Roles of Heme Insertion and the Mannose-6-Phosphate Receptor in Processing of the Human Myeloid Lysosomal Enzyme, Myeloperoxidase

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Biosynthesis of myeloperoxidase (MPO), a myeloid lysosomal hemoprotein critical for the optimal oxygen-dependent microbicidal activity of human neutrophils, is incompletely understood. The primary translation product undergoes cotranslational N-linked glycosylation with subsequent insertion of the Fe-containing prosthetic group into the peptide backbone, thereby converting the enzymatically inactive, heme-free apomPO into the peroxidatively active precursor, proMPO. Eventually, proMPO undergoes proteolytic processing into native, lysosomal MPO, with subunits of 59 and 13.5 Kd. We studied three unanswered questions regarding MPO biosynthesis: (1) At what point during MPO biosynthesis is the heme moiety inserted into the apoenzyme? (2) What consequences does heme-insertion have on subsequent processing events? (3) What role does the mannose-6-phosphate receptor (M6PR) system play in the delivery of MPO to the lysosome? Disruption of Golgi by brefeldin A (BFA) produced two major changes in MPO biosynthesis: (1) processing of the 89-Kd precursor to mature MPO was blocked and (2) constitutive secretion of the MPO precursor was inhibited. Inhibition of heme synthesis with succinyl acetone (SA) reduced peroxidase activity and profoundly blocked processing of proMPO to mature MPO. This inhibition of processing was not a generalized effect on all lysosomal enzymes, because the maturation of a non–heme-containing lysosomal enzyme, β-glucuronidase, was not altered. Electron microscopy showed that, although the normal peroxidase staining of endoplasmic reticulum was absent in SA-treated cells, there were MPO-related peptides in the ER. The role of the M6PR system was assessed by immunoprecipitating fractions obtained from M6PR affinity column chromatography. The 89-Kd proMPO failed to adhere to the M6PR affinity column, whereas the 59-Kd heavy subunit of mature MPO was specifically eluted from the column. We interpret these data to indicate that: (1) processing of proMPO to mature MPO occurs in a post-ER compartment that is itself BFA-sensitive or is distal to a BFA-sensitive compartment and (2) heme insertion into apomPO precedes and may be a prerequisite for proteolytic processing to enzymatically active mature MPO. Our analysis of the M6PR system in MPO biosynthesis led to the unanticipated finding that there were phosphomannosyl residues on mature MPO, but none on proMPO. We suggest that the bulk of proMPO at any time is not phosphorylated, but, when generated, the phosphorylated proMPO is quickly processed to the phosphorylated 59-Kd subunit of mature MPO. Thus, if the M6PR is important in the intracellular transport of MPO, it is the phosphorylated mature MPO that is directed to the lysosomal compartment by this system. We favor the alternative interpretations that MPO is targeted to lysosomes by an M6PR-independent pathway and that the observed phosphomannosyl residues on mature MPO do not play a significant role in lysosomal targeting.

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glycosyltransferases. Overall, the processing of MPO from precursor to mature, enzymatically active peptide is extremely slow. Chase periods of 5 to 8 hours are needed to detect even the smallest amount of mature lysosomal enzyme.14,18-20,24

There are three important shortcomings in our understanding of MPO biosynthesis. First, it is not clear when heme is inserted into the apoenzyme. Biosynthetic radiolabeling of HL-60 cells indicates that heme is inserted into the 89-Kd apoprotein,25 although it is not clear if this occurs in the ER or in a post-ER compartment during proteolytic processing of proMPO. Recent work by Mogullevsky et al showed that recombinant MPO expressed in Chinese hamster ovary (CHO) cells is a secreted 84-Kd heme-containing protein with peroxidase activity.24 However, glycosylation, and perhaps processing, of recombinant MPO in this system differed from that seen in cultured myeloid cells. Second, the consequences of heme-insertion on subsequent processing of proMPO have not been identified. Third, the role of the mannose-6-phosphate receptor (M6PR) system in targeting MPO to the azurophilic granule, the lysosomal compartment of the neutrophil, has not been determined.

We have used two cultured human myeloid cell lines, PLB 98526 and HL-60,27 to address specifically these three unresolved questions about MPO biosynthesis and processing.

MATERIALS AND METHODS

HL-60 cells were obtained from Dr Harry L. Malech (National Institutes of Health, Bethesda, MD) as an early passage of the line maintained in the laboratory of Dr Robert Gallo. PLB-985 cells were obtained from Dr Timothy Ley (Washington University, St Louis, MO). Lines were maintained in RPMI 1640 medium supplemented with 2 mmol/L glutamine, penicillin-streptomycin, and 10% serum (HL-60 cells) or 5% heat-inactivated fetal calf serum (FCS) with 5% serum (PLB 985 cells). Serum-plus was obtained from JRF Biosciences (Lenexa, KS). Cells were determined to be free of mycoplasma infection. Tissue culture medium was obtained from the University of Iowa Cancer Center. For biosynthetic labeling, RPMI Select Amine Kit (GTBCO, Grand Island, NY) was to prepare methionine-free medium that was supplemented with 1 mmol/L pyruvate, 1 mmol/L glutamine, antibiotics, and 10% dialyzed FCS. 35S-Methionine, (1320 Ci/mol) and 1P-orthophosphoric acid (8,500 to 9,120 Ci/mol) for radiolabeling were obtained from Amersham Life Sciences Products (Amersham Corp, Arlington Heights, IL). Brefeldin A was obtained from Epicenter Tech (Madison, WI) and stock solutions were prepared in methanol. Succinyl acetone (4,6-dioxoheptanoic acid) and reagents for the β-glucuronidase assay were obtained from Sigma Chemicals (St Louis, MO). Protein A was purchased from BRL (Gaithersburg, MD) and radiolabeled with 125I at a core facility at the Iowa City VA Medical Center. The M6PR affinity column was prepared as described by Faust et al28 and generously provided by Dr Stuart Kornfeld (Washington University, St Louis, MO).

Cell culture and biosynthetic labeling. Human leukemia cell lines, HL-60 and PLB 985, were grown in media described above and maintained at 37°C in an atmosphere of 5% CO2. For biosynthetic labeling, cells were suspended at 0.5 × 106/mL in methionine-free 1640 RPMI with 10% dialyzed heat-inactivated FCS for 1 hour. After the period of starvation, 25 μCi/mL of 35S-methionine was added and the cells maintained at 37°C for 1 hour (pulse label). At the end of the labeling period, cells were collected by centrifugation and either solubilized for subsequent analysis or resuspended in complete medium for the duration of the chase period, as previously described.18

For labeling proteins with 32P, cells were washed and resuspended in phosphate-free Eagle’s minimal essential medium for a 60-minute starvation period before labeling. 32P-Orthophosphoric acid, made isotonic by addition of 10 MOPS, was added to a final concentration of 250 μCi/mL. Cells were incubated for the designated period of time, solubilized, and processed as described.

Subcellular fractionation and separation. Subcellular fractions of radiolabeled cells were separated by centrifugation of discontinuous gradients of Percoll. Cells were isolated from the culture medium by centrifugation, washed once in phosphate-buffered saline (PBS), and resuspended in relaxation buffer [100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L ATP (Na), 3.5 mmol/L MgCl2, 10 mmol/L PIPE, pH 7.3] at 4°C. Cells were disrupted by nitrogen cavitation (350 psi for 20 minutes at 4°C) as previously described.30 Unbroken cells and nuclei were removed by centrifugation (500g for 10 minutes at 4°C), the cavitate loaded atop the Percoll gradient,29 and the gradient centrifuged (48,000g for 15 minutes at 4°C in a JA-20 rotor; Beckman Instruments, Inc, Palo Alto, CA). Fractions were collected and analyzed after the Percoll had been removed from each fraction by ultracentrifugation (twice at 100,000 rpm for 10 minutes at 4°C in a TL-100 rotor using a Beckman TL-100 table top ultracentrifuge; Beckman Instruments). We have previously shown using this method that gradient fraction 6 represents lysosomes, fractions 10 to 14 represent organelles with intermediate density, and fraction 20 represents light organelles, including Golgi and ER.30 Both lysates of whole cells and subcellular fractions were analyzed for activity of two lysosomal enzymes, MPO and β-glucuronidase, which occupy the same subcellular compartment in myeloid cells.31 MPO activity was determined spectrophotometrically as the oxidation of o-dianisidine.32,33 β-Glucuronidase activity was measured spectrophotometrically as the liberation of phenolphthalein from phenolphthalein glucuronide acid in an acetate buffer as previously described34 and after Percoll had been removed by ultracentrifugation.

Electron microscopy. Cells in suspension were pelleted and fixed for 2 to 16 hours with 2.5% glutaraldehyde in 0.1 μmol/L sodium cacodylate buffer (pH 7.2). After two buffer rinses, cells were osmicated for 30 minutes with 1% osmium tetroxide and 1.5% potassium ferrocyanide in the same buffer, dehydrated with a graded ethanol series, and embedded in Embed resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with an Hitachi H-7000 transmission electron microscope (Hitachi Scientific Instruments, Mountain View, CA) at an accelerating voltage of 75 kV.

Endogenous peroxidase stain. Cells were fixed with 0.1% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2) for 5 minutes. Cells were rinsed with buffer and incubated for 30 minutes at 37°C with 0.05 mol/L Tris buffer (pH 7.2) containing 0.2% diaminobenzidine-4HCl and 0.003% hydrogen peroxide. After two rinses with phosphate buffer, cells were processed for electron microscopy as described above.

Immunogold labeling of MPO-related peptides. Cells were fixed for 15 minutes with 0.1% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2). Cells were rinsed, dehydrated, and embedded in Lowicryl K4M resin at progressively lower temperatures. Immunogold labeling was performed by floating grids with ultrathin sections on top of drops of a series of immunoreagents. Sections were incubated with monospecific, polyclonal rabbit anti-MPO for 12 hours at 4°C followed by 2 hours at room temperature.
temperature with goat antirabbit IgG conjugated to 10 nm gold particles. Sections were counterstained with lead citrate.

**Analysis of MPO-related peptides.** MPO was immunoprecipitated from cell lysates, subcellular fractions, or column fractions using a monoclonal rabbit polyclonal antibody to human MPO as previously described. In general, 100 μL of lysed radiolabeled cells (5 x 10⁶ cell equivalents) or 700 μL of culture supernatant were used for immunoprecipitations. Briefly, samples were incubated with nonimmune rabbit serum for at least 30 minutes at 4°C, followed by a 10% suspension of washed protein A-containing S aureus to clear the sample of nonspecific radiolabeled species that might contaminate the immunoprecipitation. The cleared sample was then incubated sequentially with MPO antiserum and protein A-bearing Staphylococcus aureus. The protein A-antigen-antibody complex was then washed serially with 1 mL each of 0.5% Triton X-100 in Tris-buffered saline (10 mmol/L Tris buffer, pH 7.5, with 150 mmol/L NaCl), 2 mol/L urea in 0.5% Triton X-100 in Tris-buffered saline, 1 mg/mL bovine serum in 0.5% Triton X-100 in Tris-buffered saline, and Tris-buffered saline. After the final wash, the antigen-antibody complex was released from protein A by heating at 100°C for 5 minutes in the presence of the sodium dodecyl sulfate (SDS)-sample buffer (62 mmol/L Tris, 2 mol/L EDTA, 5% β-mercaptoethanol, 2.3% SDS, pH 6.9). A 5-μL aliquot of each sample was counted in a scintillation spectrometer and the remainder separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resultant gel was fixed, soaked in 1 mol/L sodium salicylate,35 dried, and subjected to autoradiography. In some cases, the radioactivity in specific bands was quantitated by excising the bands from the gel and counting in a β-counter. In other cases, the relative intensity of signals seen by autoradiography was quantitated using densitometry on a Shimadzu CS-9000U Dual-Wavelength Flying Spot Scanner (Shimadzu Scientific, Tokyo, Japan).

**M6PR affinity chromatography.** A 400 μL M6PR (215 Kd) affinity column was generously provided by Dr Stuart Kornfeld (Washington University, St Louis, MO) and equilibrated with column buffer (50 mmol/L imidazole, pH 7, 150 mmol/L NaCl, 0.05% Triton X-100, and 5 mmol/L sodium β-glycerophosphate, as previously described). Lysates of radiolabeled cells were applied to the column three times in sequence to guarantee sufficient exposure of the column matrix to ligand in the cell lysate. After the sample was loaded the third time, the column was washed with buffer and 1-mL fractions were collected and the counts monitored. When eluted counts reached baseline, 2 mmol/L glucose-6-phosphate in column buffer was applied to the column. This served as a control for any radiolabeled species that might elute from the column nonspecifically in the presence of a phosphorylated sugar. When eluted counts returned to baseline, 5 mmol/L mannose-6-phosphate in column buffer was applied to the column. The starting material as well as fractions (unbound, glucose-6-phosphate eluate, mannose-6-phosphate eluate) were immunoprecipitated with anti-MPO and the immunoprecipitates analyzed by SDS-PAGE and autoradiography.

**RESULTS**

**Kinetics of MPO biosynthesis.** When PLB 985 cells were pulse-chased with [35S]-methionine and the radiolabeled protein immunoprecipitated with antiserum to MPO, the earliest detectable protein was the 89-Kd proMPO. As previously noted for MPO biosynthesis in HL-60 cells,14,16,20,22,24 processing of proMPO to the mature heterodimer was relatively slow. Even at 39 hours of chase, a significant fraction of the MPO-related counts remained in the 89-Kd species. To obtain a better assessment of the kinetics of MPO processing, PLB 985 cells were pulse-labeled and chased for 3, 15, or 39 hours. Labeled cells were cavitated and subcellular organelles separated by centrifugation on a discontinuous gradient of Percoll. Previously,10 we have shown that fraction 6 represents lysosomes, fractions 10 to 14 represent organelles of intermediate density, and fraction 20 represents Golgi and ER. Fractions 6, 10, 14, and 20 from each chase period were immunoprecipitated with MPO antiserum and analyzed by SDS-PAGE and autoradiography (Fig 1). The relative amounts of precursor (89 Kd) and mature (59 Kd) MPO were assessed densitometrically (data not shown) from the autoradiogram of the gel of the immunoprecipitates. After 39 hours of chase, greater than 90% of the MPO-related signal in the lysosomal fraction was in the 59-Kd heavy subunit of mature MPO.

We have previously shown that monensin, chloroquine, or NH₄Cl, at concentrations that alkalinized lysosomes, do not inhibit processing or augment baseline secretion of proMPO.22 In like fashion, PLB 985 cells were cultured at 10 mmol/L or 50 mmol/L NH₄Cl throughout the pulse-chase period and the processing of proMPO was examined. Under control conditions, secreted proMPO represented a...
approximately 2.6% of the total proteins secreted, somewhat similar to the 10% to 14% range reported previously.\textsuperscript{3,6} In the presence of 10 mmol/L NH\textsubscript{4}Cl and 50 mmol/L NH\textsubscript{4}Cl, the relative amounts of MPO-related peptides secreted decreased to 60% and 53% of control values (data not shown). In contrast, under the same conditions, the total amount of radiolabeled proteins secreted increased to 125% and 175% of control values, respectively. Thus, it is noteworthy that alkalization decreased secretion of proMPO, in contrast to what would be predicted if the M6PR system were operating in the lysosomal targeting of MPO.

The processing of proMPO to mature MPO appeared to be somewhat slower when cells were alkalinized; at 19 hours of chase there was relatively more signal in the 89-Kd species in the presence of NH\textsubscript{4}Cl than in the absence of alkalization (Fig 2). This effect was seen to a greater extent in the presence of 50 mmol/L NH\textsubscript{4}Cl, in which the bulk of the intracellular immunoprecipitable MPO appeared as proMPO. Because the most profound effect of alkalization was on the secretion of proMPO, the resultant failure of alkalinized cells to release proMPO extracellularly likely exaggerated the observed increase in intracellular proMPO under alkalizing conditions. Identical to the results of our previous studies of MPO biosynthesis in HL-60 cells,\textsuperscript{22} longer chase periods resulted in synthesis of mature MPO even in the presence of 50 mmol/L NH\textsubscript{4}Cl (data not shown).

**Effect of breafeldin A (BFA) on processing of MPO.** BFA is a fungal product known to disrupt Golgi and thereby to block export of proteins from the ER.\textsuperscript{37-40} To localize the intracellular site of processing events in MPO biosynthesis, we studied the effects of BFA in this system.

In PLB 985 cells cultured in the presence of up to 1.0 \mu g/mL BFA for 6 hours, Golgi were no longer apparent and the ER became elongated and dilated (Fig 3c). As reported for other cells, these effects were reversed after BFA was removed from the culture medium.\textsuperscript{41} After growth in 1.0 \mu g/mL of BFA for 6 hours, PLB 985 cells were collected by centrifugation, washed, resuspended in fresh medium free of BFA, and cultured for an additional 16 hours. Under these conditions, the morphology of PLB 985 cells returned to normal with the reappearance of apparently normal Golgi (Fig 3d).

Biosynthesis of MPO was significantly altered in two major ways by treatment with BFA. First, the processing of proMPO into mature MPO was inhibited by 0.5 \mu g/mL of BFA (Fig 4). It is noteworthy that the 37/39-Kd bands were produced by BFA-treated cells, although there was no 59-Kd species detectable under the same conditions. In addition, secretion of proMPO was nearly completely inhibited by 0.5 \mu g/mL of BFA. Like the morphologic changes, the BFA-mediated changes in MPO maturation and secretion were reversible when the BFA-treated cells were resuspended in medium free of BFA (Fig 5).

**Binding of proMPO to M6PR affinity column.** To determine more directly the role of the M6PR in MPO biosynthesis, we examined the binding of proMPO and mature MPO to an M6PR affinity column (kindly provided by Dr Stuart Kornfeld). Cells were pulse-chased and the solubilized cells were passed over the M6PR affinity column. Bound proteins were eluted first with glucose-6-phosphate and then with mannose-6-phosphate. MPO-related species were immunoprecipitated from unbound as well as from eluted fractions and the immunoprecipitates separated by SDS-PAGE.

ProMPO was not detected in the immunoprecipitates of column fractions specifically eluted with mannose-6-phosphate (Fig 6). Immunoprecipitation of starting material and the unbound fractions had large amounts of proMPO, verifying that proMPO was synthesized normally under the experimental conditions. In contrast, the 59-Kd heavy subunit of mature MPO was immunoprecipitated from the mannose-6-phosphate eluate. There were no MPO-related peptides nonspecifically eluted from the col-
Fig 3. Electron microscopy of PLB 985 cells after BFA. PLB 985 cells were grown in the absence (a and b) or presence (c) of 0.5 μg/mL of BFA for 6 hours. In the presence of BFA, there was a loss of Golgi and development of dilated ER. These changes reversed and normal morphology reappeared (d) after cells were washed free of BFA and cultured an additional 16 hours without BFA.

umn by glucose-6-phosphate. In other experiments, the secreted form of proMPO also failed to bind to the M6PR affinity column (data not shown).

To determine if phosphorylated proMPO would bind to the M6PR affinity column, PLB 985 cells were radiolabeled with 32P for 12 hours, solubilized, and passed over the affinity column as performed previously. Unbound fractions as well as the peak fractions eluted with mannose-6-phosphate were immunoprecipitated with antiserum to MPO. As shown in Fig 7, the 89-Kd MPO-related species was detected in the unbound fraction only; no immunoreactive proteins were identified in the mannose-6-phosphate eluate even after prolonged exposure of the autorad.

Effect of heme synthesis on MPO processing. There is some controversy about the precise time in MPO biosynthesis at which the heme group is inserted into MPO. To determine what effect the insertion of the heme moiety might have in the processing of proMPO, we examined MPO biosynthesis in the presence of succinyl acetone (SA), an inhibitor of heme synthesis.

PLB 985 tolerated SA at concentrations as high as 500 μmol/L for periods as long as 96 hours with no change in
Fig 4. Effects of BFA on MPO biosynthesis. PLB 985 cells were pulse-labeled for 1 hour and chased for 36 hours in the absence (−) or presence (+) of 0.5 μg/mL BFA. Solubilized cells and culture supernatant were immunoprecipitated and analyzed as described earlier. In the presence of BFA, the 59-Kd heavy subunit of MPO was not synthesized, although both proMPO and the 37/39-Kd MPO-related species were detected. In addition, there was no proMPO secreted into the culture supernatant by cells grown in the presence of BFA.

viability, total protein synthesis, or doubling time. In the presence of 250 μmol/L SA for 72 hours, the activity of MPO decreased to 26% ± 0.04% of control values (mean ± SEM of 5 experiments performed in triplicate). In contrast, the activity of β-glucuronidase, a lysosomal enzyme that lacks heme but is present in the same subcellular compartment as MPO, was unchanged in the presence of SA.

Electron microscopic examination of PLB 985 cells cultured in the presence of 250 μmol/L SA showed normal cell morphology. However, the peroxidase-staining of ER (Fig 8a and b), a striking feature of control cells (Fig 8a), was nearly totally absent from cells cultured in the presence of SA (Fig 8b). In addition, there was much less intense peroxidase activity in lysosomes in the cells grown in the presence of SA. To determine if apolproMPO, presumably enzymatically inactive, was present in the peroxidase-negative ER of SA-treated cells, immunogold electron microscopy was performed (Fig 8c and d). In both control and SA-treated cells, immunogold staining of ER was intense. There was no difference of immunoreactivity in ER between control and SA-treated cells, indicating that SA did not alter synthesis of apopMPO.

In the presence of SA, there was a marked decrease in the amount of proMPO processed into mature MPO (Fig 9). Under control conditions, the majority of proMPO was processed to MPO after 39 hours of chase (Fig 1). In contrast, most of the MPO-related protein was in the 89-Kd form when pulse-chase labeling was performed in the presence of SA. When subcellular fractions of radiolabeled cells grown in the presence of SA were immunoprecipitated, the apparent maturation arrest of proMPO was distinctly seen (data not shown). Under control conditions, the ratio of 89:59-Kd species in fraction 14 was 0.24, whereas in the presence of SA this ratio was 1.50. The amount of proMPO secreted was not affected by SA (data not shown).

When PLB 985 cells labeled in the presence of SA were applied to the M6PR affinity column, the number of counts eluted and the elution profile of radioactivity were identical to those seen under control conditions, indicating that generation of the M6P ligand proceeded normally in the

Fig 5. Reversibility of the effects of BFA on MPO biosynthesis. PLB 985 cells were cultured in the absence of presence of BFA for 6 hours, washed free of BFA, and pulse-labeled for 1 hour without and with a subsequent 16-hour chase. Cells from the pulse and pulse-chase and supernatants from the pulse-chase were immunoprecipitated. After BFA (0.25 or 0.50 μg/mL) had been washed from the cells, MPO processing and secretion occurred normally.
characterized, i.e., the mechanism(s) by which MPO is targeted to the lysosome and the intracellular site of the insertion of the heme group during synthesis and its influence on subsequent processing events.

Targeting of lysosomal enzymes generally uses a transport system dependent on the generation of phosphomannose residues in the enzymes and delivery to a prelysosomal compartment via M6PR. Gabel et al. have shown that HL-60 cells possess M6PR, although at levels considerably below that of human fibroblasts. In addition, a variety of cells have targeting systems that are M6PR-independent. Based on the current understanding of M6PR-dependent lysosomal enzyme targeting, one would anticipate certain features of MPO biosynthesis if it were mediated by this pathway. First, proMPO should contain phosphorylated mannose residues by which the proMPO binds to M6PR. Second, because alkalization of the lysosome would inhibit M6PR-proMPO dissociation in the prelysosomal compartment, alkalization of cells should result in increased secretion of proMPO into the culture medium, decreased processing of proMPO to mature MPO, and decreased delivery of mature MPO to the lysosome. However, as detailed below, MPO biosynthesis deviates from these two predictions: (1) phosphorylated proMPO is short-lived and does not bind to an M6PR affinity column and (2) alkalization does not increase secretion of proMPO into the culture medium.

Based on studies by Strömberg et al. and Hasilik et al. and on our data, it is clear that MPO is phosphorylated during its biosynthesis. ProMPO is phosphorylated after 60 minutes of labeling, but the amount of this phosphorylated species does not increase during longer periods of radiolabeling. After longer periods, the most intense 32P signal is seen in the heavy subunit of mature MPO, and this species is concentrated in the lysosomal fractions on Percoll gradients. Both previously published studies showed that...
the small subunit of MPO is not phosphorylated and that the phosphorylated oligosaccharides on both proMPO and the mature heavy subunit of MPO are susceptible to digestion with endoglycosidase H.

In prior studies, we had noted that alkalinization of HL-60 cells neither increases the amount of proMPO secreted nor completely inhibits processing of proMPO to mature MPO. Similar results were reported by others. Monensin and chloroquine inhibit proteolytic processing of MPO by HL-60 cells but at concentrations much higher than those needed to alkalinize cells. Thus, the effects of monensin on MPO processing may not reflect alterations in pH, because monensin has been shown to modify organellar traffic by a mechanism independent of changes in pH. Furthermore, Hasilik et al reported that alkalinization of HL-60 cells with NH₄Cl decreased the amount of MPO secreted and concluded that "transport and maturation of constituents of azurophilic granules do
SUCINYL ACETONE

not involve acid pH-dependent mechanisms, and it may be possible that transport is not directed by mannose-6-phosphate residues. Thus, evidence from our studies and from those of other workers in the field support the hypothesis that lysosomal targeting of MPO may be M6PR-independent.

Phosphomannosyl residues are generated on lysosomal enzymes in a post-ER, pre-Golgi compartment.40 Our studies of the effects of BFA on MPO biosynthesis show that disruption of the Golgi resulted in cessation of proteolytic processing of proMPO to mature MPO, suggesting that such processing occurs in a post-ER compartment, perhaps the Golgi or some prelysosomal compartment that is also susceptible to the effects of BFA. As with the use of any inhibitor, conclusions derived from our BFA studies should be interpreted cautiously. The net effects of BFA on organellar function are complex48 and in certain cell types can include mixing of contents in the ER and Golgi,49 altering morphology in trans Golgi,50 and affecting gene expression for the ER resident protein grp 78,51 Nonetheless, it is noteworthy that the 39-Kd species was synthesized in the presence of BFA despite the absence of the 59-Kd peptide. The 39-Kd MPO-related peptide has been noted by a variety of investigators,13,14,36,52 but its significance in MPO biosynthesis is unknown. Two-dimensional peptide maps of chymotryptic digests of MPO-related peptides in human neutrophils have shown that this peptide is closely related to, but not identical with, the mature heavy subunit of MPO.52 However, whether it represents a proteolytic product of the 59-Kd subunit generated when the sample is heated in SDS before electrophoresis36,53 or an intermediate in normal MPO biosynthesis5 remains unresolved. The appearance of the 39-Kd peptide in the presence of BFA, under conditions in which no mature heavy subunit was generated, argues against the explanation that this peptide is produced by artifactual proteolysis of the 59-Kd subunit.

If the M6PR system were participating in lysosomal targeting of MPO, one would anticipate that proMPO would be present in the M6P-eluate from the M6PR affinity column. Instead, we found that proMPO did not bind to the M6PR affinity column and that only the 59-Kd heavy subunit of mature MPO was immunoprecipitated from the M6P eluate. Phosphorylated proMPO failed to bind to the M6PR affinity column. Assuming that the phosphomannosyl residues on MPO-related peptides are functionally important, we believe there are at least two possible explanations for this unanticipated result that are not mutually exclusive.

First, the phosphorylated form of proMPO may be very short-lived and may undergo proteolytic processing to mature MPO very soon after the action of phosphotransferase. This explanation is consistent with two important observations of Strömberg et al.21 that (1) the amount of phosphorylated proMPO does not increase with prolonged labeling and (2) the subcellular distribution of proMPO...
labeled with radioactive amino acids is distinctly different from that seen for phosphorylated proMPO. Analysis of the intracellular transport of phosphorylated MPO in pulse-chase experiments shows that the bulk of the 32P signal is on the mature heavy subunit and is concentrated in the lysosomal fraction.\(^{21}\) The appearance of 32P-labeled MPO in the lysosomes occurs more rapidly than the appearance of \(^{35}S\)-methionine–labeled MPO, suggesting that phosphorylation occurs during the intracellular transport.\(^{21}\) This observation is consistent with our suggestion that the bulk of proMPO at any time is not phosphorylated and that, when generated, the phosphorylated proMPO is quickly processed to the phosphorylated 59-Kd subunit of mature MPO.

Second, it is possible that the native configuration of phosphorylated proMPO does not permit its binding to the affinity column via the phosphomannosyl ligand. According to this scheme, after proteolytic processing of proMPO into mature MPO, the conformational restriction is released, thereby allowing specific binding of the mature heavy subunit to the M6PR affinity column. We do not favor this interpretation, because it assumes without basis that there are restrictions for the interactions between phosphomannoses on proMPO and the M6PR that exist in vitro but not in vivo.

If the hypothesis that phosphorylated proMPO is very short-lived is true, either phosphorylation or some event proximal to phosphorylation of proMPO must be rate-limiting for MPO processing. In this context, the effects of succinyl acetone on MPO biosynthesis are relevant. Cells tolerated succinyl acetone well; cell viability, total protein synthesis, and the M6PR affinity elution pattern were unchanged in the presence of 250 \(\mu\)mol/L SA. SA specifically inhibited the MPO activity of cells cultured in its presence without modifying the activity of \(\beta\)-glucuronidase, a non–heme-containing azurophilic granule protein. Concomitantly, SA blocked processing of the 89-Kd species to mature MPO, indicating that insertion of heme into apoproMPO precedes and may be a prerequisite for proteolytic processing to mature MPO.

Based on these studies, we propose the model shown in Fig 11, which is consistent with the data presented here and with the long-standing observation that the processing of MPO in cultured myeloid cell lines is extremely slow.\(^{14,18-20,24,45}\) The primary translation product of MPO messenger RNA (mRNA) is a single peptide of 80 Kd that undergoes cotranslational cleavage of the signal peptide and N-linked glycosylation, resulting in a 92-Kd species that is rapidly cleaved by glucosidase I to generate the 89-Kd apoproMPO. This species is located in the ER and is extremely stable, as suggested by the BFA experiments and the very slow processing of 89-Kd MPO-related species during biosynthesis. We speculate that the next event is heme insertion into apoproMPO, which we have indicated in Fig 11 to be an ER event, based on the peroxidase staining in the ER of HL60\(^{53}\) and PLB 985 cells (Fig 8), and consistent with the data of Arnjots and Olsson.\(^{25}\) ProMPO is then phosphorylated, most likely after exiting from the ER, because phosphomannosyl residues are generated in a post-ER, pre-Golgi compartment.\(^{55}\) The phosphorylated proMPO has a very short half-life and is proteolytically processed to mature MPO in a prelysosomal compartment that is either BFA-sensitive or distal to a BFA-sensitive site, because BFA disrupted proteolytic maturation (Fig 4). The phosphomannosyl residues on mature MPO are able to bind to the M6PR affinity column, although it is not known what role these potential ligands play in directing MPO to lysosomes.

We cannot currently distinguish immunohistochemically between apoproMPO and proMPO because they are both 89-Kd proteins and our antiserum is directed against the native, mature MPO. Probes specific for the pro region should provide direct information regarding the time, subcellular location, and functional consequences of heme insertion for the biosynthesis and lysosomal targeting of MPO. It is clear that human myeloid cell lines possess

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**Fig 11.** Proposed scheme for MPO processing. The 80-Kd primary translation product undergoes cotranslational cleavage of the signal peptide (square) and N-linked glycosylation (branches) at 5 sites to generate a 92-Kd apoproMPO. This form of apoenzyme is rapidly processed to the 89-Kd apoproMPO by the action of glucosidase I, an event inhibited by castanospermine.\(^{25}\) The heme moiety (hexagon) is inserted at this point, thereby allowing specific binding of the mature heavy subunit to the M6PR affinity column. We speculate that the next event is heme insertion into apoproMPO, which we have indicated in Fig 11 to be an ER event, based on the peroxidase staining in the ER of HL60\(^{53}\) and PLB 985 cells (Fig 8), and consistent with the data of Arnjots and Olsson.\(^{25}\) ProMPO is then phosphorylated, most likely after exiting from the ER, because phosphomannosyl residues are generated in a post-ER, pre-Golgi compartment.\(^{55}\) The phosphorylated proMPO has a very short half-life and is proteolytically processed to mature MPO in a prelysosomal compartment that is either BFA-sensitive or distal to a BFA-sensitive site, because BFA disrupted proteolytic maturation (Fig 4). The phosphomannosyl residues on mature MPO are able to bind to the M6PR affinity column, although it is not known what role these potential ligands play in directing MPO to lysosomes.

We cannot currently distinguish immunohistochemically between apoproMPO and proMPO because they are both 89-Kd proteins and our antiserum is directed against the native, mature MPO. Probes specific for the pro region should provide direct information regarding the time, subcellular location, and functional consequences of heme insertion for the biosynthesis and lysosomal targeting of MPO. It is clear that human myeloid cell lines possess
with I-cell disease, a disorder in which cells are unable to
of the peroxidase in human monocytes. Biochim Biophys Acta
M6PR, but our data do not support a conventional role
of M6PR in targeting proMPO to a prelysosomal compart-
ment of the lysosome. Therefore, proMPO must use
an alternative pathway and the functional significance of
phosphomannosyl residues detected on mature MPO re-
mains to be determined. That leukocytes should use target-
paths independent of M6PR is consistent with the obser-
vation that hepatocytes and leukocytes from patients with
I-cell disease, a disorder in which cells are unable to
generate the M6P recognition marker, have normal levels
of lysosomal enzymes, whereas fibroblasts from the same
patients have a profound storage disorder. Rijnboutt et
al recently described a mechanism for lysosomal targeting
of cathepsin D in hepG2 cells that is M6PR-independent.
Furthermore, Valore and Ganz have recently described
features of the lysosomal targeting of defensins in HL60
cells. The defensins are targeted to the same intracellular
compartment as is MPO and may use the same pathway.

Analogous to our findings, secretion of prodefensins was
not increased by alkalization of cells. Because defensins
and their precursors lack sites for N-linked glycosylation, it
is highly unlikely that the M6PR pathway participates in
directing the proteins to azurophilic granules. Thus, it
seems likely that lysosomal targeting in neutrophil granulo-
cyes occurs via an M6PR-independent mechanism.

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NOTE ADDED IN PROOF

While this report was in press, Castenada et al reported similar
effects of succinyl acetone on the subcellular distribution of MPO
in HL-60 cells, using electron microscopy.

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Roles of heme insertion and the mannose-6-phosphate receptor in processing of the human myeloid lysosomal enzyme, myeloperoxidase

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