissociation. The inability to dissociate ligand results in constantly occupied MPRs, which stimulate the secre-

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**Fig. 7. Proposed secretory pathway of lysosomal enzymes in Caco-2 cells.** Majority of newly synthesized soluble mannose 6-phosphate-containing lysosomal enzymes (●) accumulate in the apical medium, at least a portion of which arrive by an indirect pathway that is IGF-II/MPR dependent and involves the recapture pathway (RECAP) at the basolateral surface. Inhibition (//) of selected pathways by the indicated treatments (in italics) is shown. Enrichment of the MPRs (y-shaped receptor symbol) on the basolateral cell surface is also indicated (8). TGN, trans-Golgi network.
tion of newly synthesized acid hydrolases (14, 45). Our results show that treatment of Caco-2 cells with NH₄Cl had a slight inhibitory effect on the secretion of lysosomal enzymes at the apical surface but significantly increased (3-fold) the amount of secretion at the basolateral surface (see Table 1 and Fig. 6). A similar result was observed when cells were incubated in the presence of 10 mM Man-6-P: the amount of lysosomal enzymes in the cells, apical medium, and basolateral medium was 96%, 67%, and 348% of control, respectively, at ≈6 h of chase. These results indicate the involvement of an acidified compartment(s) in regulating the entry of lysosomal enzymes into the apical and basolateral secretory pathway, with the loss of acidified compartments having a selective inhibitory effect on apical secretion (Fig. 7).

In summary, we propose that targeting of lysosomal enzymes to the apical surface occurs in part via an indirect pathway: the IGF-II/MPR recaptures secreted lysosomal enzymes at the basolateral surface and the enzymes are subsequently delivered to an apical endosomal compartment (Fig. 7), a site where the endocytic pathways from the apical and basolateral surfaces meet (19, 25). The ability of the IGF-II/MPR to internalize secreted lysosomal enzymes solely from the basolateral surface (8) and the observed decrease in the accumulation of lysosomal enzymes in the apical medium in cells treated with NH₄Cl or Man-6-P, two treatments that result in the unavailability of the receptor, support the role of IGF-II/MPR in generating the steady-state polarized distribution (i.e., apical enrichment) of secreted phosphorylated lysosomal enzymes in intestinal epithelial cells. Further studies are required to address the issue of how lysosomal enzymes are selectively segregated into the apical secretory pathway and whether the number and/or extent of phosphorylation of N-linked oligosaccharides present on an acid hydrolase plays any role in this process.

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