Proteomic Analysis of Lysosomal Acid Hydrolases Secreted by Osteoclasts

IMPLICATIONS FOR LYTIC ENZYME TRANSPORT AND BONE METABOLISM*

Cornelia Czupalla‡, Hannu Mansukoski‡, Thilo Riedl‡, Dorothee Thiel‡, Eberhard Krause§, and Bernard Hoflack‡¶

Osteoclasts, the bone-digesting cells, are polarized cells that secrete acid hydrolases into a resorption lacuna where bone degradation takes place. The molecular mechanisms underlying this process are poorly understood. To analyze the nature of acid hydrolases secreted by osteoclasts, we used the mouse myeloid Raw 264.7 cell line that differentiates in vitro into mature osteoclasts in the presence of the receptor activator of NF-κB ligand. Upon differentiation, we observed a strong increase in the secretion of mannose 6-phosphate-containing acid hydrolases. A proteomic analysis of the secreted proteins captured on a mannose 6-phosphate receptor affinity column revealed 58 different proteins belonging to several families of acid hydrolases of which 16 are clearly involved in bone homeostasis. Moreover these acid hydrolases were secreted as proproteins. The expression of most of the identified acid hydrolases is unchanged during osteoclastogenesis. Thus, our data strongly support the notion that the polarized secretion of acid hydrolases by osteoclasts results from a reorganization of key steps of membrane traffic along the lysosomal pathway rather than from a fusion of lysosomes with the membrane facing the resorption lacuna. Molecular & Cellular Proteomics 5:134–143, 2006.

From the ‡Technical University of Dresden, 01307 Dresden, Germany and the §Institute of Molecular Pharmacology, 13125 Berlin, Germany

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fuse with the membrane of the ruffled border thereby releasing their content of mature, fully active acid hydrolases into the resorption lacuna (11).

In the present study, we analyzed the secretion of acid hydrolases by osteoclasts in detail. We observed a strong increase in the secretion of different acid hydrolases, which still contained the Man-6-P targeting signal. Thus, we applied immobilized, cation-independent MPRs as highly specific affinity reagents for the purification of the complete set of Man-6-P-containing acid hydrolases and associated proteins secreted by osteoclasts. We present an exhaustive list of ~60 proteins identified by MS, many of them being implicated in bone homeostasis. Our analysis further revealed that these Man-6-P-modified acid hydrolases are secreted as proforms thus suggesting a modification of MPR trafficking pathways during osteoclastogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Metabolic Labeling**—Soluble recombinant RANKL was produced in *Pichia* yeast as described previously (12). Raw 264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS (HyClone Laboratories, Perbio Science, Erembodegem-Aalst, Belgium) and antibiotics. In *vivo* osteoclastogenesis was induced by addition of RANKL as described previously (12). Prior to collection of conditioned medium, cells were serum-starved for 6 h or overnight. For metabolic labeling, cells were incubated for 2 h with methionine-free Dulbecco’s modified Eagle’s medium containing 1 mCi/ml [35S]methionine, 10 mM Heps, pH 7.0, and 10% dialyzed FCS followed by a 3-h chase in the presence of 10 mM Man-6-P.

**Immunoprecipitation of Cathepsin D**—Conditioned medium from [35S]methionine-labeled cells was diluted in lysis buffer (PBS supplemented with 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and complete protease inhibitors (Roche Diagnostics)). Cells were washed twice with PBS, homogenized in lysis buffer, and centrifuged at 13,000 rpm for 15 min. Cell extracts and conditioned medium were precooled with Protein A-Sepharose CL-4B (Amersham Biosciences), and cathepsin D was immunoprecipitated with a polyclonal antibody (13). Immunocomplexes were collected with Protein A-Sepharose and analyzed by SDS-PAGE followed by autoradiography.

**Measurement of Enzyme Activities**—For measurement of glycosidase activities, conditioned media were incubated for 3 h at 37 °C in assay buffer I (100 mM sodium citrate, pH 4.6, 0.2% Triton X-100) supplemented with a 1 mM concentration of the appropriate 4-methylumbelliferyl substrates. Reactions were stopped by addition of 0.5 M Na2CO3. Fluorescent reaction products were measured using a fluorescence multwell plate reader (Tecan, Grödig, Austria) with excitation at 360 nm and emission at 440 nm. Cathepsin K activity was measured using (Z-Leu-Arg)2-Rh110 bisamide (Calbiochem) in assay buffer II (100 mM sodium acetate, pH 4.6, 0.1% Triton X-100) supplemented with a 1 mM concentration of the appropriate 4-methylumbelliferyl substrates. Reactions were started by addition of assay buffer II (100 mM sodium acetate, pH 4.6, 40 mM sodium tartrate, 20 mM p-nitrophenyl phosphate) and stopped after 30 min at 37 °C by addition of 0.1 M NaOH. Colored reaction products were measured at 405 nm. Protein concentrations of corresponding cell extracts were determined using the DC protein assay (Bio-Rad), and enzymatic activities were normalized to the protein amount of the cells secreting these enzymes.

**Purification of Man-6-P-containing Enzymes**—Soluble bovine cat-ion-independent MPR was purified from FCS by affinity chromatography on immobilized phosphomannan and coupled to Affi-Gel 15 (Bio-Rad) as described previously (14). Conditioned media were diluted in column buffer (50 mM imidazole, pH 6.5, 150 mM NaCl, 5 mM sodium β-glycerophosphate, 2 mM EDTA, 0.05% Triton X-100) and applied to an MPR Affi-Gel affinity column. The column was washed with 5 volumes of column buffer followed by 3 volumes of 5 mM glucose 6-phosphate in column buffer and 5 volumes of column buffer. Bound proteins were eluted with 5 volumes of 5 mM Man-6-P in column buffer and analyzed for their enzymatic activity or by SDS-PAGE.

**Protein Identification by Mass Spectrometry**—Proteins were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. Protein bands were excised, washed, in-gel reduced, S-alkylated, and in-gel digested with trypsin (Promega, Madison, WI) as described previously (15). Peptides were extracted by addition of 0.3% trifluoroacetic acid in acetonitrile, the separated supernatant was dried under vacuum, and samples were redissolved in 0.1% (v/v) trifluoroacetic acid in water. MALDI-MS measurements were performed using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode using α-cyano-4-hydroxy-cinnamic acid as matrix. All peptide mass fingerprints were internally calibrated with trypsin autolysis peaks. MALDI-TOF/TOF fragment ion analysis was carried out in the LIFT mode of the instrument. Spectra were processed using FlexAnalysis software. Protein identification, both by peptide mass fingerprinting and fragment ion analysis, was performed using MASCOT (Matrix Science, London, UK). Search criteria were as follows: taxonomy, mouse; mass accuracy, 50 ppm for peptide mass fingerprinting and 0.5 Da for fragment analysis; modifications, carbamidomethylation and methionine oxidation; maximum one missed cleavage site. The National Center for Biotechnology Information (NCBI) non-redundant protein database (version 20041117; 2,171,938 sequences) and Swiss-Prot (version 45.5, 215,444 sequences) were searched. CapLC-MS/MS experiments were performed on the quadrupole orthogonal acceleration time-of-flight mass spectrometer Q-TOF Ultima (Micromass, Manchester, UK) equipped with a Z-spray nanoelectrospray source. A Micromas CapLC liquid chromatography system was used to deliver the peptide solution to the electrospray source. Peptides were separated using an analytical column (PepMap C18, 3 μm, 150 mm x 75-μm inner diameter; LC Packings, Sunnyvale, CA) and an eluent flow rate of 200 nl/min. Mobile phase A was 0.1% formic acid (v/v) in acetonitrile-water (5:95, v/v), and B was 1.0% formic acid in acetonitrile-water (8:2, v/v). Runs were performed using a gradient of 3–64% B in 60 min. To perform MS/MS experiments automatic function switching (survey scanning) was used. The MS survey range was m/z 300–1990, and the scan duration was 1.0 s. The collision gas was argon at a pressure of 6.0 × 10−3 millibar. The MS/MS ion search option of the MASCOT program (www.matrixscience.com) was used to search against the NCBI non-redundant protein database and Swiss-Prot. The mass tolerance of precursor and sequence ions was set to 0.1 and 0.2 Da, respectively (other search criteria were as above).

**Immunofluorescence and Confocal Laser Scanning Microscopy**—Purified MPRs were fluorescently labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR). Raw 264.7 cells and osteoclasts grown on glass were fixed with 3% paraformaldehyde in PBS for 10 min and permeabilized in 0.05% saponin in PBS for 15 min. Samples were incubated with fluorescently labeled MPR fragments and primary anti-cathepsin D antibody in 1% bovine serum albumin in PBS for 1 h followed by incubation with Texas Red-conjugated goat anti-rabbit.
secondary antibody (Molecular Probes). Samples were viewed with a Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany).

**DNA Microarray and Real Time PCR**—mRNA isolation, cDNA synthesis, and Affymetrix expression analysis were done as described previously (12). Real time PCR was performed with a Stratagene MX4000 QPCR system and Brilliant SYBR Green QPCR kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions using the following primers: cation-dependent MPR, 5’-H11032-GGAATGGAGCAGTTTCCTCA-3’ and 5’-H11032-GGCAGTGGTAGTGAAGATCA-3’; cation-independent MPR, 5’-H11032-GCTGCTGCAAGAGGTACC-3’ and 5’-H11032-GTGATATGGCCATTTTCTGC-3’; TRAcP, 5’-H11032-TTGTCAGAAGCTGTAAGC-3’ and 5’-H11032-TAGCGGACAAGCAGGACTCT-3’; cathepsin K, 5’-H11032-TGATGAAAATTGTGACCGTGA-3’ and 5’-H11032-CCTGCAAAGCCACCAAATCT-3’; cathepsin D, 5’-H11032-CCTGAAGCTAGGAGGCAAAA-3’ and 5’-H11032-AGGGTCCAGCAACACTAAGC-3’; legumain, 5’-H11032-TATGTGCTGGCATACTCTG-3’ and 5’-H11032-CCACCCAAACTGGCTTCTTA-3’; and β-glucuronidase, 5’-H11032-TGAATGGGATTCATGTGGTG-3’ and 5’-H11032-TGCTTGAAGCCTTTTTCTCC-3’.

**RESULTS**

**Osteoclasts Secrete Acid Hydrolases Still Containing Mannose 6-Phosphate**—As a model system of osteoclastogen...
thesis, we have used the mouse myeloid cell line Raw 264.7. Treatment of Raw 264.7 cells with RANKL for 4 days results in cultures mainly consisting of multinucleated osteoclasts (12, 16). We first followed the secretion of enzymatic activities of known acid hydrolases such as β-galactosidase and β-glucuronidase as well as of the bound material eluted with 5 mM Man-6-P were determined: β-galactosidase (β-gal), β-glucuronidase (β-gluc), β-hexosaminidase (β-hex), β-mannosidase (β-man), and cathepsin K (cTsk). Shown are results from one representative experiment of three performed in duplicate.

FIG. 2. Osteoclasts secrete Man-6-P-modified acid hydrolases. Raw 264.7 cells (1.3 × 10⁴/cm²) were treated with RANKL for 5 days to generate osteoclasts. Conditioned media collected from osteoclasts grown for 6 h in serum-free medium were passed over an MPR affinity column. The following enzymatic activities of the conditioned media as well as of the bound material eluted with 5 mM Man-6-P were determined: β-galactosidase (β-gal), β-glucuronidase (β-gluc), β-hexosaminidase (β-hex), β-mannosidase (β-man), and cathepsin K (cTsk). Shown are results from one representative experiment of three performed in duplicate.

FIG. 3. Protein profile of Man-6-P-containing proteins secreted by osteoclasts. Raw 264.7 cells (1.0 × 10⁷) were differentiated into osteoclasts in the presence of RANKL during 5 days and then grown overnight in serum-free medium. Conditioned medium was collected, and Man-6-P-containing proteins were purified on an MPR affinity column and separated by SDS-PAGE. Coomassie-stained bands were excised and analyzed by MALDI-TOF/TOF mass spectrometry. Molecular mass markers are indicated.

Osteoclast-secreted Lysosomal Proteins

whereas ≈40–55% of the secreted β-galactosidase, β-glucuronidase, β-hexosaminidase, and β-mannosidase bound to the receptor. Thus, a significant fraction of these secreted enzymes still contains the Man-6-P marker as a typical hallmark of newly synthesized acid hydrolases.

Identification of Acid Hydrolases Secreted by Osteoclasts—Our results showing that several acid hydrolases are secreted as Man-6-P-containing proteins prompted us to identify the complete set of Man-6-P-modified proteins secreted by osteoclasts. Proteins were purified by their affinity to immobilized MPRs and further fractionated by SDS-PAGE as shown in Fig. 3. First the major Coomassie-stained bands were analyzed by peptide mass fingerprinting, and identified proteins were confirmed by MALDI-TOF/TOF peptide sequencing. This led to the identification of 14 proteins (see Fig. 3 and Table I). The major secreted Man-6-P-containing proteins are peptidases such as cathepsins A, B, D, S, and Z; legumain (asparaginyl endopeptidase); and tripeptidyl-peptidase I. Moreover glycosidases such as α-mannosidase, α-N-acetylglucosaminidase, β-hexosaminidase, and β-glucuronidase, enzymes involved in lipid metabolism, i.e. palmitoyl-protein thioesterase 1 and the epididymal secretory protein E1 (Niemann-Pick C2 protein homolog), and another lysosomal protein, interferon γ-inducible protein 30 were found. To identify additional, less abundant Man-6-P-containing proteins, the gel...
### Proteins secreted by osteoclasts captured on an MPR affinity column

| Protein                                      | NCBI gene identifier | Predicted molecular mass | No. of sequenced peptides | Man-6-P | Propeptide sequence (position, MS/MS score) | Change in mRNA expression | Bone phenotype (syndrome)                  |
|----------------------------------------------|----------------------|--------------------------|---------------------------|---------|--------------------------------------------|---------------------------|-------------------------------------------|
| **Peptidases**                               |                      |                          |                           |         |                                            |                           |                                           |
| Dipeptidyl-peptidase I                       | 3023454              | 52,343                   | 10                        | Yes     | 140VNMNAHLGLGLOER162                      | −2.7                      | Yes (Papillon-Lefevre, Haim-Munk)         |
| Dipeptidyl-peptidase II                      | 13626390             | 56,234                   | 9                         | Yes     | 167LYTHHNNFVK176                         | 1.3                       |                                           |
| Tripeptidyl-peptidase I                      | 12644085             | 61,304                   | 12                        | Yes     | 184SWTATAYK191                           | 1.3                       |                                           |
| Retinoid-inducible serine carboxypeptidase   | 48474586             | 50,934                   | 13                        | Yes     |                                            | −1.9                      |                                           |
| Angiotensinase C-like                        | 33469015             | 55,764                   | 1                         | Yes     |                                            | −2.3                      | Yes (Goldberg)                           |
| Cathepsin A                                  | 131082               | 53,809                   | 13                        | Yes     |                                            | −2.3                      |                                           |
| Cathepsin Z                                  | 12585209             | 33,974                   | 10                        | Yes     |                                            | −2.3                      |                                           |
| **Glycosidases**                             |                      |                          |                           |         |                                            |                           |                                           |
| Di-N-acetylchitobiase                        | 27229204             | 41,504                   | 4                         | Yes     |                                            | −1.6                      |                                           |
| Sialidase 1                                  | 17367967             | 44,563                   | 5                         | Yes     |                                            | −1.6                      | Yes (Morquio)                            |
| Lysosomal α-glucosidase                      | 51338793             | 106,180                  | 10                        | Yes     |                                            | −1.6                      |                                           |
| α-Galactosidase A                            | 1703210              | 47,611                   | 1                         | Yes     |                                            | −1.6                      |                                           |
| β-Galactosidase                              | 114944               | 73,074                   | 14                        | Yes     |                                            | −1.6                      | Yes (Morquio)                            |
| Lysosomal α-mannosidase                      | 17380364             | 114,532                  | 34                        | Yes     |                                            | −1.6                      |                                           |
| Epidydism-specific α-mannosidase             | 17367999             | 115,551                  | 31                        | Yes     |                                            | −1.6                      |                                           |
| β-Mannosidase                                | 13310141             | 101,320                  | 2                         | Yes     |                                            | −1.6                      | Yes (Sanfilippo)                        |
| β-Glucuronidase                              | 114964               | 74,192                   | 23                        | Yes     |                                            | −1.6                      | Yes (Sanfilippo)                        |
| β-Glucosidase                                | 121284               | 57,585                   | 2                         | No      |                                            | −1.3                      | Yes (Sanfilippo)                        |
| α-N-Acetylgalcosaminidase                    | 7305299              | 82,115                   | 4                         | Yes     |                                            | −1.3                      | Yes (Sanfilippo)                        |
| Tissue α-L-fucosidase                        | 31541781             | 53,452                   | 2                         | Yes     |                                            | −1.5                      | Yes (Sanfilippo)                        |
| β-Hexosaminidase α chain                    | 232255               | 60,560                   | 16                        | Yes     | 37TYLYPNNFQFR47                          | 1.8                       |                                           |
| β-Hexosaminidase β chain                    | 1346280              | 61,077                   | 20                        | Yes     | 31LOPALWPFFR47                          | 1.8                       |                                           |
| α-L-Lidonidase                               | 1352424              | 71,135                   | 11                        | Yes     |                                            | 1.8                       | Yes (Hurler)                             |
| N-acetylglucosaminidase-β-glucosaminidase    | 2498163              | 36,998                   | 5                         | Yes     |                                            | 1.8                       |                                           |
| **Sulfatases**                               |                      |                          |                           |         |                                            |                           |                                           |
| Galactosamine (N-acetyl)-6-sulfatase         | 31980654             | 57,989                   | 4                         | Yes     |                                            | −1.6                      | Yes (Morquio)                            |
| Arylsulfatase A                              | 1703420              | 53,742                   | 4                         | Yes     |                                            | −1.6                      | Yes (Morquio)                            |
| Arylsulfatase B                              | 33302601             | 42,868                   | 5                         | Yes     |                                            | −1.6                      | Yes (Maroteau-Lamy)                      |
| Glucosamine (N-acetyl)-6-sulfatase           | 29789239             | 62,042                   | 5                         | Yes     |                                            | −1.5                      | Yes (Sanfilippo)                        |
was cut into 50 slices of equal size, and each of them was subjected to protein identification by tryptic in-gel digestion followed by CapLC-ESI tandem mass spectrometry. Analysis of 11,191 MS/MS spectra revealed a total number of 58 different proteins belonging to several families of acid hydrolases including the known osteoclast markers cathepsin K and TRAcP (see Table I). This includes 15 peptidases, 16 glycosidases, five sulfatases, 10 enzymes involved in lipid metabolism, and 12 others. Among those, 33 proteins have been described previously as lysosomal acid hydrolases carrying the typical Man-6-P recognition marker such as cathepsins A, B, and D or α- and β-mannosidase (18). Our analysis also revealed lysosomal enzymes such as dipeptidyl-peptidase II, angiotensinase C-like protein, γ-glutamyl hydrolase, cathepsins F and Z, legumain, di-N-acetylchitobiase, and lysosomal phospholipase 2A for which a Man-6-P modification has not been described yet, indicating that they might be transported to lysosomes by a Man-6-P-dependent pathway in normal cells. Moreover we identified a third group of proteins that have not been classified as lysosomal acid hydrolases so far, namely sphingomyelinase-like phosphodiesterase 3a, interleukin-4-induced protein 1, and dentin matrix protein 4. It is interesting to note that β-glucosidase (β-glucocerebrosidase) and lipoprotein lipase, which do not contain Man-6-P residues, were also detected, probably reflecting their interaction with other Man-6-P-containing ligands. This probably holds true for four other proteins that are clearly not lysosomal enzymes, i.e. lysozyme C and three glycosyltransferases (N-acetylgalactosaminyltransferase 6, β-1,4-galactosyltransferase 5, and CMP-N-acetyl-poly-α,2,8-sialyltransferase), which were counted as possible contaminants. These glycosyltransferases are type II transmembrane proteins, which could...
be released as a soluble form after proteolytic cleavage.

To determine whether the Man-6-P-containing acid hydrolases were also secreted as proproteins, the MS/MS data were evaluated with respect to the presence of tryptic peptides overlapping with propeptide sequences. Although not all acid hydrolases contain a propeptide, such prosequences were found for 12 acid hydrolases, in particular for peptidases such as cathepsins B, D, F, K, L, S, and Z; dipeptidyl-peptidase I; tripeptidyl-peptidase I; and legumain as well as for β-hexosaminidase α and β chains (see Table I). For some other prosequence-containing acid hydrolases such as dipeptidyl-peptidase II, β-galactosidase, and lysosomal α-glucosidase we were not able to identify tryptic peptides spanning the propeptide sequence because they were either too short or too long to be detected by MS. Angiotensinase C was identified with only one peptide, which did not overlap with the prosequence. The other identified acid hydrolases are known not to contain a propeptide.

To confirm the finding that osteoclasts secrete proforms of acid hydrolases, we metabolically labeled osteoclasts and immunoprecipitated cathepsin D, one of the major secreted Man-6-P-containing proteins from cell extracts and conditioned media. Fig. 4 shows that cathepsin D is synthesized as a ~55-kDa proform that is matured into a 45-kDa form in murine osteoclasts. In contrast, secreted cathepsin D was exclusively detected as 55-kDa proform. This is in accordance with our MS/MS analysis of the propeptide sequence of cathepsin D and further underlines that acid hydrolases are secreted as proforms by osteoclasts. The presence of mature 45-kDa cathepsin D in intracellular compartments implicates that the enzyme reaches acidic compartments, i.e. late endosomes/lysosomes in osteoclasts. Indeed we also analyzed the intracellular distribution of cathepsin D in precursor Raw 264.7 cells and osteoclasts and compared it with the pattern of Man-6-P-modified proteins. For that purpose we fluorescently labeled a soluble form of MPRs. With this probe we detected Man-6-P-containing proteins mostly in perinuclear compartments, presumably Golgi structures, both in Raw 264.7 cells and osteoclasts (Fig. 5). Moreover more peripheral structures reminiscent of endosomes were labeled with the fluorescent MPR probe in osteoclasts. Binding of the MPR probe was quenched by the addition of 10 mM Man-6-P, thus proving the specificity of the detection of Man-6-P-containing proteins (data not shown). Cathepsin D was detected in scattered intracellular compartments both in precursor cells and osteoclasts. Only part of the cathepsin D-positive structures also contained Man-6-P-modified proteins, but a significant cathepsin D staining was also seen in structures that are clearly not labeled with the fluorescent MPR probe. This indicates that cathepsin D reaches acidic compartments where it loses the Man-6-P modification both in Raw 264.7 cells and osteoclasts. Taken together, our data suggest that two pools of cathepsin D and probably other acid hydrolases exist in osteoclasts, one of which is delivered to lysosomes and matures in this compartment, whereas the other is directly transported to the ruffled border and secreted as Man-6-P-modified proproteins.

**Expression Levels of Acid Hydrolases and MPRs during Osteoclastogenesis**—Our finding of a drastic increase in secretion of Man-6-P-containing proteins during osteoclastogenesis could be explained by an increase in the expression levels of at least some of these proteins, which could result in an overload of the receptors in the trans-Golgi network. On the other hand, down-regulation of MPRs could explain the increased secretion of acid hydrolases. Therefore, expression of both MPRs, i.e. cation-dependent and cation-independent Man-6-P receptors, was analyzed in Raw 264.7 cells and osteoclasts by quantitative real time PCR. Table II shows that the expression of neither of the receptors was significantly changed during osteoclastogenesis. This is in accordance with our DNA microarray analysis of mRNA expression in osteoclasts (12). Similarly the mRNA expression levels of most of the secreted acid hydrolases were not dramatically increased. Some Man-6-P-modified proteins such as dipeptidyl-peptidase I, legumain, and lysosomal acid lipase were even expressed at lower levels in osteoclasts (see Table I). These findings were confirmed by quantitative real time PCR analysis of selected gene products (see Table II). The only exceptions are the two known osteoclast markers cathepsin K and TRACP, which are expressed at much higher levels in osteoclasts but are not major secreted proteins (see Fig. 3). Therefore, it is not likely that increased secretion of acid hydrolases is caused by their increased expression or by changes in the expression of the MPRs.

**DISCUSSION**

The results of our present study show that osteoclasts acquire the property of secreting large amounts of Man-6-P-containing precursor forms of acid hydrolases during their differentiation process. In our proteomic analysis, we captured Man-6-P-modified proteins secreted by osteoclasts on immobilized MPR affinity columns and thus could analyze the complete set of Man-6-P-containing acid hydrolases and associated proteins involved in bone degradation. We identified ~60 proteins that mostly belong to the subfamilies of peptidases, glycosidases, sulfatases, and enzymes involved in lipid
metabolism. This demonstrates the complexity of the pool of Man-6-P glycoproteins secreted by osteoclasts, which is much higher than for other cell types such as monocytic or breast cancer cells where only two secreted proteins have been identified (19). So far, only the brain Man-6-P glycoproteome has been shown to consist of a comparable number of proteins (18).

Most of the proteins found in our analysis are known as typical lysosomal acid hydrolases targeted to lysosomes by a Man-6-P-dependent pathway in many other cell types (see Table I) (18, 20–24). Among these proteins, we detected the osteoclast marker cathepsin K thus proving the validity of our approach. In contrast, in a previous proteomic analysis of proteins secreted by osteoclasts that identified only a dozen proteins, this cathepsin was not found (25). In addition to well characterized Man-6-P-modified proteins we identified several lysosomal acid hydrolases, such as dipeptidyl-peptidase II, cathepsin Z, di-N-acetylglucosaminidase, and others, that are targeted to lysosomes by a yet unknown mechanism. Because the same enzymes isolated from brain tissue have also been found to interact in vitro with immobilized MPRs it is very likely that they contain the Man-6-P recognition marker (18). In contrast, β-glucosidase and lipoprotein lipase have been classified as enzymes following a Man-6-P-independent targeting pathway because they are reported as enzymes not being Man-6-P-modified (26, 27). Their detection among the Man-6-P-containing proteins in our screen could be explained in a way that a minor fraction of these enzymes might interact with other Man-6-P-containing ligands. This is underlined by the low abundance of these enzymes in our samples as indicated by the low number of detected peptides (see Table I). Finally we identified some proteins, namely sphingomyelinase-like phosphodiesterase 3a, interleukin-4-induced protein 1, and dentin matrix protein 4, that have not been found before to bind to immobilized MPRs. Secretion of these enzymes by osteoclasts might reflect their potential role in bone degradation.

A set of different acid hydrolases is required for the degradation of the organic bone matrix, mostly consisting of collagens, sulfated proteoglycans, and heavily sialylated glycoproteins such as osteocalcin or osteopontin. The substrate specificities of the acid hydrolases identified here would allow the digestion of these different components. Support of the notion comes from phenotypes observed in several naturally occurring human lysosomal storage disorders or in knock-out mice characterized by the monogenic defect of given acid hydrolases (see Table I). Accordingly several human lysosomal storage disorders are characterized by abnormalities of the skeleton (28). For example, mucopolysaccharidoses I, IIIA, B, D, IVA, and VI due to a deficiency in α-iduronidase, N-sulfoglucosamine sulfohydrolase, α-N-acetylglucosaminidase, N-acetylglucosamine-6-sulfatase, N-acetylgalactosamin-6-sulfatase, and arylsulfatase B, respectively, are all characterized by abnormal development of many bones including the spine and skeletal deformations such as thickened skulls or oval vertebrae (29). Similarly the Goldberg syndrome due to a lack of cathepsin A results in dwarfism (30). Sialidosis due to a lack of sialidase 1 is characterized in part by osteopenia (31). Some other enzyme deficiencies, not reported in humans, have been produced in animal models. For example, cathep-

\[
\text{TABLE II}
\]

Changes in gene expression during osteoclastogenesis detected by real time PCR

| Gene                  | -Fold changea |
|-----------------------|---------------|
| Cation-dependent MPR  | 1.59 ± 0.32   |
| Cation-independent MPR| 1.91 ± 0.45   |
| TRACP                 | 319.57 ± 57.55|
| Cathepsin K           | 135.30 ± 36.25|
| Cathepsin D           | −1.31 ± 0.30  |
| Legumain              | −1.84 ± 0.49  |
| β-Glucuronidase       | 1.60 ± 0.31   |

a Average -fold change ratio in mRNA expression in Raw 264.7 cells vs. osteoclasts (p < 0.05).
Although a lower trabecular bone volume has been reported in humans or animals. Mouse genetics could be the simplest explanation, and a list of genes for which no natural occurring defect has been reported in humans or animals. Moreover, our analysis of acid hydrolases secreted by osteoclasts provides an exhaustive list of genes for which no natural occurring defect has been reported in humans or animals. Mouse genetics could be used to evaluate their functional importance in bone metabolism.

How acid hydrolases are secreted into the osteoclast resorption lacuna is largely unknown. From morphological studies examining the distribution of MPRs and acid hydrolases in osteoclasts, a constitutive pathway for enzyme transport toward the resorption lacuna has been proposed (7). However, the presence of late endosomal markers on the ruffled border membrane facing the resorption lacuna led to the proposal that late endocytic compartments could fuse with this membrane, resulting in the release of mature, fully active acid hydrolases (9, 11). Here we provide for the first time a biochemical study to address this question. Our data show that most of cathepsin K and about 50% of other acid hydrolases are secreted as Man-6-P-containing precursor forms. Moreover, our immunoprecipitation experiments of cathepsin D demonstrate that this protease is exclusively secreted as proprotein. Therefore, our findings indicate that these enzymes have not encountered the acidic environment of late endocytic compartments, and they would lose their Man-6-P modification resulting in the observed binding efficiency of about 50% to immobilized MPRs. This could simply reflect our incapability of efficiently blocking phosphatase activities and in particular TRAcP activity, which is also secreted in large amounts by osteoclasts and has been proposed to be involved in the dephosphorylation of Man-6-P recognition markers (34). Enhanced synthesis of MPRs as suggested by Baron et al. (7) does not seem to be the reason for increased routing of Man-6-P-containing acid hydrolases to the cell surface because we could not detect a drastic change in MPR expression. Moreover, our findings also exclude a down-regulation of MPRs as explanation for the increased acid hydrolase secretion as has been described for cells lacking one of the two MPRs (35). It is also unlikely that an overload of the sorting capacity of the MPRs as observed in several cancer cells such as MCF-7 and CaCo-2 (36) causes the increased secretion of acid hydrolases by osteoclasts because none of the major secreted proteins are expressed at higher levels during osteoclastogenesis (see Fig. 3). In fact, only for the known osteoclast markers TRAcP and cathepsin K did we observe a highly increased expression in osteoclasts. Taken together, our data suggest that the secretion of acid hydrolases into the osteoclast resorption lacuna is caused by a modification of MPR trafficking pathways during osteoclastogenesis rather than by a fusion of acidic endocytic compartments containing fully processed enzymes with the ruffled border membrane. This is in accordance with previous studies showing changes in the steady state distribution of MPRs during the different phases of bone resorption (7, 9). Further support for this notion comes from our studies of the intracellular localization of cathepsin D that led to the proposal of two different pools of this enzyme and possibly other acid hydrolases of which only the Man-6-P-containing pool is directly transported to the ruffled border. This is also consistent with our DNA microarray analysis of mRNA expression revealing that several effectors of GTPases of the ADP-ribosylation factor and Rab subfamilies functioning along the MPR and lysosomal membrane-associated glycoproteins transport routes are differentially regulated during osteoclastogenesis. Further studies will address their implication in the polarized secretion of acid hydrolases by osteoclasts.

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To whom correspondence should be addressed: Technical University of Dresden-Bioinnovation Centre, Tatzberg 47-51, 01307 Dresden, Germany. Tel.: 49-351-463-40235; Fax: 49-351-463-40244; E-mail: bernard.hoflack@biotec.tu-dresden.de.

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