Osteopetrosis Mutation R444L Causes Endoplasmic Reticulum Retention and Misprocessing of Vacular H⁺-ATPase a3 Subunit*

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Osteopetrosis is a genetic bone disease characterized by increased bone density and fragility. The R444L missense mutation in the human V-ATPase a3 subunit (TCIRG1) is one of several known mutations in a3 and other proteins that can cause this disease. The autosomal recessive R444L mutation results in a particularly malignant form of infantile osteopetrosis that is lethal in infancy, or early childhood. We have studied this mutation using the pMSCV retroviral vector system to integrate the cDNA construct for green fluorescent protein (GFP)-fused a3R444L mutant protein into the RAW 264.7 mouse osteoclast differentiation model. In comparison with wild-type a3, the mutant glycoprotein localized to the ER instead of lysosomes and its oligosaccharide moiety was misprocessed, suggesting inability of the core-glycosylated glycoprotein to traffic to the Golgi. Reduced steady-state expression of the mutant protein, in comparison with wild type, suggested that the former was being degraded, likely through the endoplasmic reticulum-associated degradation pathway. In differentiated osteoclasts, a3R444L was found to degrade at an increased rate over the course of osteoclastogenes. Limited proteolysis studies suggested that the R445L mutation alters mouse a3 protein conformation. Together, these data suggest that Arg-445 plays a role in protein folding, or stability, and that infantile malignant osteopetrosis caused by the R444L mutation in the human V-ATPase a3 subunit is another member of the growing class of protein folding diseases. This may have implications for early-intervention treatment, using protein rescue strategies.

V-ATPases2 are evolutionarily conserved proton pumps that, in eukaryotic cells, are responsible for acidification of intracellular compartments, such as the Golgi apparatus, endosomes, lysosomes, secretory vesicles, and vacuoles (1–5). In some specialized cells, like bone-resorbing osteoclasts, they are also targeted to the plasma membrane (6–9).

Mammalian V-ATPases contain 14 different subunits organized into a cytoplasmic Vι sector (subunits A–H), and a membrane-embedded V0 sector (subunits a, c, c', d, e, and a45) (10). Several of the V-ATPase subunits have multiple isoforms that may be expressed in a tissue, cell-type, or organelle-specific manner. In yeast, for example, the a subunit, which plays a direct role in proton translocation, has two isoforms, Vph1p and Stv1p. Vph1p is localized to the vacuole, whereas Stv1p is found primarily in Golgi (11, 12). The mammalian a subunit has four isoforms, a1–4; a1, a2, and a3 are ubiquitously expressed, but to different degrees in different tissues and organelles, whereas a4 expression appears to be specific to plasma membranes of renal intercalated cells (9, 13–15). Although ubiquitously expressed, a3 appears to be most highly enriched in osteoclasts (9). In actively bone-resorbing osteoclasts, V-ATPases containing the a3 subunit isoform are specifically targeted to the osteoclast ruffled border, where they are involved in acidifying the resorption lacuna to demineralize bone (15).

The importance of the a3 subunit in bone biology has been demonstrated in mouse models: a3 knock-out (16), the oc/oc truncation mutant (17), or point mutations, e.g. R740S, at the Arg residue critical for proton translocation (18), lead to severe osteopetrosis, a bone disease characterized by increased bone density and fragility due to the inability of osteoclasts to secrete acid to resorb bone. This illustrates that, despite its ubiquitous expression, the critical function of a3 is its role in proton transport within the osteoclast ruffled border. Other a3 functions, such as its involvement in lysosomal acidification

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§The abbreviations used are: V-ATPase, vacuolar-type H⁺-ATPase; LAMP2, lysosome-associated membrane protein 2; RANKL, receptor activator for nuclear factor-κB ligand; TPCK, tosyl phenylalanyl chloromethyl ketone; TM, transmembrane-α-helix; CFTR, cystic fibrosis transmembrane conduc-
tance regulator; ARO, autosomal recessive osteopetrosis; PNGase F, peptide N-glycosidase F; Endo H, endo-β-N-acetylglucosaminidase H; BMM, bone marrow mononuclear; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation.

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V-ATPase a3R444L Misfolding Causes Osteopetrosis

apparently can be complemented by V-ATPases with alternate a subunit isoform composition (19).

In humans, type 1 infantile malignant osteopetrosis (OPTB1; OMIM #259700) is caused by autosomal recessive mutations in the TCIRG1 gene (ATP6V0A3; 11q13.2), which codes for the a3 subunit isoform of the V-ATPase complex. This particular form of autosomal recessive osteopetrosis (ARO) is a rare and severe disease caused by a defect in competence of osteoclasts for resorption of bone (20). It results in dense, brittle bone with severe encroachment of marrow cavities of the long bones, leading to anemia and thrombocytopenia with compensatory hepatosplenomegaly. Associated craniofacial bone abnormalities also lead to hydrocephalus, nasal obstruction, and nerve compression resulting in progressive deafness and blindness. Functions of monocytes and macrophages are also compromised and most children with this affliction die in infancy, or early childhood, often of infection, unless early intervention by bone marrow transplantation is implemented (21–23).

Many ARO mutations in TCIRG1 are in regulatory motifs or splice sites, or result in large deletions or truncations; however, the missense mutation R444L has been reported in the Costa Rican population (24). Study of this mutation could shed light on the underlying cause of a3R444L-mediated ARO, but also has more general implications for understanding the role of the highly conserved Arg-444 in the structure and function of V-ATPase a subunits. Indeed, a homologous mutation in the kidney-specific a4 isoform (R449H) is also disease causing, resulting in autosomal recessive distal renal tubular acidosis (25). Thus, we constructed the homolog of the R444L mutation (R445L) in a mouse expression system to further elucidate its effect on a subunit protein structure and function, and the consequent cell biological mechanisms leading to disease.

EXPERIMENTAL PROCEDURES

**Reagents, Enzymes, and Antibodies**—TPCK-trypsin (catalog number T1426), mammalian protease inhibitors (P8340), phosphatase inhibitors (P5726), and puromycin (P7255) were from Sigma. Peptide N-glycosidase F (PNGase F; P0704S) and endo-β-N-acetylglucosaminidase H (Endo H; P0702S) were from New England Biolabs. Site-directed mutagenesis was performed using the QuikChange Lightning Site-directed Mutagenesis Kit from Agilent Technologies. Antibodies were from: anti-GFP (sc-8334), Santa Cruz; anti-LAMP2 (ABL-93), the Developmental Hybridoma State Bank, IA; anti-calnexin (MA3–27), BD Biosciences; and Alexa Fluor 568 goat anti-rabbit IgG (A11011), Invitrogen.

**Mammalian Primary Cells, Cell Lines, and Constructs**—Bone marrow mononuclear (BMM) cells obtained from femurs of 2-month-old male (C3H/H11003) is caused by autosomal recessive mutations in the subunit isoform composition (19).

in the absence of nucleosides (Invitrogen, 12561) supplemented with 10% FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. For differentiation into osteoclasts, RAW cell lines were plated in 100-mm tissue culture dishes at 7 x 10^5 cells/dish and cultured for 5 days in the presence of 100 ng/ml of recombinant, soluble RANKL. For protein rescue experiments RAW cells were grown to 85% confluence, then the medium was replaced with regular medium (control), medium containing 10% glycerol, or medium containing 5% DMSO. Cells were then cultured for an additional 24 h at 37°C (control and 10% glycerol), or at 26°C (5% DMSO), prior to protein extraction.

The mouse a3 cDNA-containing vector, pcDNA3.1-a3, was a kind gift of Dr. Beth S. Lee (Ohio State University). The insert was subcloned into the pEGFP-N1 vector (Clontech) between EcoRI and SacII sites, generating the construct, pEGFP-N1-a3. Site-directed mutagenesis was used to introduce the R445L mutation into a3, yielding the construct, pEGFP-N1-a3R444L. PCR was subsequently used to generate inserts from these constructs, with wild type, or mutant, cDNA inserts fused with GFP cDNA and flanked by Xhol and EcoRI restriction sites. These PCR products were subcloned into Xhol/EcoRI-cut pMSCV-puro vector (Clontech). The control GFP-expressing vector, pMSCV-EGFP, was a kind gift of Drs. Helen Sarantis and Scott D. Gray-Owen (University of Toronto). To construct this, EGFP was amplified from pEGFP-N1 with flanking BamHI and EcoRI restriction sites. The PCR product was subcloned into BamHI/EcoRI-cut pMSCVpuro. Construct inserts and flanking regions were confirmed by DNA sequencing.

Retroviruses were generated by cotransfecting GP-293 (Clontech) cells (30–40% confluent in 6-well tissue culture plates) with 2 µg of pVSV-G (Clontech) and 2 µg of pMSCV-a3-GFP, pMSCV-a3R444L-GFP, or pMSCV-GFP, a plasmid DNA with FuGENE HD (Roche Applied Science). The resulting cell supernatants were used to infect RAW cells (passage 3), which were selected in 7 µg/ml of puromycin, 48 h post-infection. This resulted in establishment of the stable cell lines, RAW-a3-GFP, RAW-a3R444L-GFP, and RAW-GFP, which were maintained in medium containing 4 µg/ml of puromycin. Cloning primers, constructs, and cell lines are listed in Table 1.

**Reverse Transcription and PCR**—RNA was extracted from RAW-derived osteoclasts using TRIzol reagent (Invitrogen). The purified total RNA was digested with DNase I (Invitrogen) at a concentration of 1 unit per microgram of RNA and reverse transcription was performed using RevertAid H Minus reverse transcriptase (Fermentas). PCR was performed using HotStar Taq DNA polymerase (Qiagen) with primers for GFP and GAPDH (Table 1).

**Whole Cell and Membrane Protein Extraction**—To obtain whole cell lysates from retrovirally transduced RAW cell lines and osteoclasts derived from them, cells were washed twice with PBS and lysed in RIPA buffer (Cell Signaling Technologies, 9806) according to the supplier’s instructions. To obtain whole cell lysates from BMM-derived osteoclasts, the cells were washed twice with ice-cold PBS and lysed in buffer containing 300 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4, at 25°C, with protease inhibitors, phosphatase...
inhibitors, and 1 mM PMSF (from 100 mM stock in anhydrous ethanol), added prior to use.

To isolate membrane proteins from transduced RAW cells in 100-mm tissue culture dishes, cells were washed twice with ice-cold PBS and scraped on ice in membrane protein collection buffer. The latter buffer consisted of 50 mM Na2HPO4, 10 mM KCl, 1.5 mM MgCl2, 2 mM DTT, 10 mM Tris-HCl, pH 7.4, at 25 °C, with addition of mammalian protease inhibitor mixture (1:200, v/v) and 1 mM PMSF prior to use. The cell suspension was homogenized by passage 15–20 times through a 27.5-gauge syringe needle and then centrifuged at 100,000 × g for 1 h and the membrane pellet was resuspended in 150 μl of membrane protein collection buffer.

Deglycosylation, SDS-PAGE, and Immunoblotting of a3 and a3-GFP—Glycoproteins were deglycosylated with PNGase F, and Endo H, according to the supplier’s instructions. Briefly, membrane pellets or whole cell lysates (15–20 μg of total protein) were diluted to a final volume of 30 μl in glycoprotein denaturing buffer. Samples were denatured at 65 °C for 10 min, then 0.1 volume each of 10% Nonidet P-40 and ×10 G7 reaction buffer were added, followed by 1,000 units of PNGase F. The final volume was adjusted to 40 μl with distilled water. The reaction mixture was incubated for 1 h at 37 °C. Proteins were then solubilized by addition of 0.25 volume of 5× SDS-PAGE gel-loading buffer, resolved by SDS-PAGE, and immunoblotted. Anti-GFP antibodies were used at a dilution of 1:500, and anti-GAPDH at 1:10,000, followed by appropriate HRP-conjugated secondary antibody. Blots were developed with Western Lightning ECL detection solution (PerkinElmer Life Sciences) and images were acquired using the Bio-Rad Molecular Imager ChemiDoc XRS system.

Immunostaining, Confocal Microscopy, and Image Analysis—Cells expressing wild type a3-GFP, mutant a3R444L-GFP, or control GFP, were cultured on glass coverslips for 48 h and then fixed in 2% paraformaldehyde in microtubule stabilizing buffer (MTSB; 127 mM NaCl, 5 mM KCl, 1.1 mM Na2HPO4, 0.4 mM K2HPO4, 2 mM MgCl2, 5.5 mM glucose, 20 mM PIPES, pH 7.4, at 25 °C) for 20 min. Cells were permeabilized with 0.1% saponin in MTSB containing 100 mM glycine for 20 min, then blocked in 5% FBS and 0.05% saponin in MTSB at room temperature for 1 h. Coverslips were incubated with anti-LAMP2 (1:200), or anti-calnexin (1:100) antibodies in blocking buffer overnight, then washed with 0.05% saponin in MTSB and incubated with fluorescent secondary antibodies (1:500). The cells were then incubated in PBS with 0.1 μg/ml of DAPI for 10 min for nuclear staining. Images were obtained with a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera and spinning disk confocal scan head.

Quantitative Confocal Image Analysis—Quantitative image colocalization analysis was performed with Velocity 5.2 software (PerkinElmer Life Sciences). Values shown in the present work are Pearson’s correlation coefficients (r). A threshold of r > 0.6 (“better than moderately positive”) was used to define colocalization, as described (26). Data were compared using two-tailed t tests to judge significance of differences, as appropriate.

RESULTS

Aberrant Expression of Mouse a3R445L In Macrophages and Osteoclasts—To investigate the functional effects of the human R444L mutation on the V-ATPase a subunit, the equivalent mutation, R445L, was engineered into a mouse a3 subunit expression system. As osteoclasts are the cells primarily affected by the R444L mutation, the mouse RAW macrophage cell line was chosen as the expression host. RAW cells can be differentiated into mature osteoclasts in the presence of RANKL, thereby providing an established in vitro osteoclastogenesis model (27). Murine wild type a3 and mutant a3R445L constructs were expressed in RAW cells as C-terminal fusions with GFP to allow them to be distinguished from the native, endogenously expressed V-ATPase a3 subunit. Stable expression was obtained by pMSCV-derived retroviral transduction, followed by puromycin selection (see “Experimental Procedures”). Details of constructs and cell lines are summarized in Table 1.

RAW cell lines derived by this method were grown in the presence of RANKL to obtain mature osteoclasts. That full-length a3 was being transcribed was demonstrated by reverse transcription of mRNA extracted from the RAW cell lines, followed by PCR using GFP primers, as shown in Fig. 1A. Significant differences in fused or control GFP expression among the cell lines were not apparent, relative to housekeeping GAPDH gene expression. Protein bands and expressed as percentages. Band intensities were normalized to GAPDH. Protein band intensities obtained after trypsinolysis and deglycosylation were normalized to control (untreated) bands and expressed as percentages. Band intensities were compared using two-tailed t tests to judge significance of differences, as appropriate.

V-ATPase a3R444L Misfolding Causes Osteopetrosis

Limited Proteolysis—Membrane fractions harvested as described above were lyophilized and then reconstituted in 100 μl of proteolysis buffer (50 mM Na2HPO4, 10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.4, at 25 °C). A final concentration of 2.5 μg/ml, or 5 μg/ml, of TPCK-trypsin (prepared in 20 mM CaCl2, 1 mM HCl, pH 3) was added to 15 μg of membrane protein. The solution was incubated for 1 h at 37 °C and trypsinolysis was stopped by adding 0.1 volume of 100 mM Np'-tosyl-l-lysine chloromethyl ketone hydrochloride (prepared in 1 mM HCl, pH 3). Trypsinolysed proteins were deglycosylated using PNGase F, as described above.

Quantitative Protein Band Analysis—Protein bands in immunoblots images were quantified using Bio-Rad Quantity One 4.6.9 software. Background was subtracted using the rolling disk method, and band intensities were normalized to GAPDH. Protein band intensities obtained after trypsinolysis and deglycosylation were normalized to control (untreated) bands and expressed as percentages. Band intensities were compared using two-tailed t tests to judge significance of differences, as appropriate.
on a single luminal loop of the membrane domain, and Lee et al. (28) have shown that the mouse a1 subunit is also glycosylated. Based on these observations, the diffuse 152-kDa band has been identified here as the mature glycoprotein, whereas the 134-kDa band is the core-glycosylated protein, a biosynthetic intermediate that is expected to be present at lower steady-state levels in the endoplasmic reticulum (ER). For the RAW-a3R445L-GFP cell line, expression of the mutant protein was observed only as the 134-kDa band, with no diffuse band apparent at 152 kDa. The mutant 134-kDa band was also present at a much lower intensity than was seen for the combined wild type bands.

Because the effects of the human R444L mutation are manifested in osteoclasts, it was of interest to determine whether the fate of the mutant a3R445L-GFP protein was affected by osteoclastogenesis in the RAW cell system. Thus, whole cell lysates were collected from the three transduced cell lines after 5 days of culture in the presence, or absence, of RANKL. Immunoblots probed with anti-GFP antibodies are shown in Fig. 1, which demonstrated again that wild type a3-GFP protein was expressed as 134- and 152-kDa bands, both in undifferentiated cells and osteoclasts. In RAW-a3R445L-GFP-derived osteoclasts, however, protein was observed only at 152 kDa. The mutant 134-kDa band was also present at a much lower intensity than was seen for the combined wild type bands.

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3 N. Kartner, Y. Yao, A. Bhargava, and M. F. Manolson, submitted for publication.

### TABLE 1

Primers, plasmids, and cell lines used in this study

| Primer (5’→3’)* | Application |
|-----------------|-------------|
| acctcctggggtcatcctgtccgc | R445L mutation of mouse a3 |
| ggcgctgagagcatctgcaagttgccgctgta | XhoI-a3 (pMSCV cloning) |
| ggcgctgagagcatctgcaagttgccgctgta | EcoRI-a3 (pMSCV cloning) |
| ggttggacctgtgacctgtaa | BamHI-3GFP (pMSCV cloning) |
| ggttggacctgtgacctgtaa | EcoRI-GFP (pMSCV cloning) |
| ggttggacctgtgacctgtaa | GAPDH detection (upper) |
| ccaccgtgtagtag | GAPDH detection (lower) |
| ccaccgtgtagtag | a3-GFP detection |
| ccaccgtgtagtag | a3c-GFP detection |
| aacctgctgctggctgtgga | GFP detection (lower) |

| Plasmid | Construct | Expression product |
|---------|-----------|--------------------|
| pMM780 | pEGFP-N1 | a3-GFP |
| pMM919 | pEGFP-N1-a3 | a3-GFP |
| pMM928 | pEGFP-N1-a3R445L | a3R445L-GFP |
| pMMS81 | pMSCV-a3-GFP | a3-GFP |
| pMMS82 | pMSCV-a3R445L-GFP | a3R445L-GFP |
| pMMS83 | pMSCV-GFP | GFP |

| Cell Line | Description |
|-----------|-------------|
| RAW-a3-GFP | RAW 264.7 cells expressing integrated a3-GFP |
| RAW-a3R445L-GFP | RAW 264.7 cells expressing integrated a3R445L-GFP |
| RAW-GFP | RAW 264.7 cells expressing integrated GFP |

* Underlining corresponds to target amino acid or restriction site indicated in “Application.”

* Designations refer to the strain collections of the communicating author (M.F.M.).

* See “Experimental Procedures” for details of plasmid construction. The constructs were all in pMSCVpuro and used to produce retroviral supernatants to stably integrate the constructs into RAW 264.7 cells, resulting in the listed cell lines.

![FIGURE 1](image-url)

**A**. PCR products representing detection of GFP (upper row), relative to GAPDH (lower row). cDNA from total RNA of osteoclasts that were RANKL-differentiated from RAW-a3-GFP, RAW-a3R445L-GFP, or RAW-GFP (Table 1) cells, or extracted from untransduced control cells was amplified with primers for GFP or GAPDH detection (Table 1) and PCR products were run on agarose gels.

**B.** 20 μg of microsomal membrane protein per lane, from cells described for panel A, was separated by SDS-PAGE and immunoblotted with anti-GFP antibody. Specific bands were mature, processed glycoprotein at 152 kDa and immature, core-glycosylated protein at 134 kDa. Smaller bands were ubiquitous and nonspecific.

**C.** Cells described for panel A were cultured for 5 days in standard medium, or were RANKL-differentiated into osteoclasts. Cell lysate proteins (20 μg/lane) were immunoblotted with anti-GFP or anti-GAPDH antibody. GFP staining revealed a3-GFP with a diffuse, mature glycosylated band at 152 kDa and a core-glycosylated band at 134 kDa (upper left panel). Arrowheads indicate 134- and 152-kDa band positions in lanes. Note that the 152-kDa band was absent in the lanes containing a3R445L-GFP. For both mutant and wild type a band was also visible at 128 kDa, which likely represents the unglycosylated biosynthetic intermediate. GAPDH staining provided a loading standard (lower panels). D, proteins from panel C were quantified as described under “Experimental Procedures.” The 128- and 134-kDa bands were used to quantify a3R445L-GFP protein. Additionally, the 27-kDa GFP band (not shown) from RAW-GFP cells was quantified. Results were plotted as ratios normalized to GAPDH. Data were taken from three blots derived from independent experiments.
LTRs normally serve as promoters that constitutively drive expression of the insert. Despite this up-regulation, the mutant protein was observed at reduced levels. This was quantified by normalizing band intensities to GAPDH levels, obtained by staining the same blots with anti-GAPDH antibodies. Fig. 1D graphically confirms the observations of Fig. 1C. The amount of $a3^{R445L}$-GFP protein was significantly decreased in mature osteoclasts (+ RANKL) compared with that in undifferentiated cells (− RANKL) ($p < 0.05; n = 3$), despite the fact that $a3$-GFP and GFP expression were significantly up-regulated (both $p < 0.05; n = 3$).

**Misprocessing of the $a3^{R445L}$-GFP Glycoprotein**—There are numerous post-translational modifications that can alter protein mobility in SDS-PAGE. To verify that the shifts in mobility of $a3^{R445L}$-GFP protein were due to differences in glycosylation, deglycosylation experiments were done with PNGase F, an amydase that cleaves between the initial oligosaccharide N-acetylglucosamine (GlcNAc) and the polypeptide Asn residue of N-linked glycoproteins. Additional experiments were done with Endo H, which cleaves mannose-rich, N-linked oligosaccharides between the initial two GlcNAc residues, leaving a single GlcNAc attached to the polypeptide Asn of the glycosylation site.

Initial experiments were done using lysates of mouse BMM-derived osteoclasts to observe carbohydrate cleavage from endogenous, native $a3$. Immunoblot analysis using anti-$a3$ antibody is shown in Fig. 2A, where the mature glycosylated $a3$ subunit is seen as a diffuse 116-kDa band. PNGase F treatment yielded an $a3$ band of ~94 kDa. This corresponds well to the predicted polypeptide size of the subunit, confirming that the observed mobility shift for untreated $a3$ is due to N-linked glycosylation. A weak band was also seen in the untreated lane at 102 kDa. This likely represents the core-glycosylated glycoprotein, the biosynthetic intermediate in the ER.

To learn more about the effect of the R445L mutation on $a3$, lysates were collected from transduced RAW cells, expressing GFP fusion proteins, and subjected to PNGase F digestion. As seen in Fig. 2B, fusion proteins from RAW-$a3$-GFP cells immunoblotted with anti-GFP antibody gave the expected bands at 134 and 152 kDa, whereas RAW-$a3^{R445L}$-GFP lysates yielded a major band only at 134 kDa. Upon PNGase F treatment, both constructs yielded bands only at 128 kDa, the unglycosylated fusion protein. To test whether the 134-kDa band indeed represents core-glycosylated $a3$, lysates were treated with Endo H. Endo H cannot cleave complex oligosaccharide that has been processed in the Golgi; thus, it can be used to discriminate ER-retained, mannose-rich, core-glycosylated glycoproteins from glycoproteins that have trafficked to the Golgi (29, 30). Fig. 2C shows immunoblots where $a3$-GFP and $a3^{R445L}$-GFP lysates were treated with Endo H, followed by immunoblotting with anti-GFP antibodies. At first glance, the mature glycoprotein appears to be unaltered by Endo H treatment, whereas the 134-kDa band is eliminated and a corresponding increase is seen in the 128-kDa band. The latter observation reiterates that the 134-kDa band is the core-glycosylated intermediate and the 128-kDa band is the unglycosylated protein. Closer examination of the 152-kDa band, however, reveals a reproducible reduction in size by 2 kDa, to 150 kDa ($n = 3$). The observation that the 152-kDa band was reduced slightly in size closely resembles what has been seen for Endo H treatment of CD4, which also has two glycosylation sites. Shin et al. (29) have explained this by suggesting that one of the oligosaccharide chains in the mature CD4 molecule is biantennary and not converted to the complex type in the Golgi, whereas the other site is complex and therefore Endo H-resistant. A similar explanation may hold for the $a3$ subunit, but the nature of its oligosaccharide chains has yet to be investigated in detail. The 128-kDa deglycosylated protein bands are reasonably close in size to the
V-ATPase $a_3^{R445L}$ Misfolding Causes Osteopetrosis

predicted polypeptide size of 121 kDa, given that hydrophobic membrane proteins, and fusion proteins with strikingly different domains, often migrate with anomalous mobility in SDS-PAGE.

Taken together, these observations suggest that the 134-kDa band is the mannose-rich, core-glycosylated form of the $a_3$ subunit fusion protein, which would go on to be processed in the Golgi to the mature 152-kDa glycoprotein. Lack of the latter band in $a_3^{R445L}$-GFP-expressing cells supports the notion that the mutation causes misfolding or instability in the nascent subunit, leading to retention of the immature, core-glycosylated intermediate in the ER, without further trafficking and processing. Direct proof of this was sought in cellular localization experiments.

Retention of $a_3^{R445L}$-GFP within the ER—In macrophages, V-ATPases containing the $a_3$ subunit are targeted to lysosomal membranes (15). GFP fusion protein constructs of $a_3$ can be localized by virtue of their innate fluorescence, so it was of interest to determine the fates of wild type and mutant constructs by fluorescence microimaging. Fig. 3A shows RAW-$a_3$-GFP macrophages illuminated to show GFP fluorescence (green) and Alexa Fluor-labeled anti-LAMP2 fluorescence (red). LAMP2 is predominantly a lysosomal marker, and the merged image reveals a strong colocalization of $a_3$-GFP with LAMP2-positive compartments. Fig. 3B shows RAW-$a_3^{R445L}$-GFP cells, where $a_3^{R445L}$-GFP was observed in the perinuclear region and, in contrast with the wild type protein, it did not colocalize with LAMP2-positive compartments. Fig. 3C demonstrates that wild type $a_3$ does not colocalize with calnexin, a marker for ER, and Fig. 3D, in contrast, shows a strong colocalization of mutant $a_3$ and calnexin. Quantification of LAMP2 and calnexin colocalization with either $a_3$-GFP or $a_3^{R445L}$-GFP (Fig. 3E) showed that there was a significantly greater ($p < 0.001$) association of wild type $a_3$-GFP with lysosomes (rather than ER), and a significantly greater ($p < 0.001$) association of the mutant $a_3^{R445L}$-GFP with ER (rather than lysosomes). Values of $r > 0.6$ generally indicate a moderate to high degree of colocalization. These observations support the notion that the primary defect of the mutant $a_3^{R445L}$ protein lies in its misfolding and consequent retention in the ER.

Altered protein conformation of $a_3^{R445L}$-GFP—Misfolding of mutant protein has been elucidated in other systems by using the method of limited proteolysis to demonstrate altered protease susceptibility (31). To confirm the conclusions of the above work, it was of interest to determine directly if the R445L mutation had an effect on the protein conformation of $a_3^{R445L}$-GFP. To test this, limited trypsinolysis was performed on microsomal membrane proteins derived from RAW-$a_3$-GFP and RAW-$a_3^{R445L}$-GFP cells. Intact membranes containing $a_3$-GFP or $a_3^{R445L}$-GFP protein were treated with 2.5 and 5 µg/ml of trypsin and then detergent-denatured and deglycosylated with PNGase F. Fig. 4A shows immunoblots of these digests, probed with anti-GFP antibodies.

The $a_3^{R445L}$-GFP mutant protein was found to be significantly more susceptible to trypsinolysis in comparison with $a_3$-GFP, and also to have some alterations in banding pattern. A closer comparison of band patterns is shown in Fig. 4B, revealing new 26- and 60-kDa bands for the mutant protein (lane h).
Mutant lane d, as expected, and the trypsin banding was somewhat altered (lane h), comparing lane h expected, and the trypsin banding was somewhat altered (lane h). Comparing lanes d and h, the only novel bands are those of a3R445L. In untreated 

PNGase F deglycosylation shifted the band to 128 kDa (lane f), as determined by limited proteolysis. Treatment the major band shifted to 128 kDa (lane e, asterisk glycylated) in the absence of enzyme (lanes d and h), but on trypsinolysis the band intensity was reduced almost to background (lane f). After PNGase F treatment the major band shifted to 128 kDa (lanes c, white arrowhead), as expected, and the trypsin banding was somewhat altered (lane d). Mutant a3R445L-GFP showed a major uncleaved band at 134 kDa (core-glycosylated) in the absence of enzyme (lane e, asterisk), but on trypsinolysis the band intensity was reduced almost to background (lane f). After PNGase F treatment the major band shifted to 128 kDa (lanes c, white arrowhead), as expected, and the trypsin banding was somewhat altered (lane d). Comparing lanes d and h, the only novel bands are those of a3R445L.

Different, the only novel bands are those of a3R445L. In the absence of enzyme treatment (lane a, black arrowhead), but on trypsinolysis the band intensity was reduced substantially (lane b). PNGase F deglycosylation shifted the band to 128 kDa (lane c, white arrowhead), as expected, and the trypsin banding was somewhat altered (lane d). Mutant a3R445L-GFP showed a major uncleaved band at 134 kDa (core-glycosylated) in the absence of enzyme (lane e, asterisk), but on trypsinolysis the band intensity was reduced almost to background (lane f). After PNGase F treatment the major band shifted to 128 kDa (lanes c, white arrowhead), as expected, and the trypsin banding was somewhat altered (lane d). Comparing lanes d and h, the only novel bands are those of a3R445L.

Partial Rescue of a3R445L with Osmolyte—Similar observations have been made for a number of disease-causing mutations in other proteins, most notably in cystic fibrosis transmembrane conductance regulator (CFTR), where the deletion of Phe-508 (ΔF508) results in cystic fibrosis. The ΔF508 CFTR is retained in the ER due to misfolding and is misprocessed in a manner very similar to what we describe here for the a3R444L mutant in the mouse V-ATPase system (33). In experimental systems ΔF508 CFTR can be partially rescued at low temperature, or in the presence of DMSO or glycerol (34). It would be of great interest to know if, in principle, the human a3R444L mutant could be similarly rescued. We show in Fig. 5 that in the experimental mouse system such rescue is possible. Although, unlike what is seen for CFTR, low temperature and DMSO, even in combination do not appear to rescue a3R444L, the osmolyte, glycerol, at 10% concentration is able to achieve partial rescue of the protein. As has been shown for ΔF508 CFTR expression in C127 cells (34), higher or lower concentrations of glycerol were not effective (data not shown), 10% being optimal. These observations suggest that protein rescue with chemical chaperones might be worth considering as a therapeutic strategy.
**V-ATPase a3R444L Misfolding Causes Osteopetrosis**

**DISCUSSION**

The R444L Mutation in a3 Confers a Protein Misfolding/Misprocessing Phenotype—We have shown elsewhere that wild type mouse a3 is N-glycosylated at two sites, Asn-484 and Asn-504, located on a single luminal loop within its membrane domain.3 Consistent with this, in the present work, mouse a3, with a predicted size of 93.4 kDa, was observed in immunoblots as an Endo H-sensitive, immature, core-glycosylated band migrating at 102 kDa, and an Endo H-insensitive, mature, glycosylated band at 116 kDa. The processing and maturation of the a3 glycoprotein is indicative of its biosynthetic pathway, involving ER biosynthesis and assembly into the V-ATPase complex, trafficking to the Golgi for processing, and ultimately trafficking to lysosomes and, in active osteoclasts, to the plasma membrane.

It was of interest to determine whether the disease-associated R444L mutant protein is similarly processed. To investigate the fate of the homologous mutant protein, a3R444L, in the mouse system, a retroviral vector was used to integrate GFP-tagged wild type or mutant cDNAs into the genomic DNA of RAW macrophage cells. Stable expression of a3R444L-GFP in this system demonstrated that the primary phenotypic defect resulting from the mutation was the retention of immature a3R444L-GFP glycoprotein in the ER, presumably due to misfolding, and its ultimate degradation. That this is not simply an artifact of the heterologous expression system was demonstrated by the apparently normal processing and trafficking of the wild type a3-GFP fusion protein. Further evidence of correct targeting of the C-terminal GFP-tagged a subunits, which requires functional assembly into the V-ATPase complex, has been demonstrated also in our work with the Vph1p homolog in yeast,3 and by others in mouse macrophages (35) and in the slime mold, Dictyostelium (36).

In the context of the dysfunctional osteoclasts seen in a3R444L ARO patients, the above observations would imply that the mechanism behind the dysfunction is that the mutant a3R444L subunit is incapable of being incorporated into the V-ATPase complex and subsequently trafficking to the plasma membrane of the osteoclast ruffled border. V-ATPases that specifically incorporate the a3 subunit isoform need to be localized to the osteoclast ruffled border to acidify the external resorption lacuna; without this process, bone cannot be resorbed (37, 38).

Four lines of evidence presented here support the notion that a3R444L is misfolded and retained in the ER. First, the mutant fusion protein was observed only as 128- and 134-kDa bands, lacking the 152-kDa mature glycosylated band of the wild type fusion protein (PNGase F treatment reduced all bands to 128 kDa, i.e., the deglycosylated protein). Lack of a 152-kDa a3R444L-GFP band, and reduced intensity of the 128- and 134-kDa bands (compared with the combined 134- and 152-kDa bands of a3-GFP), suggested misprocessing of the mutant glycoprotein, due to lack of trafficking to the Golgi, and degradation of the retained protein by the endoplasmic reticulum-associated degradation (ERAD) pathway of the ER quality control machinery (39), respectively.

Second, we showed by colocalization, after fluorescence immunostaining of LAMP2, or calnexin, that a3-GFP colocalized with LAMP2, not calnexin, suggesting that it was associated primarily with the lysosomal compartment. In contrast, a3R444L-GFP colocalized with calnexin, not LAMP2, suggesting that it was associated with the ER. This supports the notion that, like native a3, a3-GFP can traffic normally to the lysosome, whereas the a3R444L-GFP mutant protein does not leave the ER.

Third, upon limited proteolysis, the mutant protein was observed to be more sensitive to protease degradation, and novel proteolytic fragments were obtained, strongly suggesting increased protease accessibility due to global conformational changes caused by misfolding. Finally, the observation that the mutant protein was amenable to osmolyte rescue by growth of cells in 10% glycerol strongly suggests similarities with other systems, such as ΔF508 CFTR, where protein misfolding, misprocessing, and aberrant trafficking have been well characterized (33).

Recent studies have suggested that presenilin 1 is a mandatory chaperone required for N-linked glycosylation of the a1 subunit, working in conjunction with oligosaccharyltransferase and the Sec61α subunit of the translocon complex (28). If a3 glycosylation has the same requirements, we can conclude that the interaction between presenilin 1 and a3R444L-GFP, and the subsequent core glycosylation step by oligosaccharyltransferase is not impaired, as we have shown that a mannose-rich, biosynthetic-intermediated state of glycosylation is achieved for a3R444L-GFP. It is possible that interaction with calnexin, or Bip, is ultimately responsible for retention of a3R444L-GFP, as both are known to bind V-ATPase during assembly in plant cells (40), but the precise mechanism of retention remains unknown. In yeast, the a subunit homolog, Vph1p, is rapidly degraded if it fails to assemble into the V₀ complex within the ER. Hill and Cooper (41) have suggested that it is degraded via an alternative ER quality control pathway. In mammalian cells, entry into the ERAD pathway (42) must come into play, as significant degradation of the retained mutant protein was observed, and trafficking to Golgi and lysosomes clearly does not occur. The significance of the increased degradation in mature osteoclasts is presently unclear, but many cellular processes are induced during osteoclastogenesis, some of which might impact protein turnover. Whether the heterologous expression of misfolded mutant a3R444L-GFP protein might induce up-regulation of ERAD as part of the unfolded protein response (39, 43) remains to be determined.

The Structural Significance of Arg-444—The current structural topology model for the yeast V-ATPase a subunit, Vph1p, suggests that the Arg-462 residue is found within a hydrophobic transmembrane α-helix, TM3 (32). The topology model places the Arg residue within 1–2 helical turns of the cytoplasmic interface. Because Vph1p and human a3 are highly conserved orthologs, sharing 36% identity, it is likely that the homologous human Arg-444 and mouse Arg-445 are similarly placed within putative TM3 helices. In the yeast system, the R462L mutation has a relatively mild phenotype (44), suggesting that, unlike Arg-735, where any missense mutation completely disrupts V-ATPase activity (45), Arg-462 is not involved directly in proton transport.
Arginine in hydrophobic membrane domains of proteins is not uncommon, and can have important implications for protein structure and disease (46). Asymmetric distribution of basic amino acids, with respect to the lipid bilayer, is a well known factor influencing membrane topology, as has been generalized in the “positive inside rule” of von Heijne (47), and may assist TM membrane insertion (48). Furthermore, Vostrikov et al. (49) have shown that Arg, Gly, and aromatic residues near the ends of membrane-spanning α-helices may be determinants of helical tilt within the membrane bilayer. Arginine is the most effective, determining tilt direction and inducing tilt angles as high as 24° in synthetic peptides incorporated into artificial membrane systems. It is intriguing, in this regard, that the sequence, GRYL, which contains all of the aforementioned artificial membrane systems. It is intriguing, in this regard, that the sequence, GRYL, which contains all of the aforementioned residues (R being the human Arg-444), is highly conserved at the cytoplasmic end of the putative TM3 of the α subunit. Thus, one possible effect of Arg-444 mutation may be failure to maintain a critical tilt angle or tilt direction for TM3 in the membrane domain of the α3 subunit, resulting in misalignment with other membrane-spanning helices and consequent misfolding or instability of folded a3.

Conclusion—In summary, in this study it has been demonstrated that the R444L point mutation responsible for a type 1 infantile malignant osteopetrosis is caused by protein misfolding that results in retention of the V-ATPase α3 subunit in the ER. The misfolded protein consequently does not traffic to the plasma membrane, where it needs to be to make its functional contribution to the process of bone resorption. Furthermore, we have shown experimentally that conditions that are known to rescue misfolded proteins can partially rescue the α3<sup>R444L</sup> mutant glycoprotein.

Hematopoietic stem cell transplantation (HSCT) is performed in severe cases of ARO (23), but there are associated high risks (25%) of disease progression and poor 5-year survival (24%) for recipients of HLA-haplotypemismatched hematopoietic stem cell transplantation. Furthermore, preservation of vision requires intervention prior to 3 months of age (22). Radiological prenatal diagnosis can be obtained at 25 weeks gestation (50), but already encroachment of marrow cavities is obvious, suggesting that earlier fetal DNA testing is required. Pharmacological intervention, if available, would likely have to begin in neonates, if not in utero, to prevent development of severe manifestations of disease.

The severe ARO phenotype seen with the R444L α3 point mutation resembles what is seen in oc/oc mice where death occurs at 3 weeks of age (51). The oc/oc model results from a naturally occurring deletion within the Tcirg1 gene, in the N-terminal domain of the protein, which prevents the expression of the α3 subunit. The osteoclasts of these mice lack a ruffled border, and cannot secrete acid. The R445L point mutation causes ER retention and degradation of mouse α3, and this prevents the localization of α3-containing V-ATPases to lysosomal and plasma membranes. The functional outcome for critical plasma membrane expression of V-ATPase, for the human R444L mutation, would likely be the same as is seen in oc/oc mice; however, unlike the oc/oc model, some steady-state level of intact, core-glycosylated protein is observed in the experimental mouse system.

In another protein misfolding disease, ΔF508 CFTR-mediated cystic fibrosis, the ER-retained ΔF508 CFTR can under some circumstances be induced to traffic to the plasma membrane in mammalian cells, where it is observed to have significant function despite the mutation (52). Thus, much effort has been expended in attempting to find a clinically viable pharmacological means of achieving trafficking of the mutant protein to the plasma membrane as a potential cure for a large percentage of cystic fibrosis cases (53, 54). In a similar manner, a pharmacological chaperone that can rescue α3<sup>R444L</sup>, especially if usable during early infancy, or in utero, might be of benefit to patients afflicted with α3<sup>R444L</sup>-mediated ARO. This remains a tall order, but the work described here, elucidating the disease mechanism of the α3 R444L mutation and showing the potential for protein rescue, is a first step in addressing this need.

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Note Added in Proof—The findings of Lee et al. (28), suggesting that presenilin 1 is a mandatory chaperone for the V-ATPase α1 subunit, has recently been disputed by Zhang et al. (55).

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