Separation and Analysis of Subcellular Organelles in a Human Promyelocytic Leukemia Cell Line, HL-60: Application to the Study of Myeloid Lysosomal Enzyme Synthesis and Processing

By William M. Nauseef and Robert A. Clark

We describe a system for analysis of the intracellular pathways in the biosynthesis and packaging of functionally important proteins in human myeloid cells. The human promyelocytic cell line HL-60 was used since peripheral blood neutrophils are terminally differentiated and do not actively synthesize protein. Cells were disrupted by nitrogen cavitation and subcellular organelles in postnuclear supernatant separated on a discontinuous gradient of Percoll modified to resolve organelles important in protein synthesis. This Percoll gradient separated azurophilic granules from less dense organelles and partially separated the less dense organelles from one another. Approximate densities of organelles identified by electron microscopy and by biochemical markers are azurophilic granules, 1.02 g/mL; endoplasmic reticulum, 1.039 g/mL; Golgi apparatus, 1.032 g/mL; and plasma membrane, 1.027 g/mL. We validated the utility of this method of subcellular fractionation by examining intracellular transport of myeloperoxidase, a myeloid lysosomal enzyme present in azurophilic granules. The subunits of mature myeloperoxidase [molecular weight (mol wt) = 89,000 and 13,500] cosediment with biochemical markers for lysosomes, whereas the large mol wt (89,000) precursor forms cosediments with biochemical markers of less dense organelles. Within the limits of assay sensitivity, the 89,000-mol wt precursor is enzymatically inactive and has no spectral evidence for a heme group, suggesting that precursors of myeloperoxidase may undergo proteolytic maturation in a prelysosomal compartment with concomitant incorporation of a heme group and acquisition of enzymatic activity. This system of analysis should be suitable for the identification, subcellular localization, and maturational analysis of other myeloid lysosomal enzymes as well as functionally important membrane proteins.

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The MICROBICIDAL and tumoricidal capacity of human polymorphonuclear neutrophils (PMNs) depends on the interaction of toxic oxygen radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radical with granule proteins, most notably myeloperoxidase (MPO), to generate a toxic milieu within the phagolysosome.1 Many aspects of this complex system have been characterized in detail, although studies of the biosynthesis and intracellular transport of myeloid lysosomal enzymes have been few in number.2,4 The major limitation for such studies has been the paucity of protein synthesis in cells as terminally differentiated as are human PMNs.7 For this reason, investigators have recently used cell lines derived from human tumors for study of various processes in myeloid cells.9 One such line, the human promyelocytic leukemia line HL-60,10 is especially suited for such studies because these cells resemble promyelocytes and can be chemically induced to develop the morphologic and functional characteristics of mature myeloid cells such as PMNs11 or macrophages.12 HL-60 cells contain azurophilic granule proteins such as MPO,13 elastase,13 β-glucuronidase,14 and cathepsin B14 and after induction display functional membrane receptors,13,16 respond to a chemotactic stimulus,17 carry out phagocytosis,18 and exhibit a stimulus-dependent respiratory burst.19

To dissect the subcellular pathway for transport of nascent lysosomal enzymes and functionally important integral membrane proteins, we have developed a method of subcellular fractionation using nitrogen cavitation and density centrifugation on a Percoll gradient. This method allowed clear separation of lysosomes and less dense organelles important in protein synthesis and posttranslational modification of nascent proteins. We illustrate the utility of this method of analysis by examining MPO synthesis, demonstrating that the enzymatically inactive, large-mol wt precursor form of MPO cosediments with Golgi apparatus, whereas enzymatically active, heme-containing mature MPO cosediment with biochemical markers for lysosomes.

MATERIALS AND METHODS

HL-60 cells. The HL-60 cell line was a gift from Dr H.L. Malech of Yale University and was originally obtained from Dr R.C. Gallo at the National Institutes of Health. Cells were cultured in RPMI 1640 (provided by the Cancer Center, University of Iowa) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L glutamine, and penicillin-streptomycin. Cells were determined to be free of mycoplasma infection.

Disruption of cells. Between 1 and 2 × 10⁶ HL-60 cells were harvested, washed once in phosphate-buffered saline (150 mmol/L NaCl, 10 mmol/L potassium phosphate, pH 7.0, PBS), and suspended in 20 mL of ice-cold relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L adenosine triphosphate–Na₂, 3.5 mmol/L MgCl₂, 10 mmol/L PIPES, pH 7.3). The cells were pressurized with N₂ for 20 minutes at 350 psi in a nitrogen bomb (Parr Instrument Co, Moline, Ill.) at 4 °C and the cavitate collected dropwise into ethylene glycol tetra-acetic acid (EGTA), pH 7.4 (final concentration, 1.25 mmol/L), as previously described.20

Subcellular fractionation. The cavitate was centrifuged (500 g for ten minutes at 4 °C) to separate nuclei and unbroken cells (P₁) from the supernatant (S₁). S₁ was layered atop the preformed Percoll gradients.

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**MYELOID LYOSOMAL ENZYME SYNTHESIS**

Density centrifugation. Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) was made isotonic by adding one tenth the final volume of a tenfold-concentrated solution of solution buffer (10× relaxation buffer) containing 12.5 mmol/L EGTA. Two Percoll solutions, a light and a dense, were prepared. To prepare 100 mL of light Percoll (final density, 1.040 g/mL), 10 mL of 10× relaxation buffer with EGTA, 26.4 mL of undiluted Percoll (density, 1.130 g/mL), and 63.6 mL of deionized water were mixed. To prepare 30 mL of dense Percoll (final density, 1.120 g/mL), 3.0 mL of 10× relaxation buffer, 26.4 mL of undiluted Percoll, and 0.6 mL of deionized water were mixed. To form the gradient for separation of subcellular organelles, 22 mL of light Percoll were layered atop 6 mL of dense Percoll and the tube centrifuged at 48,000 g (JA-20 rotor, Beckman Instruments, Inc, Palo Alto, Calif) for 15 minutes at 4 °C. Subsequently, S1 was layered atop the preformed gradient and centrifugation continued for an additional 15 minutes at 48,000 g. Two additional gradients were run in parallel: one (blank gradient) loaded with a volume of relaxation buffer equal to that of S1, loaded on the sample gradient, and one (marker gradient) loaded with a volume of relaxation buffer equal to that of S1, loaded on the marker gradient. The distances from the bottom of the tube to bands seen in the sample gradient and in the blank gradient were measured. The distances and corresponding densities for the standard density beads were plotted and the densities of the visible bands in the sample gradient estimated from the density gradient profile. Approximately 1-mL fractions were collected from each gradient as previously described.29 Because Percoll interferes with some assays (β-glucuronidase,30 polynacrylamide gel electrophoresis in sodium dodecyl sulfate [SDS-PAGE], and electron microscopy31), it was depleted from a portion of each fraction by ultracentrifugation in an Airfuge (Beckman) at 180,000 g for 10 minutes. As previously shown,29 the density of Percoll in any given fraction must be greater than that of the biologic material to prevent trapping of the subcellular organelles. For that reason centrifuge tubes were balanced with dense Percoll (p = 1.122). After centrifugation, the biologic material was pelleted atop a dense layer of Percoll.

**Enzyme assays.** MPO activity was determined spectrophotometrically as the oxidation of o-dianisidine.27 The change in absorption at 460 nm was measured and international units of MPO activity calculated as 2.655 × 103 times the rate of change in absorption at 460 nm%/min and data are expressed as international units per milliliter. Elastase activity was measured by the digestion of tritiated elastin as described by Stone et al32 using perillyl human neutrophil elastase (HNE) as a standard; 1H-elastin and purified elastase were the generous gifts of Dr P.J. Stone, Boston University, and data are presented as cpm/h/mL. We established that 3 μg of purified HNE solubilizes 2.417 ± 0.436 cpm/h (n = 11). β-Glucuronidase activity was measured spectrophotometrically as the liberation of phenolphthalein from phenolphthalein glucuronide acid (Sigma Chemical Co, St Louis) in an acetate buffer as previously described29 and after Percoll was removed by ultracentrifugation. Data are presented as units per milliliter where 1 unit is the quantity of β-glucuronidase that hydrolyzes 1 μmol of substrate per minute using a millimolar extinction coefficient of 33 (mmol/L)-1·cm-1 for phenolphthalein.

Vitamin B12-binding protein was measured as binding of 32Pvitamin B12 (Amersham Corp, Arlington Heights, Ill) as described by Gottlieb et al.34 Cytochrome c oxidase was measured spectrophotometrically as oxidation of cytochrome c in the manner described by Cooperstein and Lazarow.35 Cytochrome c (Sigma) was reduced with sodium dithionite prior to the assay. Alkaline phosphatase activity was measured spectrophotometrically as the liberation of p-nitrophenol from p-nitrophenylphosphate in a sodium barbital buffer (pH 10.5) as described by DeChatelet and Cooper.36 Data are presented as U/mL where 1 unit is the amount of alkaline phosphatase that hydrolyzes 1 μmol of p-nitrophenylphosphate per minute, using a millimolar extinction coefficient of 18.6 (mmol/L)-1·cm-1 for p-nitrophenol. For samples containing Percoll, an identical sample without p-nitrophenylphosphate was run in parallel to quantitate the turbidity at 410 nm produced by Percoll. This value was subtracted from that obtained with substrate. Sulfatase C activity was measured fluorometrically as the release of 4-methylumbelliferyl sulfate (Sigma) as previously described.37 Data are presented as units per milliliter where 1 unit is the amount of sulfatase C that produces 1 μmol of 4-methylumbelliferyl sulfate. Galactosyl transferase activity was determined by the method of Beaufay et al38 as the transfer of uridine diphosphate (UDP)-4C-galactose to ovalbumin. The labeled UDP-galactose (Amersham) had a specific activity of 337 mCi/mmol, and approximately 0.2 μCi (590 picomoles or 6.5 × 105 cpm) were added per reaction. Data are presented as cpm/mL. Protein was measured according to the method of Lowry et al.39

**Spectroscopy.** Absorption spectra were measured from 400 to 600 nm, using a Perkin-Elmer (Norwalk, Conn) 320 dual-beam spectrophotometer equipped with a head-on photomultiplier tube. Portions of the fractions were divided into two cuvettes, a background and detection was run, and the spectrum subtracted and to density bands were added to the second cuvette. The reduced minus oxidized spectrum was run and examined for peaks characteristic of cytochrome b (559, 529, 428 nm). Triton X-100 (0.1%) (Sigma, St Louis) was added to both cuvettes and the scan repeated to visualize the characteristic MPO peaks at 472 and 428 nm. As reported for PMNs,39 HL-60 granules separated on Percoll gradients displayed the MPO spectrum only when detergent was added (ie, the granule membrane restricted passage of dithionite). The amount of MPO was quantitated using an extinction coefficient of 75 (mmol/L)-1·cm-1 at 472 nm,37 and cytochrome b was quantitated using an extinction coefficient of 21.6 (mmol/L)-1·cm-1 at 559 nm.37 The limit of spectroscopic detection of MPO in our hands is approximately 0.050 μmol/L.

**PAGE.** Percoll-depleted fractions were solubilized in gel sample buffer (62.5 mmol/L Tris-HCl, 190 mmol/L NaCl, 6 mmol/L EDTA, 2.3% SDS, 2.5% Triton X-100, 5% 2-mercaptoethanol, pH 6.8) and placed in a boiling water bath for two minutes. Bromophenol blue in 50% glycerol was added to samples prior to electrophoresis. Samples were electrophoresed into a 10% acrylamide resolving gel with a 3.5% acrylamide stacking gel. Electrophoresis was carried out overnight at 40 V (constant voltage) and voltage increased the following morning until the dye front had migrated 10.5 cm into the resolving gel.

**Immunooautoradiographic detection of MPO peptides.** Proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) according to the technique of Towbin et al,33 blotting for 1.75 hours at 35 V in a Tris-glycine aqueous methanol buffer (0.025 mol/L Tris base, 0.192 mol/L glycine, 20% methanol, pH 8.3). Peptides immunochemically related to MPO were identified using anti-MPO rabbit antisem according to a method previously described.34 The antiserum was raised to highly purified human MPO (A4/A2n 0.82) and has been shown to provide a sensitive and specific means for detection of MPO-related peptides.34 The nitrocellulose paper was placed in Burridge buffer (50 mmol/L Tris base, 150 mmol/L NaCl, 0.005% NaNO2, pH 8.7) containing 3% bovine serum albumin (BSA, radiodioummassay grade, Sigma) at 4 °C overnight. The paper was then placed in anti-MPO antiserum (1:40 in 3% BSA in Burridge buffer) for two hours at room temperature. The paper was washed extensively with multiple changes of Burridge buffer for a period of one to two hours and then placed in 50 mL of 3% BSA in Burridge buffer containing 125I-protein A (1 to 3 × 106 cpm/mL) for one hour at room temperature. The paper was extensively washed for several hours with multiple changes of Burridge buffer, left overnight in the
same buffer, and again washed well the next morning. The paper was dried and autoradiographed using Kodak XAR-5 x-ray film (Eastman Kodak Co, Rochester, NY) and a fluorescence-intensifying screen. The gel was fixed and stained with R-250 Coomassie brilliant blue in 50% methanol–10% acetic acid. Mol wts were calculated from plots of $R_v$ log mol wt, using myosin (205 kilodaltons [kDa]), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) as standards (Sigma).

**Electron microscopy.** Percoll was removed from samples by ultracentrifugation prior to electron microscopy. Samples were fixed overnight in suspension in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.2, then pelleted in microfuge tubes. Pellets were washed with cacodylate buffer, then postfixed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer for 45 minutes. Samples were washed twice with cacodylate buffer and dehydrated by graded ethanol series and propylene oxide followed by embedment in Spurr's low viscosity medium.37 Polymerization was carried out in a vacuum oven at 70°C for eight hours. Resultant blocks were sectioned on a Ultracut E ultramicrotome (Reichert, Buffalo). Glass knives were used for sectioning, since any residual Percoll may damage diamond knives.11 Sections were placed on 200-mesh copper grids, stained with 5% uranyl acetate for ten minutes, washed, counterstained with lead citrate for eight minutes, and washed again. Specimens were viewed and photographed in a H-600 electron microscope (Hitachi, Mountain View, Calif) at an acceleration voltage of 50 kV in a magnification range of 5,000 to 17,000.

**RESULTS**

In contrast to PMNs, HL-60 cells actively synthesize protein. To identify the subcellular components of HL-60 cells involved in protein synthesis, a Percoll gradient was generated to separate these elements from lysosomes. The density profile of the gradient was determined by using colored marker beads of known density and measuring the distance of the colored bands separated in the gradient from the bottom of the tube (data not shown). When the supernatant from the low-speed centrifugation of the cavitate ($S_1$) was separated on the preformed gradient of Percoll, five bands were seen (indicated in the text by numbers 1 to 5), although one band (no. 4) was visible only when the gradient was heavily loaded. The resultant bands segregated into a single high-density band and four less dense bands, the latter bands visibly distinct from each other and easily aspirated from the gradient using a Pasteur pipette.

After cavitation most of the enzyme activity was present in $S_1$, the supernatant from low-speed centrifugation of the cavitate, indicating nearly complete disruption of the cells (Table 1). For example, 93% of the MPO activity detected in the cavitate was recovered in $S_1$, whereas only 7% was in $P_1$, the pellet of unbroken cells and nuclei. In addition, most of the enzyme activity loaded on the gradient in $S_1$ was recovered subsequently in fractions from the gradient. Of note, there was neither vitamin $B_12$-binding protein nor cytochrome b found in $S_1$, consistent with previous reports that HL-60 cells lack specific granules.12,13,17

The profiles of biochemical markers in the 1-mL fractions collected from the gradient are shown in Fig 1. Fraction 1 was from the bottom of the gradient and was the most dense fraction. Since the top of the gradient was at fraction 25, activity in fractions lighter than 25 represented soluble enzyme activity (cytosol) that failed to enter the gradient. Peak activity of the lysosomal markers MPO, elastase, and β-glucuronidase, was in the dense part of the gradient, fractions 4 to 8. This corresponded to the most dense visible band, no. 1, with $\rho = -1:102$. In contrast, the enzyme markers for other organelles equilibrated in much lighter regions of the gradient. Peak activities for sulfatase C and galactosyl transferase, identified as biochemical markers for endoplasmic reticulum and Golgi apparatus, respectively,38 cosedimented with visible bands 3 and 4 having densities of 1.039 and 1.032 g/mL, respectively. Alkaline phosphatase, a marker for plasma membrane in neutrophils,39 had two broad peaks of activity, one in fractions 16 to 21 and one in fractions 23 to 25, the latter cosedimenting with visible band no. 5 with a density of 1.027 g/mL. Fraction 15 contained visible membrane aggregates and had substantial MPO, sulfatase C, and alkaline phosphatase activity as well as a small amount of galactosyl transferase activity at this intermediate density. This fraction also contained peak activity of cytochrome oxidase, a marker for mitochondria22 (data not shown) and represented visible band no. 2 with a density of 1.050 g/mL. Thus it appeared that centrifugation on this gradient afforded wide separation of lysosomes from less dense organelles and provided resolution of the less dense organelles into three visible bands, cosedimenting with biochemical markers for Golgi apparatus, endoplasmic reticulum, and plasma membrane.

To confirm that lysosomes were separated from less dense organelles, we examined fractions 6, 15, and 21 by transmission electron microscopy. Electron micrographs of fraction 6 (Fig 2A), an area of the gradient rich in enzymatic activity for lysosomal markers, displayed numerous electron-dense membrane-bound lysosomal like organelles. In contrast, electron micrographs of fraction 21 (Fig 2B), a region in the gradient free of biochemical markers for lysosomes but rich in enzymatic activity of markers for other organelles, showed no lysosomal granules but had numerous membrane vesicles, presumably derived from plasma membrane, endoplasmic reticulum, and Golgi apparatus. Electron micrographs of fraction 15 (Fig 2C) contained numerous mitochondria, membrane vesicles, and lysosomes, consistent with the bio-

<table>
<thead>
<tr>
<th>Marker</th>
<th>Activity in $S_1$ (Percentage of Total in Cavitate)</th>
<th>Activity in Gradient Fractions† (Percentage of Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>93.0</td>
<td>85.3</td>
</tr>
<tr>
<td>Elastase</td>
<td>89.0</td>
<td>97.0</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>72.0</td>
<td>75.0</td>
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<tr>
<td>Vitamin $B_12$-binding protein</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>97.0</td>
<td>83.0</td>
</tr>
<tr>
<td>Sulfatase C</td>
<td>89.0</td>
<td>72.6</td>
</tr>
<tr>
<td>Galactosyl transferase</td>
<td>75.0</td>
<td>92.5</td>
</tr>
</tbody>
</table>

*Represents the percentage of enzyme activity present in the whole cavitate that is recovered in $S_1$, where $S_1 + P_1$ equals 100%.

†Represents the enzyme activity recovered from the gradient as a percentage of the activity in $S_1$, loaded atop the gradient.

Marker enzyme recovery is based on the results of three cavitations.
Fig 1. Profile of biochemical markers in fractions from Percoll gradient of HL-60 cells. Fraction 1 was from the bottom of the gradient and the most dense, whereas fraction 25 was at the top of the gradient; so activity in less dense fractions represented cytosolic activity that failed to enter the gradient. Peak activity of lysosomal markers (MPO, elastase, and β-glucuronidase) were in the dense part of the gradient (fractions 6 to 8) and corresponded to visible band 1 with a density of 1.102 g/mL. In contrast, biochemical markers for plasma membrane (alkaline phosphatase), endoplasmic reticulum (sulfatase C), and Golgi apparatus (galactosyl transferase) equilibrated in much less dense regions of the gradient. These less dense subcellular organelles were separated by this gradient into three visible bands, with densities of 1.039 (endoplasmic reticulum), 1.032 (Golgi apparatus), and 1.027 g/mL (plasma membrane).

We have applied this analytical method to determine the subcellular location of intermediate molecular species generated during the biosynthesis of MPO (Fig 3). MPO was analyzed in three ways. First, enzymatic activity was determined in fractions from the gradient and is shown in the histogram plotting activity against fraction number. Second, MPO in selected fractions was quantitated by assessing the spectral properties of the intact heme group in mature MPO. Third, the immunochemical reactivity of the protein backbone of MPO was assessed by immunoblotting. Fractions 4 to 6, the area of the gradient where the activities of all three lysosomal enzyme markers were maximal, displayed peak MPO activity, had the highest concentration of heme-containing MPO as determined spectrally, and contained mature MPO, represented in the autoradiograph as the 59-kDa α-subunit. The 13.5-kDa β-subunit ran with the dye front in this 10% acrylamide gel and is not shown. In contrast, fractions 22 to 23, taken from the area of the gradient with peak galactosyl transferase activity, had no MPO enzymatic activity and no spectral evidence of MPO heme, but contained the 89-kDa precursor of MPO as the predominant species seen in the autoradiograph. Fractions 15 and 16 had some MPO activity, trace spectral evidence of MPO, and both mature and pro-MPO in the autoradiograph. These data are consistent with the hypothesis that pro-MPO is present in the Golgi apparatus but is devoid of enzymatic activity and without a heme group, at least within the limits of the detection assays used. Moreover, they suggest that during processing there is concomitant insertion of heme into the peptide backbone and proteolytic maturation of pro-MPO to yield enzymatically active MPO.

DISCUSSION

There is a great deal of interest in the synthesis and intracellular transport of lysosomal enzymes. There is considerable evidence that nascent lysosomal enzymes are cotranslationally inserted into the lumen of endoplasmic reticulum and glycosylated at selected asparagines in the peptide backbone of the proenzyme during subsequent transport through the endoplasmic reticulum and Golgi apparatus, the proenzyme undergoes extensive modification of the carbohydrate side chains, including phosphorylation of mannose side chains in many cases. The final transfer of the phosphorylated glycosylated proenzyme to the lysosome involves interaction with mannose-6-phosphate receptors present in the lysosomal membrane, although there is evi-
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Fig 3. Subcellular localization of MPO processing. The presence of MPO species was evaluated in three distinct ways: enzymatic activity (shown in histogram) was measured using the o-dianisidine assay to assess peroxidative activity, the presence of a heme group was assessed spectrally using oxidation-reduction difference spectroscopy, and the MPO peptide backbone was analyzed immunochemically using electroblotting and anti-MPO antiserum. Fractions 4 to 6, the area of the gradient where the lysosomal markers cosedimented, displayed peak MPO activity, had the highest concentration of the heme-containing MPO, and contained mature MPO, identified in the autoradiograph by the 59-kDa α-subunit. In contrast, fractions 22 to 23, which cosedimented with markers for less dense organelles, lacked MPO activity and spectral evidence of heme, but contained the 89-kDa precursor of MPO as shown by immunoautoradiography. Fractions 15 and 16 contained MPO activity, trace amounts of heme-containing MPO, and immunochemical evidence of mature and precursor forms of MPO.

The modified gradient described here separated the lysosomal compartment from organelles of lower density. The high-density fractions contained azurophilic granules, whereas the low-density fractions contained endoplasmic reticulum, Golgi apparatus, and plasma membrane. These latter fractions had no activity for any lysosomal enzymes and by electron microscopy were free of lysosomes but rich in membrane vesicles. Thus, our method of subcellular fractionation allows separation of lysosomes from less dense organelles important for the synthesis and proper targeting of lysosomal enzymes.

We applied this method of subcellular fractionation to HL-60 cells to determine to what extent synthesis and transport of a myeloid lysosomal enzyme such as MPO...
follow the schema derived from studies of other cells. MPO is a glycoprotein containing high mannose side chains and is synthesized in vivo as an 89-kDa proenzyme and subsequently packaged into azurophilic granules as a heterodimer (αβ) composed of two heavy-light protomers of 59,000 and 13,500 mol wt as determined under reducing conditions in SDS-PAGE. As predicted, mature MPO cosedimented with lysosomal enzyme markers whereas the 89-kDa pro-MPO cosedimented with elements of the Golgi apparatus and plasma membrane. Of note, both mature MPO and pro-MPO were found in one intermediate area of the gradient (fractions 15 and 16) where there was activity of alkaline phosphatase and sulfatase C as well as MPO. This may represent a prelysosomal compartment in which the pro-MPO undergoes proteolytic maturation. However, further studies to improve resolution of organelles at this density are needed to exclude the possibility of contamination of these fractions by soluble MPO nonspecifically adherent to endoplasmic reticulum. Within the limits of oxidative-reduction difference spectroscopy, our data suggest that pro-MPO lacked heme, whereas the mature MPO present in lysosomes contained a prominent heme group. Thus, maturation of pro-MPO appears to require insertion of a heme as well as proteolytic processing into the native MPO. Currently, studies directed at identifying the timing and subcellular location for heme incorporation are under way.

This method of subcellular fractionation of HL-60 cells provides a useful system to dissect further the processing and intracellular transport of myeloid lysosomal enzymes. Understanding these events may provide insight into defects in lysosomal enzymes in myeloid cells (eg, MPO deficiency) as well as identify mechanisms by which the normal myeloid cell sorts proteins destined for azurophilic granules from those destined for specific granules and for plasma membrane.

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REFERENCES

51. Chaplinski TJ, Niedel JE: Cyclic nucleotide-induced matura
Separation and analysis of subcellular organelles in a human promyelocytic leukemia cell line, HL-60: application to the study of myeloid lysosomal enzyme synthesis and processing

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