Effects of Human a3 and a4 Mutations That Result in Osteopetrosis and Distal Renal Tubular Acidosis on Yeast V-ATPase Expression and Activity*

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V-ATPases are multimeric proton pumps. The 100-kDa “a” subunit is encoded by four isoforms (a1–a4) in mammals and two (Vph1p and Stv1p) in yeast. a3 is enriched in osteoclasts and is essential for bone resorption, whereas a4 is expressed in the distal nephron and acidifies urine. Mutations in human a3 and a4 result in osteopetrosis and distal renal tubular acidosis, respectively. Human a3 (G405R and R444L) and a4 (P524L and G812R) mutations were recreated in the yeast ortholog Vph1p, a3 (G424R and R462L), and a4 (W520L and G812R). Mutations in a3 resulted in wild type vacuolar acidification and growth on selective media with no detectable V-ATPase activity. However, a4 W520L mutation had defective growth on selective media with no detectable V-ATPase activity and reduced expression of a, A, and B subunits. The a4 W520L mutation phenotypes were dominant negative, as overexpression of wild type yeast a isoforms, Vph1p, or Stv1p, did not restore growth. However, deletion of endoplasmic reticulum assembly factors (Vma12p, Vma21p, and Vma22p) partially restored a and B expression. That a4 W520L mutation affects both Vo and V1 subunits is a unique phenotype for any V-ATPase subunit. Yeast V-ATPases fail to assemble when any of the genes that encode subunits are deleted, except for subunits H and c" (16–17). Previous studies have revealed that deletion of any V\textsubscript{o} subunit, except for the c" subunit (16), results in the loss of all V\textsubscript{o} subunits from the vacuole (18). The loss of any V\textsubscript{1} subunit, with the exception of subunit H (17), leads to an absence of all V\textsubscript{1} subunits at the vacuole (18). Without subunit H, the assembled V-ATPase is not active (17, 19), and loss of the c" subunit results in uncoupling of enzymatic activity (16).

Vo domain assembly depends on the presence of three assembly factors, Vma12p, Vma21p, and Vma22p. Phenotypes of cells that lack Vma12p, Vma21p, or Vma22p resemble those lacking a V\textsubscript{o} structural subunit, in that V\textsubscript{o} does not assemble properly, V\textsubscript{o} subunits are not found at the vacuole, and Vph1p is rapidly degraded (22, 24). Two of these proteins, Vma12p and Vma22p, form a complex that binds transiently to Vph1p and aids in Vph1p assembly and maturation (21–23, 25, 26).
A recent study has shown that Vma21p is required for the assembly of the \( V_\alpha \) domain (27).

An important question is how V-ATPase can function in the wide variety of locations and physiological processes listed above. In yeast, V-ATPases have two organelle-specific isoforms of the “\( \alpha \)” subunit, Vph1p and Stv1p (28). Vph1p localizes to the vacuole, whereas Stv1p localizes to the Golgi (28, 29). In humans, four different \( \alpha \) isoforms have been identified, \( \alpha_1 \) to \( \alpha_4 \), which are expressed in a tissue- and organelle-specific manner (30, 31). The \( V_\alpha \) domain also contains tissue-specific subunit isoforms, including B, C, E, and G (2, 31–33). Mutations in the \( \alpha_3 \), \( \alpha_4 \), and B1 subunits are known to lead to human disease (32, 34–37). There are no known human diseases associated with mutations in \( \alpha_1 \) or \( \alpha_2 \). The \( \alpha_3 \) isoform is highly enriched in osteoclasts (38), and mutations within \( \alpha_3 \) result in autosomal recessive osteopetroses (35–37, 39). The \( \alpha_4 \) isoform is specific to renal intercalated cells of the kidney (31), and mutations within the \( \alpha_4 \) gene lead to distal renal tubule acidosis (dRTA) (5, 34, 40).

The bone disease osteopetrosis demonstrates the essential function of V-ATPases in bone resorption. The osteopetroses are a group of heritable conditions characterized by defects in osteoclast bone resorption. Osteoclasts resorb bone by first creating a sealed zone, the resorption lacuna, between the osteoclast ruffled border membrane and the bone surface. The process of V-ATPase-mediated proton transport into resorption lacunae (41) is an essential component of bone remodeling.

The importance of V-ATPase in renal proton secretion is highlighted by inherited dRTA. V-ATPases in the plasma membranes of renal intercalated cells of the distal nephron pump protons from the blood into the urine, an essential step in the removal of metabolic acid. dRTA is characterized by impaired renal acid secretion, resulting in metabolic acidosis. Autosomal recessive dRTA involves mutations to the \( \alpha_4 \) or the B1 subunit isoforms of V-ATPase. Mutations within the V-ATPase B1 and \( \alpha_4 \) genes in some cases also result in sensorineural deafness (34, 42).

In humans, 26 mutations have been identified in \( \alpha_3 \) that result in autosomal recessive osteopetrosis (36, 37, 43, 44), and 21 mutations have been identified in \( \alpha_4 \) that result in dRTA (5, 40). The majority of these mutations result in frameshifts, abnormal splicing, and insertion of stop codons and as such are uninformative, except to further illustrate the essential roles of \( \alpha_3 \) and \( \alpha_4 \). However, missense and small deletion mutations were identified that could pinpoint critical domains. Two missense mutations, G405R and R444L, were identified in the V-ATPase \( \alpha_3 \) subunit isoform that account for all the defects in nine unrelated families in Costa Rica (36, 45). We have recreated these \( \alpha_3 \) mutations in the yeast a subunit ortholog, Vph1p, as G424R and R462L. Of the 21 \( \alpha_4 \) mutations, only three were missense mutations. We recreated two of them, P524L and G820R, in Vph1p as W520L and G812R. Characterizing the effect of these missense mutations can identify critical domains within \( \alpha_3 \) and \( \alpha_4 \) that are essential for assembly, targeting, or retention and retrieval of V-ATPases to and from the plasma membrane. Technically, it is difficult to characterize these mutations in humans. The only biochemical information is from fibroblast and lymphoblast cell lines from patients with frameshift and abnormal splicing mutations, and those studies confirm the expected null phenotype (37, 44).

Yeast V-ATPases are an attractive model for the study of the biochemistry of \( \alpha_3 \) and \( \alpha_4 \) mutations because the \( \alpha \) subunit is remarkably conserved across species. The subunit sequences of human \( \alpha_3 \) and \( \alpha_4 \) isoforms have ~55% similarity to the yeast ortholog, Vph1p. Characterizing the mechanistic outcome of \( \alpha_3 \) and \( \alpha_4 \) mutations in yeast could reveal critical amino acids involved in V-ATPase assembly, targeting, or activity. Here we have recreated four missense mutations, two from \( \alpha_3 \) and two from \( \alpha_4 \) mutations in the yeast Vph1p subunit, and report on their respective V-ATPase assembly and activity phenotypes.

**EXPERIMENTAL PROCEDURES**

**Materials—Escherichia coli** and yeast culture media were purchased from Difco. General chemicals and protease inhibitors were purchased from Sigma. Restriction endonucleases, T4 DNA ligase, and other molecular biology reagents were from Fermentas Life Sciences (Burlington, Canada). Zymolyase 100T was obtained from Seikagaka Corp. (Rockville, MD). The monoclonal antibodies, 8B1-F3 against the yeast V-ATPase 69-kDa A subunit, 13D-11 against the yeast V-ATPase 60-kDa B subunit, and 10D7 against the 100-kDa \( \alpha \) subunit, were purchased from Molecular Probes, Inc. (Eugene, OR). A polyclonal serum against Vma12p was the kind gift from Dr. Tom H. Stevens (University of Oregon), and a serum against Vma22p was the kind gift from Dr. Antony A. Cooper (University of Missouri). Standard YPD medium was formulated as 20 g of Difco peptone, 10 g of yeast extract, and 20 g of D-glucose/liter, with the pH adjusted to 5.8.

**Strains and Plasmids—**For strains and plasmids, see Table 1.

**Mutagenesis—**Yeast strain MM53 \( MATa \) ura3-52 \( \Delta vph1:: \) LEU2 (28) and plasmids MM322 pRS316 + \( VPH1 \) Sall Sma1 pRS316 + Sall Scal pVPI1-78 (28), and MM623 pRS316 + \( VPH1 \) containing a SacI site were used to generate and study \( VPH1 \) mutants. A PCR strategy, gene splicing by overlap extension (gene SOEing), was used to create the mutations (46). Mutagenesis was performed on the EcoRI-NotI fragment of pRS316 (MM322) or the SacI fragments of pRS316 (MM623). Primers used for mutagenesis are listed in Table 1, with substitution sites underlined.

For \( VPH1 \) mutation G812R, first round PCR primers were MO30 and MO155 with MO154 and T7. Primers MO30 and T7 were used for second round PCR. The PCR product was cut with EcoRI and NotI and cloned into pRS316 from MM322 cut with EcoRI and NotI. For \( VPH1 \) mutation R462L, first round PCR primers were MO23 and MO157 with T7 and MO156. Primers MO30 and T7 were used for second round PCR. The PCR product was cut with SacI and cloned into pRS316 from MM623 cut with SacI. For \( VPH1 \) mutation W520L, first round PCR primers were MO23 and MO154 with T7 and MO155. Primers MO23 and T7 were used for second round PCR. The PCR product was cut with SacI and cloned into pRS316 from
MM623 cut with SacI. DNA sequencing confirmed the mutations in these plasmids.

Vph1p_W520L was overexpressed by inserting the Sall-NotI fragment of Vph1p into pRS424 and pRS426 with SacI and NotI cut pRS424 This study

Preparation of Whole Cell Extracts—Yeast whole cell extracts were prepared by growing yeast cells to stationary phase, washing with water, and chilling the pellet for 30 min on ice. Just before homogenization the pellet was resuspended in 1 ml of 100 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1 mg/ml leupeptin), transferred to a 4 °C medium containing 0.7M sorbitol, 2 mM dithiothreitol, 100 mM serum albumin, and 5 mM Tris-MES, pH 7.6) and then homogenized with 20 strokes of a tightly coated 0.5-mm diameter glass beads to four-fifths of the final volume and chilled to 4 °C. The supernatant was centrifuged at 100,000 g for 15 min at 4 °C. The supernatant was centrifuged at 15,000 g for 15 min at 4 °C to remove whole cell debris. Cell homogenates were centrifuged in a Beckman GS-6R centrifuge at 15,000 × g for 10 min at 4 °C to remove whole cell debris. The supernatants (yeast whole cell extract) were denatured for SDS-PAGE in 63 mM Tris-HCl, pH 6.8, containing 1% (w/v) SDS, 0.6 mM 2-mercaptoethanol, and 5% (w/v) glycerol for 3 min at 70 °C.

Recreation of Human a3 and a4 Mutations in the Yeast Ortholog, Vph1p—See Fig. 1 for information.

Scoring Growth Phenotypes—Exponentially growing cells were suspended at 5 × 10⁷ cells/ml in 1 well of a standard 96-well plate. Five serial dilutions were made from the original stock into adjacent wells of the 96-well plate such that the final well held 1 × 10⁵ cells/ml with each well containing 130 µl. A 48-prong inoculating manifold (Dan-Kar Corp., Reading, MA) was used to transfer cells to solid medium. Each prong of the inoculating manifold deposits ~10 µl; thus the cell number per spot in the serial dilution ranged from ~5 × 10³ to 1 × 10⁴. Growth phenotypes were scored after incubating the plates at 30 °C for 5 days on uracil-minus media supplemented with 200 mM CaCl₂, 4 mM ZnCl₂, or buffered to pH 7.5 with 50 mM Tris-MES.

Isolation of Vacuolar Membrane Vesicles—Vacuolar membrane vesicles were isolated as described previously (47). Yeast cells were grown overnight at 30 °C to 1 × 10⁹ cells/ml in 1 liter of selective medium. Cells were pelleted, washed, and incubated for 15 min at 30 °C in 50 ml of 10 mM dithiothreitol, 100 mM Tris-HCl, pH 9.4. Cells were then converted to spheroplasts by incubating with gentle shaking for 60–90 min in YPD medium containing 0.7 M sorbitol, 2 mM dithiothreitol, 100 mM Tris-MES, pH 7.5, and 5 mg of Zymolase 100T. The spheroplasts were washed twice with ice-cold 1.2 M sorbitol and pelleted at 3,500 × g for 15 min at 4 °C. The pellet was resuspended in 40 ml of homogenization buffer (10% glycerol, 1.5% polyvinylpyrrolidone (M, 40,000), 0.25 M MgCl₂, 2 mg/ml bovine serum albumin, 50 mM Tris ascorbate, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin), transferred to a Dounce homogenizer, homogenized with 20 strokes of a tightly fitting pestle, and then centrifuged at 3,500 × g for 15 min at 4 °C. The supernatant was centrifuged at 100,000 × g (4 °C) in a Beckman L-70 Ultracentrifuge with a Beckman Ti-45 rotor. The pellet was resuspended in 8 ml of overlay medium (1 mM glycerol, 2 mM dithiothreitol, 0.25 mM MgCl₂, 2 mg/ml bovine serum albumin, and 5 mM Tris-MES, pH 7.6) and then homog-
enzized with 10 strokes of a tightly fitting pestle in a Dounce homogenizer. The homogenate was overlaid onto a 30-ml 10–30% discontinuous sucrose gradient and centrifuged in the same ultracentrifuge listed above, using an SW-20 (Beckman) rotor at 100,000 g, 4 °C for 2 h. Material at the 10–30% inter-
face was collected, diluted 10-fold with overlay medium, and centrifuged for 45 min at 100,000 × g at 4 °C. The pellets were resuspended in 1 ml of overlay medium and stored at −80 °C until used.

ATPase and Proton Translocating Activities—Concanamycin A-sensitive ATPase activities were measured using a coupled spectrophotometric assay (48). ATP-dependent proton

FIGURE 1. Multiple sequence alignment identifies conserved residues corresponding to human a3 and a4 mutations within the yeast ortholog Vph1p.

A multiple sequence alignment of the four 100-kDa mouse V-ATPase a isoforms and the yeast ortholog Vph1p was created using the DNAstar Corp. (Madison, WI) Lasergene ClustalW algorithm. Amino acids within gray-shaded areas indicate the corresponding positions in mouse (Mu) a3 and a4, and in the yeast Vph1p, in which human (Hu) a3 and a4 missense mutations have been found to result in osteopetrosis and dRTA. Human mutations are above alignments. Boxes delineate 8 putative transmembrane helices predicted by in silico analysis (Y. Yao, unpublished data).

Effects of Human “a” Mutations on V-ATPase Function

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TABLE 2

Effects of a3 (G424R and R462L) and a4 (W520L and G812R) mutations on V-ATPase activity as assayed by growth on selective media

Exponentially growing cells transformed with the single copy plasmid pRS316, with wild type VPH1 (VPH1), without wild type (∆vph1), or with VPH1 bearing the a3 mutations G424R and R462L, or the a4 mutations, W520L and G812R, were assayed for growth on uracil-minus plates with or without 200 mM CaCl2, 4 mM ZnCl2, or buffered to pH 7.5, and incubated as described under "Experimental Procedures." Three separate growth assays were conducted that gave similar results. Growth was scored relative to the VPH1 strain as follows: +++, 100% compared with wild type; ++, 75% to 50% growth relative to VPH1; +, 25 to 0% growth relative to VPH1; −, no detectable growth.

| ZnCl2 4 mM | CaCl2 200 mM | pH 7.5 | −URA |
|-----------|-------------|--------|------|
| VPH1      | +++         | +++    | ++++ |
| ∆vph1     | −           | −      | −    |
| R462L (a3)| ++          | ++++   | +++  |
| G424R (a3)| +++         | +++    | +++  |
| W520L (a4)| −           | +      | +++  |
| G812R (a4)| +           | +      | +++  |

Quinacrine Labeling—Cells grown to exponential phase in YPD or selective medium were washed and then incubated for 5 min in 200 mM quinacrine as described previously (48). The cells were then washed twice and viewed within 15 min of quinacrine labeling.

RESULTS

To investigate the effect of human a3 and a4 mutations that lead to recessive malignant osteopetrosis and dRTA, we recreated four missense mutations as follows: two a3 and two a4 in conserved residues of the yeast ortholog Vph1p. We selected a3 and a4 missense mutations to further characterize the roles of these residues in the basic function of the a subunit in V-ATPases. Recreating these point mutations in yeast Vph1p was possible because of the high degree of conservation between mammalian and fungal a subunits (see Fig. 1). As seen in Fig. 1, the human a3 mutations, G405R and R444L, and the a4 mutation, G812R, all reside within highly conserved regions. Identification of these a3 and a4 mutations was clear, as the glycine and arginine residues are perfectly conserved among all four mammalian a subunit isoforms and the yeast Vph1p. The assignment of the a4 mutation, P524L, was not as apparent because the proline was not conserved within Vph1p. Nevertheless, amino acids both upstream and downstream of a4 Pro-524 are perfectly conserved, making our alignment in this region unambiguous and indicating that the a4 Pro-524 is a tryptophan residue in Vph1p. As proline and tryptophan are both nonpolar amino acids, the change (P524L in a4 and W520L in Vph1p) is considered to be conservative.

The a3 Osteopetrosis-inducing Mutations in Yeast (G424R and R462L) Had No Effect on V-ATPase Assembly yet Had Decreased Activity—Growth of yeast cells on media supplemented with cations, or buffered at a high pH, is dependent on V-ATPase activity. As seen in Table 2, growth of yeast containing the a3 R462L and G424R mutations ranged from 75 to 100% compared with wild type on selective media supplemented with 4 mM ZnCl2, 200 mM CaCl2, or buffered to pH 7.5. Despite the slight growth defect, the yeast vacuoles appear to be acidified as shown by the accumulation of quinacrine within the vacuoles (Fig. 2). Considering that vma− strains can be rescued with mutant V-ATPases that only have 20% wild type activity (50), it was necessary to measure the hydrolytic and proton pumping activity from purified vacuolar vesicles from each mutant. Fig. 3 shows that the G424R mutation retained 74% of ATP-dependent proton pumping and 67% ATPase activity, whereas the R462L mutation demonstrated 64% of proton pumping and 62% of ATPase activity relative to wild type. Furthermore, immunoblots of these membranes reveal that both a3 mutations had wild type levels of the Vα subunit Vph1p (a ortholog) and Vα subunits Vma1p (A ortholog) and Vma2p (B ortholog), indicating that neither mutation affected the assembly or targeting of the V-ATPase complex to the vacuole (Fig. 4). These results suggest that the human a3 Gly-405 and Arg-444 residues are not essential to V-ATPase assembly and
reduce ATP hydrolytic and proton pumping activity by ~30–35%.

The a4 Mutation G812R Affects V-ATPase Activity but Not Assembly or Vacular Targeting—The G812R mutation resulted in 25–50% growth compared with wild type on selective media supplemented with 4 mM ZnCl₂, 200 mM CaCl₂, or buffered to pH 7.5 (Table 2). It also resulted in reduced accumulation of quinacrine in the vacuole (Fig. 2) and only 17% of proton pumping and 14% of ATPase activity relative to wild type (Fig. 3). Fig. 4 shows expression of V₁ subunit Vph1p (a) and V₁ subunits Vma1p (A) and Vma2p (B) on the vacuolar membrane at wild type levels. These results indicate that the human a4 Gly-820 residue is not required for assembly or targeting of V-ATPases to the vacuole but is critical for V-ATPase activity.

The a4 W520L Mutation Results in Decreased Expression of Both V₁ and V₂ Subunits—The W520L mutation resulted in no detectable V-ATPase activity as assayed by growth on selective media supplemented with 4 mM ZnCl₂, 200 mM CaCl₂, and 25–0% growth on media buffered to pH 7.5 (Table 2). Furthermore, there was no detectable accumulation of quinacrine in the yeast vacuole (Fig. 2). No measurable proton pumping or ATPase activity was detected on purified vacuolar vesicles (Fig. 3) as well as barely detectable levels of Vph1p, Vma1p, and Vma2p on vacuolar membranes (Fig. 4).

To ask whether the W520L mutation affected vacuolar targeting or complex assembly, whole cell extracts were immunoblotted. Surprisingly, the results for W520L differed not only from Δvph1 but from any other published VPH1 mutation; not only was Vph1p expression compromised but the V₁ subunits, Vma1p and Vma2p, were barely detectable, even when 4-fold more protein was immunoblotted (Fig. 5A, 80 μg for W520L compared with 20 μg for VPH1 and Δvph1). Overexpressing VPH1 in the presence of W520L did not rescue Vma1p or Vma2p expression (Fig. 5B), suggesting that the W520L mutation had a dominant effect.

The a4 W520L Mutation Is Dominant—Single or multicycopy plasmids bearing Vph1p_W520L, when expressed in Δvph1 or wild type (VPH1) yeast strains, eliminated V-ATPase activity as assayed by growth on 4 mM ZnCl₂ (Table 3). Previously, we showed that overexpressing one “a” isoform (Stv1p) could compensate for the absence of the other (Vph1p) (28). To this end, we asked whether we could rescue the W520L phenotype by overexpressing Vph1p or Stv1p. Table 3, top, shows that in the presence of the W520L mutation, expressing Vph1p or Stv1p on single or multicopy plasmids in a Δvph1 strain did not restore growth on 4 mM ZnCl₂. Only in a wild type strain, bearing a single copy plasmid of Vph1p_W520L, and only when Vph1p and Stv1p were overexpressed was there a slight complementation of the growth phenotype detectable (Table 3, bottom). These results indicate that the W520L mutation is dominant. The dominant phenotype, together with the rapid subunit degradation, suggests a possible block of
assembly in the endoplasmic reticulum (ER). To test this, we asked whether the ER V-ATPase assembly factors Vma12p, Vma21p, and Vma22p were affected by the Vph1p_W520L mutation or, alternatively, whether they could affect the Vph1p_W520L phenotype.

Immunoblots of whole cell extracts obtained from wild type, Δvph1, and Vph1p_W520L transformed into a Δvph1 strain all had similar levels of Vma12p and Vma22p (Fig. 6), indicating that the W520L mutation did not affect the stability of these ER assembly factors. We next asked whether deletions of the assembly factors could affect the Vph1p_W520L phenotype.

Δvph1 yeast (MM57) transformed with the following plasmids

| Plasmid Control | Zn²⁺ 4 mM | YPD |
|-----------------|----------|-----|
| No plasmid control | +++ | ++ |
| Single copy Vph1p_W520L | +++ | ++ |
| Overexpressing Vph1p_W520L | +++ | ++ |
| Single copy Vph1p_W520L and overexpressing Vph1p | +++ | ++ |
| Overexpressing Vph1p_W520L and overexpressing Vph1p | +++ | ++ |

The a mutation Vph1p_W520L does not affect the stability of Vma12p and Vma22p. Whole cell extracts (20 μg of protein for all lanes) from VPH1, Δvph1, Δvma12, Δvma22, and Vph1p_W520L transformed into Δvph1 were separated by SDS-PAGE and immunoblotted with polyclonal sera against Vma12p and Vma22p. Three independent immunoblots revealed similar results.

Where the V-ATPase complex interacts with Vma21p downstream of the Vma12p and Vma22p interactions (27).

**DISCUSSION**

Mutations in human a3 and a4 result in osteopetrosis and dRTA, respectively. We hypothesize that these human mutations are highlighting critical amino acids within the “a” subunits that are essential for V-ATPase assembly, activity, or targeting. The assignment for three of the four mutations between the human “a” subunits and the yeast ortholog Vph1p was straightforward (Fig. 1). Assigning human Pro-524 to yeast Trp-520 was speculative. Although the residue itself was not perfectly conserved, the alignment in this region appears unambiguous due to identity both upstream and downstream of this residue. This led us to believe that the mammalian a4 proline residue was conservatively altered to tryptophan, another nonpolar residue, in the yeast Vph1p. Although assigning a4 Pro-524 to Vph1p Trp-520 is controversial, the Vph1p_W520L mutation resulted in the most interesting phenotype.

**The Human a3 Mutations G405R and R444L Are Not Critical for Folding, Targeting, or Assembly of Yeast V-ATPases**—In humans, the osteopetrotic G405R and R444L point mutations are phenotypically identical to full a3 deletions (35, 52). Also, the human Gly-405 and Arg-444 residues are perfectly conserved among canine, bovine, murine, and yeast subunits, further suggesting a critical role for these residues. Considering all this, we expected these missense mutations, when recreated in yeast, to have a profound phenotype. Unexpectedly, recreating the human a3 mutations in yeast resulted in only a slight impairment of growth on selective media. V-ATPase assembly on the vacuolar membrane and vacuolar acidification were not noticeably affected, although proton pumping and ATP hydrolysis were reduced by 30–35%.

Considering that Gly-405 and Arg-444 are critical in human osteoclasts but only slightly affect activity in yeast, one could conclude that yeast is not a good model for studying human mutations. Alternatively, one could hypothesize that the difference between the yeast phenotype and the null phenotype in humans is highlighting an essential osteoclast function for
these two residues distinct from V-ATPase assembly or activity. For example, these point mutations may affect targeting to and from the osteoclast-ruffled border during the resorptive cycle. A mammalian trafficking defect would likely not affect Vph1p targeting to the yeast vacuole, particularly because the default pathway for integral membrane proteins in yeast is to the vacuolar membrane (53). Although the a3 isoform has been shown to be highly enriched in the osteoclast plasma membrane (38), it is currently thought that an actin-binding domain in the V1 subunit (54) controls recycling from the osteoclast-ruffled border. As we have shown that the 50-kDa N-terminal domain of the a subunit interacts with V1 subunits (55), one can speculate that conformational changes in a could affect the availability of the actin-binding sites in B2. Further elucidation of mechanisms involving these a mutations clearly requires a mammalian osteoclast culture system.

Specific to yeast, the results here suggest that Gly-424 is not critical for assembly. Nevertheless, another study has shown that the adjacent residue Gly-425, when mutated to an asparagine, did in fact reduce assembly (47).

The Human “a” Mutation G820R Is Required for Activity of V-ATPases—In contrast to the recreated a3 mutations, recreating the human dRTA a4 mutation, G820R (G812R in yeast Vph1p), resulted in severely defective growth on selective media and barely detectable vacuolar acidification with 17% of ATP-dependent proton pumping and 13% ATPase activity relative to wild type. Nevertheless, there were wild type expression levels of V-ATPase subunits Vph1p (a), Vma1p (A), and Vma2p (B) on the vacuolar membrane, indicating that folding, assembly, and targeting were unimpaired. These results suggest that Gly-820 is a key residue for activity of all V-ATPases. Gly-812 is found within the C-terminal tail domain of Vph1p, a region for which orientation within the vacuolar lumen (57) or cytoplasm (23, 58) is still in dispute. Previous studies have demonstrated that the C-terminal region is essential for V-ATPase assembly (59). Our present results, together with the results of Leng et al. (59), suggest that the C-terminal tail domain of the “a” subunit has an essential function in V-ATPase assembly and activity.

The Human “a” Mutations P524L and P524F, When Recreated in Yeast, Results in a Dominant Mutation That Affects the Stability of Both V0 and V1 Subunits—The P524L mutation, when recreated in yeast Vph1p as W520L, affects activity, stability, and expression of all V-ATPase subunits. Vph1p bearing the W520L mutation was similar to W520L with respect to inability to grow on selective plates, absence of vacuolar acidification, no detectable V-ATPase activity, and absence of V1 subunits on the vacuolar membrane. Surprisingly, when subunit levels in whole cell extracts were examined, Vph1p W520L had a more deleterious effect than the complete absence of Vph1p (W520L). Not only was the expression of Vph1p W520L reduced but expression of the two V1 subunits, Vma1p (A) and Vma2p (B), was attenuated. This is the first observed instance of a mutation within a V0 subunit affecting the stability of a V1 subunit, and it challenges the theory that the two complexes assemble independently in vivo.

To test if overexpression of one “a” isoform could complement mutations to the other “a” isoform, with respect to W520L, we asked whether single copy expression or overexpression of Vph1p or Stv1p could complement the Vph1p W520L phenotype. We found that when Vph1p W520L was expressed from a single copy plasmid in a wild type strain, it was necessary for Vph1p or Stv1p to be overexpressed to achieve even partial complementation with respect to growth on selective media. These results illustrate the dominant nature of the W520L mutation. This is in contrast to the recessive nature of the human a4 P524L mutation (5), suggesting that the biochemical basis for the resulting phenotype may differ between yeast and human.

The Dominant Negative Vph1p W520L Phenotypes Are Partially Rescued in the Absence of the ER Assembly Factors, Vma12p, Vma21p, and Vma22p—A common reason for dominant negative phenotypes for mutant proteins present in a single copy in a complex (such as the a subunit) is that the mutant protein has a higher affinity for a crucial interacting factor and inappropriately sequesters it from wild type proteins (60). The dominant negative nature of Vph1p W520L could result from its sequestering the V-ATPase assembly proteins Vma12p, Vma21p, and Vma22p. To this end, we asked whether Vph1p W520L affected the ER V-ATPase assembly factors or, alternatively, whether they could affect the Vph1p W520L phenotype.

Immunoblot of whole cell extracts revealed wild type levels of both Vma12p and Vma22p in the presence or absence of the Vph1p W520L mutation (Fig. 6), indicating that this mutation does not affect the steady state stability of these two V-ATPase
assembly factors. In contrast, deletion of either Vma12p or Vma22p partially restored Vph1p_W520L and Vma2p expression (Fig. 7). The similar results arising from an absence of Vma12p and Vma22p (∆vma12 and ∆vma22) are consistent with these assembly factors forming a single complex in the ER that transiently binds to Vph1p (with a half-life of ~5 min) to aid in its assembly and maturation (25). We speculate that the dominant negative phenotypes arising from the Vph1p_W520L mutation result from aberrant, possibly inappropriately long interactions with the Vma12p-Vma22p complex. A tight association between Vph1p_W520L and Vma2p/Vma22p could exclude wild type Vph1p or Stv1p, explaining their inability to complement, even when overexpressed (Table 3 and Fig. 5).

Deleting Vma21p also partially restored Vph1p_W520L and Vma2p expression, but not as effectively as ∆vma12 and ∆vma22, with respect to Vph1p_W520L expression itself (Fig. 7). This is in agreement with a model where Vma21p acts downstream of Vma12p and Vma22p by escorting the Vo complex from the ER to the Golgi (27).

Vph1p_W520L Provides Evidence for the Concerted Pathway for V-ATPase Assembly—The precise mechanisms by which V-ATPases assemble are still controversial with evidence suggesting two different possibilities. Mutational analysis and in vitro assays have shown that preassembled Vo and V1 domains can combine to form one complex in a process called independent assembly. Support for independent assembly includes the findings that the assembled Vo domain can be found at the vacuole in the absence of the V1 domain, whereas free V1 domains can be found in the cytoplasm and not at the vacuole (61). In contrast, in vivo pulse-chase experiments have revealed early interactions between Vo and V1 subunits, specifically Vph1p (a) and Vma1p (A), suggesting that subunits are added in a stepwise fashion to form a single complex in a concerted assembly process (56).

Previously, all deletions and mutations of Vo subunits did not affect V1 subunit expression, stability, or assembly, supporting the independent pathway model reviewed in Ref. 1. The Vph1p_W520L phenotype is unique in that it affects the expression of V1 subunits Vma1p (A) and Vma2p (B). We speculated that the W520L mutation affects how Vph1p_W520L interacts with the Vma12p-Vma22p complex within the ER, possibly by inappropriately extending the interaction. The degradation of Vma1p (A) and Vma2p (B) suggests that these V1 subunits also associate with this early ER complex and, if true, would be further evidence of a concerted pathway.

In summary, the osteopetrotic a3 mutations, when recreated in yeast, result in 65–70% of wild type activity with wild type expression of V-ATPase subunits. In contrast, the human a4 dRTA mutation G820R, when recreated in yeast, resulted in an inactive but assembled complex within the vacuole membrane suggesting that this conserved residue is essential for activity, but not assembly, of all V-ATPases. The human a4 dRTA P524L mutation in yeast resulted in a dominant negative phenotype with degradation of a, A, and B subunits. Deletion of the ER assembly factors partially rescued this phenotype, whereas overexpressing yeast a isoforms Vph1p or Stv1p were ineffective. We hypothesize that this a4 mutation results in prolonged interactions with the ER assembly factors, sequestering these assembly factors from wild type a subunits and leading to the degradation of V1 subunits. To test this hypothesis, we plan to delineate precisely when and where Vph1p_W520L, Vma1p (A) and Vma2p (B) are being degraded, through pulse-chase experiments and by using various sec mutants blocked in ER-Golgi transport.

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