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Disease-causing missense mutations in the *PHEX* gene interfere with membrane targeting of the recombinant protein

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PHEX is homologous to the M13 zinc metallopeptidases, a class of type II membrane glycoproteins. Although more than 140 mutations in the *PHEX* gene have been identified in patients with X-linked hypophosphatemia (XLH), the most prevalent form of inherited rickets, the molecular consequences of disease-causing *PHEX* mutations have not yet been investigated. We examined the effect of *PHEX* missense mutations on cellular trafficking of the recombinant protein. Four mutant *PHEX* cDNAs were generated by PCR mutagenesis: C85R, G579R and S711R, identified in XLH patients, and E581V, previously engineered in neutral endopeptidase 24.11, where it abolished catalytic activity but not plasma membrane targeting. Wild-type and mutant *PHEX* cDNAs were transfected in HEK(293) cells and PHEX protein expression was characterized. In contrast to the wild-type and E581V PHEX proteins, the C85R, G579R and S711R mutants were completely sensitive to endoglycosidase H digestion, indicating that they were not fully glycosylated. Sequestration of the disease-causing mutant proteins in the endoplasmic reticulum (ER) and plasma membrane localization of wild-type and E581V PHEX proteins was demonstrated by immunofluorescence and cell surface biotinylation. Of the three mutant PHEX proteins, the S711R was the least stable and the only one that could be rescued from the ER to the plasma membrane in cells grown at 26°C. The chemical chaperone glycerol failed to correct defective targeting of all three mutant proteins. Our data provide a mechanism for loss of PHEX function in XLH patients expressing the C85R, G579R and S711R mutations.

INTRODUCTION

X-linked hypophosphatemia (XLH), the most prevalent form of inherited rickets in humans, is a dominant disorder of phosphate homeostasis characterized by growth retardation, rachitic and osteomalacic bone disease, hypophosphatemia, and renal defects in the reabsorption of filtered phosphate and the metabolism of vitamin D (1,2). The gene responsible for XLH was identified by positional cloning and designated *PHEX* (formerly *PEX*) to depict a phosphate regulating gene with homology to endopeptidases on the X chromosome (3). Large deletions in the *Phex* gene were also identified in X-linked *Hyp* (4,5) and *Gy* (5) mice, which have served as models to study the pathophysiology of the human disease (6).

The *PHEX* gene spans ~243 kb, comprises 22 exons and translates into a 749 amino acid protein (7) (Fig. 1). The latter exhibits significant homology to the M13 family of zinc metallopeptidases, which includes neutral endopeptidase 24.11 (NEP) (8) and endothelin converting enzymes-1 (9) and -2 (10) (ECE-1 and ECE-2). These are type II membrane glycoproteins characterized by a short N-terminal cytoplasmic domain, a single transmembrane hydrophobic region and a large extracellular domain (Fig. 1) (11). The latter includes ten highly conserved cysteine residues and a zinc-binding motif (Fig. 1), which in the case of NEP and ECE-1 are essential for conformational integrity and catalytic activity, respectively (11).

NEP functions as an ectoenzyme, with its active site exposed at the cell surface, and can degrade several small peptides including substance P, bradykinin, atrial natriuretic peptide and enkephalin (8). NEP has been implicated in blood pressure regulation and pain control, and the specificity of NEP action *in vivo* has been attributed to both its tissue distribution and that of its substrates (8). ECE-1 and -2 are involved in the proteolytic activation of big endothelin-1 to endothelin-1 (9,10) and play a role in the regulation of vascular tone (11).

PHEX is expressed predominantly in osteoblasts (12–15) and odontoblasts (13,15), but not in kidney (4). These findings are consistent with data derived from the murine *Hyp* homolog of XLH, in which both a primary mineralization defect (16) and a renal phosphate leak which is dependent on a circulating factor (17,18) have been identified. On the basis of these observations, it has been suggested that PHEX may be involved in the processing or inactivation of paracrine or autocrine factors which play a role in skeletal mineralization and the regulation of renal phosphate reabsorption (6). Although crude membrane preparations from PHEX-transfected cells hydrolyze parathyroid hormone (PTH) (19), the relevance of this finding to

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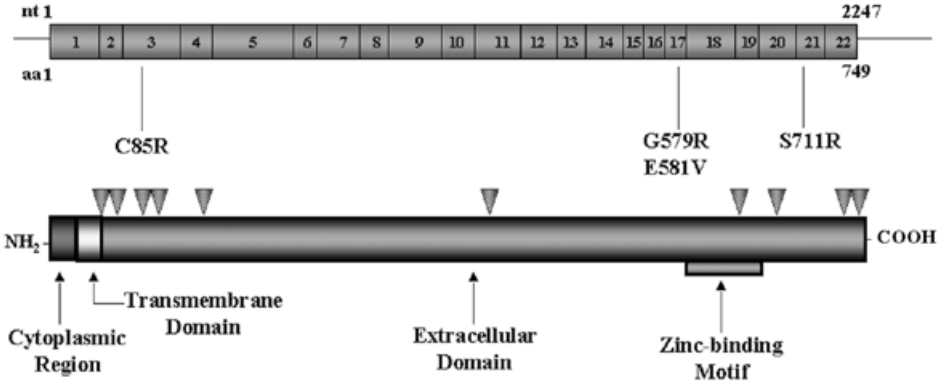

Figure 1. PHEX cDNA and protein. Shown are the 22 exons of the PHEX cDNA (top) and the structural components of the PHEX protein (bottom). These include the N-terminal cytoplasmic region, the transmembrane domain, the extracellular domain and the zinc-binding motif. Conserved cysteine residues are indicated by the triangles. The positions of the C85R, G579R, E581V and S711R missense mutations, investigated in the present study, are shown. aa, amino acid; nt, nucleotide.

the pathophysiology of XLH is not clear since PTH is not necessary for the clinical and biochemical manifestations of XLH patients (20) and *Hyp* mice (17,21). Degradation of $PTHrP_{107–139}$ by a genetically engineered soluble and secreted form of PHEX was recently reported (22). However, the physiological significance of this activity remains to be determined.

To date, 141 mutations in the *PHEX* gene have been identified in XLH patients and in sporadic cases of the disorder and, to centralize information on *PHEX* mutations, we established an online locus-specific mutation database (23) (http://data.mch.mcgill.ca/phexdb). The mutations are scattered throughout the gene and most are consistent with loss of function of the PHEX protein. The mutations include deletions (from 1 to 55 kb), splice junction and frameshift mutations, as well as duplications, insertions, and missense and nonsense mutations (23). However, the mechanism whereby loss of PHEX function elicits the XLH phenotype is not understood.

The present study was undertaken to elucidate the effect of three *PHEX* missense mutations (C85R, G579R and S711R) (Fig. 1), identified in XLH patients, on glycosylation, cellular trafficking and localization of the recombinant proteins. The C85R mutation occurs in a cluster of five cysteine residues just C-terminal of the transmembrane domain that is conserved in NEP and ECE-1 (3). The G579R mutation is found adjacent to the highly conserved zinc-binding motif (HEFTH), a major fingerprint for the M13 family of zinc metallopeptidases (24). This mutation was identified in five different families of German, Polish and Spanish origin, either as X-linked or sporadic. The S711R mutation is found in the HSP consensus sequence, conserved in all members of the endopeptidase family (23), and the histidine residue adjacent to this serine appears to be involved in the stabilization of the transition-state in NEP (25). We also examined the effect of a fourth mutation, E581V, in the conserved catalytic domain (H**E**XXH). This mutation, when engineered in NEP, abrogated catalytic activity but had no effect on the transport of the protein to the cell surface (26). This mutation was also introduced in a soluble and secreted form of PHEX, where it interfered with catalytic activity but not with terminal glycosylation of the recombinant protein (22). Using a variety of approaches, including cell surface biotinylation and immunofluorescence, we demonstrate that the wild-type and E581V PHEX proteins are targeted to the plasma membrane, whereas the disease-causing mutations, C85R, G579R and S711R, result in proteins that are not fully glycosylated and remain trapped in the endoplasmic reticulum (ER). We also show that defective targeting of the S711R is corrected by growing the cells at 26°C.

RESULTS

Expression of wild-type and mutant PHEX proteins in HEK(293) cells

Lysates from HEK(293) cells, transfected with wild-type or mutant PHEX cDNAs, were examined by immunoblotting. The PHEX-specific monoclonal antibody (15) recognized a faster (93 kDa) and a slower migrating protein band (97 kDa) in cells expressing the wild-type PHEX or E581V mutant proteins, whereas only the faster migrating species was detected in cells expressing the C85R, G579R and S711R mutants (Fig. 2). Differences in PHEX protein expression can be ascribed to differences in protein loading (see actin immunoblotting, Fig. 2).

Glycosylation pattern of wild-type and mutant PHEX proteins

To determine whether the lower molecular weight species represent an incompletely processed form of the enzyme, we characterized the glycosylation state of wild-type and mutant (C85R, G579R, S711R and E581V) PHEX proteins. This was accomplished by analyzing lysates from PHEX cDNA-transfected HEK(293) cells by western blotting, before and after treatment with either peptide:N-glycosidase F (PNGase F) or endoglycosidase H (endo H). This treatment allows discrimination between core-glycosylated proteins present in the ER and fully glycosylated proteins which have transited through the compartments of the Golgi apparatus. The wild-type and E581V mutant PHEX proteins were completely sensitive to digestion with PNGase F and a single protein band of ~87 kDa was apparent following the removal of N-linked oligosaccharide side chains (Fig. 3). However, when treated with endo H, most of the wild-type and E581V mutant PHEX proteins were resistant. In contrast, proteins harboring the disease-causing C85R, G579R and S711R mutations were completely susceptible

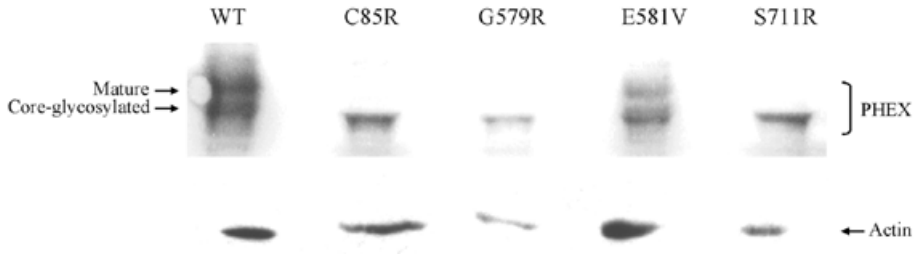

Figure 2. Expression of recombinant wild-type (WT) and mutant PHEX proteins in HEK(293) cells. HEK(293) cells were transiently transfected with wild-type or mutant PHEX cDNAs. Whole cell lysates were electrophoresed on 10% SDS–PAGE and analyzed by immunoblotting with anti-PHEX antibodies. The arrows depict the migration of the mature and core-glycosylated forms of PHEX. As a control for loading, the membrane was also probed with an anti-actin antibody.

to digestion with both PNGase F and endo H (Fig. 3). Because core-glycosylated proteins are sensitive to endo H digestion, our data suggest that the faster-moving species of wild-type and E581V PHEX proteins (Figs 2 and 3) represent the core-glycosylated forms that reside in the ER, whereas the major 97 kDa species (Figs 2 and 3) represent the fully processed, mature form of the protein. Since only the faster moving species were observed in extracts of cells expressing the C85R, G579R and S711R PHEX mutants, our data also suggest that these mutant proteins are incompletely glycosylated and retained in the ER, consistent with their sensitivity to endo H digestion.

Cellular localization of wild-type and mutant PHEX proteins

The cellular localization of wild-type and mutant (C85R, G579R, S711R and E581V) PHEX proteins in transfected HEK(293) cells was examined by immunofluorescence analysis of non-permeabilized and permeabilized cells. In non-permeabilized cells, a fluorescent PHEX signal was detected only in cells expressing the wild-type and E581V mutant proteins (Fig. 4A and E, left panel), consistent with cell surface expression for both PHEX proteins. In contrast, no fluorescent signal was evident in non-permeabilized cells transfected with either the empty vector (data not shown) or the C85R, G579R and S711R mutant cDNAs (Fig. 4B–D, left panel). Upon permeabilization, however, the C85R, G579R and S711R mutant proteins were detected intracellularly, with a perinuclear labeling pattern which is characteristic of proteins residing in the ER (Fig. 4B–D, right panel). Similar findings were observed with permeabilized wild-type and E581V PHEX-expressing cells (Fig. 4A and E, right panel). Positive controls for cell surface and ER labeling are shown in Figure 5. Non-permeabilized HEK(293) cells incubated with an antibody to the plasma membrane Na^+/K^+-ATPase yielded a pattern similar to that observed for the wild-type and E581V mutant proteins detected in non-permeabilized cells (Fig. 5A). On the other hand, permeabilized HEK(293) cells incubated with an antibody to calnexin, which resides in the ER, yielded a pattern that was similar to the perinuclear fluorescence observed in permeabilized cells transfected with the C85R, G579R and S711R cDNAs (Fig. 5B), confirming that the corresponding mutant proteins were indeed trapped in the ER.

The plasma membrane localization of wild-type and E581V PHEX proteins was confirmed by cell surface biotinylation. When HEK(293) cells expressing either the wild-type or the E581V mutant protein were treated with an impermeant biotin, immunoblotting revealed a PHEX signal in the particulate fraction containing the NeutrAvidin resin (data not shown), consistent with cell surface localization of these proteins. The absence of immunoreactive actin in this fraction indicated that intracellular proteins were not biotinylated under the conditions used. These results are consistent with the demonstration that both wild-type and E581V PHEX proteins are resistant to digestion with endo H (Fig. 3).

Interaction of PHEX proteins with calnexin

Given that the C85R, G579R and S711R mutant PHEX proteins are retained in the ER, we examined their interaction with calnexin, a molecular chaperone involved in the proper folding of proteins exiting the ER (27). Lysates from HEK(293) cells, transfected with either wild-type or mutant PHEX cDNAs, were immunoprecipitated with either anti-PHEX or anti-calnexin antibodies and analyzed by immunoblotting with the anti-PHEX antibody. As seen in Figure 6, anti-PHEX antibodies immunoprecipitated a slower and faster migrating species, corresponding to the fully glycosylated and core-glycosylated forms of the wild-type PHEX protein. For C85R, G579R and S711R proteins, only one species representing the core-glycosylated form was immunoprecipitated with the PHEX antibody (Fig. 6), in agreement with data shown in Figure 2. Immunoprecipitation of cell lysates with anti-calnexin antibody revealed that calnexin is co-immunoprecipitated with the mutant C85R, G579R and S711R PHEX proteins (Fig. 6) as well as the ER core-glycosylated forms of the wild-type PHEX protein (Fig. 6). No evidence for

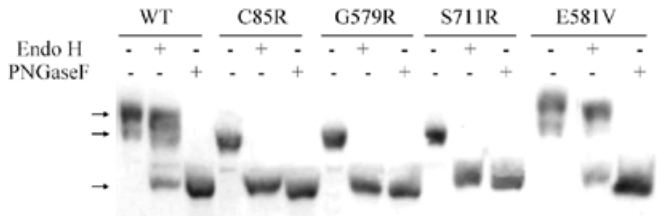

Figure 3. Glycosylation of recombinant wild-type (WT) and mutant PHEX proteins. Whole cell lysates from HEK(293) cells that were transiently transfected with wild-type or mutant PHEX cDNAs were incubated with either buffer, PNGase F or endo H, as described in Materials and Methods. The samples were fractionated on 10% SDS–PAGE and analyzed by immunoblotting with anti-PHEX antibodies. The arrows depict the migration of the mature, core-glycosylated and deglycosylated forms of PHEX protein.

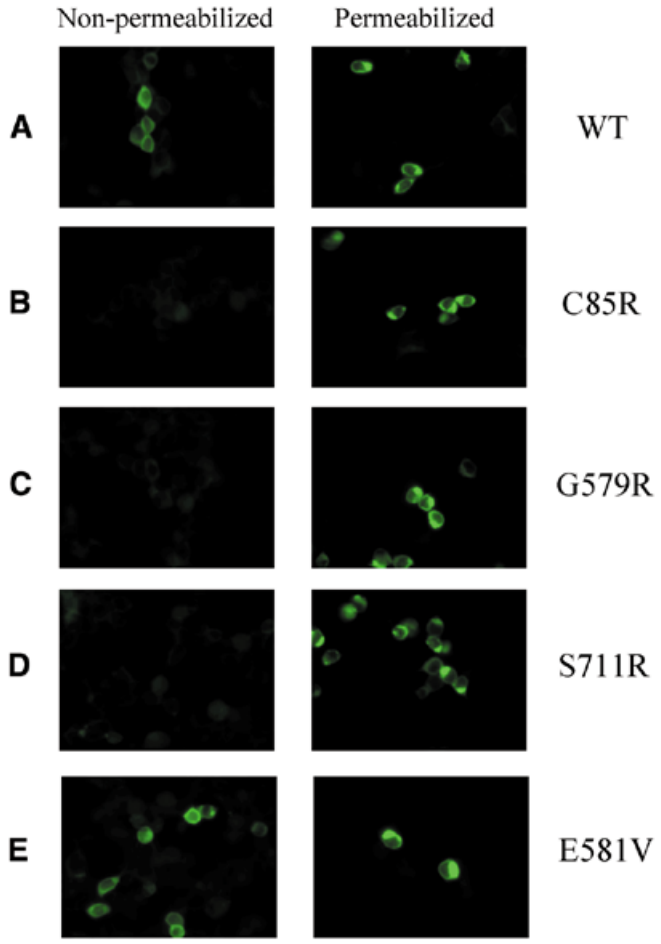

Figure 4. Immunofluorescent labeling of HEK(293) cells expressing wild-type and mutant PHEX proteins. Cells expressing wild-type (**A**) and mutant C85R (**B**), G579R (**C**), S711R (**D**) and E581V (**E**) PHEX proteins were grown on coverslips and incubated in the absence (left, non-permeabilized) or presence (right, permeabilized) of 0.5% Triton X-100. The cells were fixed and incubated sequentially with anti-PHEX antibody and an FITC-conjugated mouse IgG, as described in Materials and Methods. Slides were visualized on a Zeiss fluorescent microscope.

co-immunoprecipitation of PHEX with calnexin was obtained with pre-immune serum (data not shown). Since the immunoprecipitations were carried out with total HEK(293) cell extracts, our results strongly suggest a specific interaction between the ER form of PHEX and calnexin.

Biosynthesis and processing of wild-type and mutant PHEX proteins

Pulse-chase experiments demonstrated that initially the core-glycosylated form of wild-type and E581V PHEX proteins was synthesized and by 60 min a more slowly migrating form was evident (Fig. 7), consistent with maturation of the proteins through the late Golgi compartment where terminal glycosylation occurs. In contrast, only core-glycosylated forms of the C85R, G579R and S711R mutant PHEX proteins were synthesized and a gradual decrease in their abundance was apparent with increasing time, indicating that the mutant proteins were degraded (Fig. 7). The estimated half-lives for the C85R and

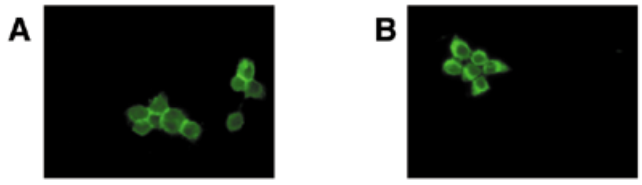

Figure 5. Immunofluorescent labeling of plasma membrane and ER markers in HEK(293) cells. HEK(293) cells grown on coverslips were fixed and incubated with antibodies raised against the Na^+/K^+-ATPase, a plasma membrane marker (**A**), or fixed, permeabilized and then incubated with antibodies raised against calnexin, an ER marker (**B**). In each case, this was followed by the addition of FITC-conjugated mouse IgG. The slides were visualized on a Zeiss fluorescent microscope.

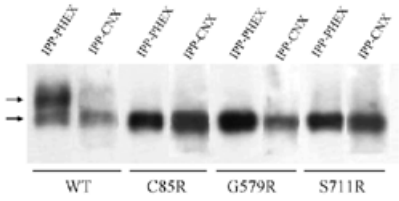

Figure 6. Co-immunoprecipitation of wild-type and mutant PHEX proteins with calnexin. Immunoprecipitation of cell extracts expressing either wild-type (WT) or C85R, G579R and S711R mutant PHEX proteins was achieved by incubation with anti-PHEX antibodies (IPP-PHEX) or anti-calnexin antibodies (IPP-CNX), as described in Materials and Methods. Immune complexes were precipitated with Protein A–Sepharose beads and immunoprecipitated material was fractionated on 10% SDS–PAGE. Immunoprecipitated proteins were detected by western blotting with anti-PHEX antibodies. No protein bands were detected when immunoprecipitation was performed with pre-immune serum (data not shown).

G579R mutants were 88 ± 11 and 106 ± 12 min, respectively, whereas that for the S711R mutant was substantially lower (57 ± 1 min). Thus, although all three XLH mutations result in the expression of unstable proteins, differences in their stability suggest different misfolded intermediates.

Rescue of mutant PHEX proteins from the ER

Previous studies demonstrated that protein processing defects arising from mutations in the genes encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (28) and the aquaporin receptor (29) can be rescued by chemical chaperones such as glycerol, at low temperature (26°C). To test the effect of glycerol on cellular trafficking of the C85R, G579R and S711R mutant PHEX proteins in HEK(293)-transfected cells, PHEX proteins in cell lysates were analyzed by immunoblotting before and after treatment with endo H. Incubation with 1 M glycerol for 48 h had no effect on endo H sensitivity of the mutant PHEX proteins or the endo H resistance of the wild-type PHEX protein (data not shown), indicating that cellular trafficking of the mutant proteins was not even partially corrected by the chemical chaperone. However, when the cells were grown at 26°C, the appearance of an endo H-resistant band was clearly evident for the S711R mutant protein (Fig. 8), consistent with partial rescue of the protein from the ER. Neither the C85R nor the G579R mutant proteins acquired endo H resistance (Fig. 8). Immunofluorescence of

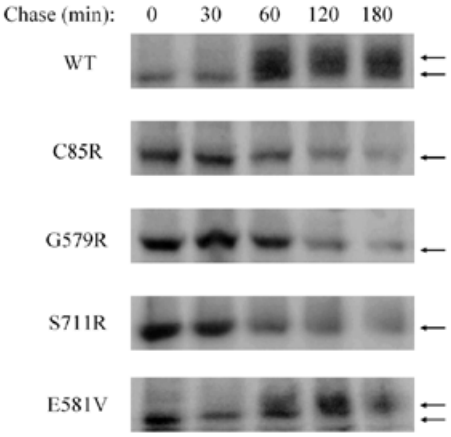

Figure 7. Biosynthesis and processing of wild-type (WT) and mutant PHEX proteins. HEK(293) cells transfected with wild-type and mutant PHEX cDNAs were pulsed for 30 min with [^{35}S]methionine and chased with unlabeled methionine for various times. Cells lysates were subjected to immunoprecipitation with PHEX antibody and analyzed by SDS–PAGE and fluorography. Arrows indicate the core-glycosylated and mature forms of the protein.

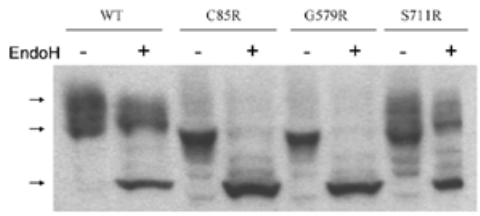

Figure 8. Glycosylation of recombinant wild-type (WT) and mutant PHEX proteins expressed at low temperature. HEK(293) cells were transiently transfected with wild-type or mutant PHEX cDNAs and grown at 26°C. Whole cell lysates derived therefrom were incubated with either buffer or endo H, as described in Materials and Methods. The samples were fractionated on 10% SDS–PAGE and analyzed by immunoblotting with anti-PHEX antibodies. The arrows depict the migration of the mature, core-glycosylated and deglycosylated forms of the protein.

cells expressing S711R mutant PHEX protein grown at 26°C confirmed the cell surface localization, thereby providing evidence for partial rescue of the mutant protein out of the ER to the plasma membrane (Fig. 9).

DISCUSSION

In the present study we demonstrate that three disease-causing missense mutations in the PHEX gene identified in XLH patients interfere with membrane targeting of the recombinant mutant proteins. In contrast to wild-type PHEX protein, the C85R, G579R and S711R mutants are susceptible to endo H digestion, indicating that they are not fully glycosylated and likely trapped in the ER. We also provide evidence for ER sequestration of the disease-causing mutants and for cell surface expression of wild-type PHEX protein by immunofluorescence and biotinylation studies. In addition, we show that treatment of mutant cDNA-transfected cells with a chemical chaperone is not sufficient to rescue the mutant PHEX proteins from the ER. However, when the cells are incubated at 26°C, partial rescue of the S711R PHEX protein out of the ER to the plasma membrane was evident. Taken together, our data provide a mechanism for loss of PHEX function in XLH patients expressing the C85R, G579R and S711R mutations.

We also examined the production and cellular trafficking of the E581V mutant PHEX protein. Although this mutation in the catalytic domain (HEXXH) has not been identified in XLH patients, the equivalent mutation in NEP was shown to abrogate catalytic activity without interfering with cell surface expression (26). Consistent with these findings, we demonstrate that the E581V mutant, like wild-type PHEX protein, is resistant to endo H digestion and expressed at the cell surface, as assessed by cell surface immunofluorescence and biotinylation. Thus, the features of the E581V mutant protein are identical to wild-type PHEX and differ substantially from the C85R, G579R and S711R mutant proteins. These findings demonstrate that manipulations associated with site-directed mutagenesis are not responsible for abnormal targeting of the disease-causing PHEX mutations.

The C85R, G579R and S711R mutations occur in residues that are conserved in the zinc metallopeptidase gene family. C85 is found in a cluster of cysteines and is likely to be involved in disulfide bond formation, making it an important residue for proper folding of the protein. Indeed, our data indicate that the C85R mutant is misfolded and trapped inside the cell. G579 lies one amino acid upstream of the conserved zinc-binding domain, HEXXH, which is necessary for catalytic activity, and S711 lies in the conserved HSP motif, shown to be important in the stabilization of the transition state of NEP (25). Therefore, mutations in these residues are expected to abrogate catalytic activity rather than interfere with cellular processing. However, in each case, the amino acid substitutions, G→R and S→R, involve the replacement of a small amino acid residue with a large, positively charged residue. Thus, these mutations are also likely to affect protein folding, leading to sequestration of mutant proteins inside the cell, and our data clearly provide evidence that this is indeed the case.

We demonstrate that the faster migrating PHEX protein which is susceptible to endo H digestion is co-immunoprecipitated with calnexin, a chaperone that binds monoglucosylated protein substrates and promotes initial folding reactions in the ER (27). The removal of the terminal glucose by glucosidase II releases the ligand from calnexin. When glucosyltransferase no longer recognizes the ligand as incompletely folded, the protein is allowed to exit the ER (30). If protein folding is incomplete, a second round of calnexin binding is initiated to drive the folding process toward completion. Thus, misfolded mutant proteins that are sequestered in the ER are often inappropriately associated with calnexin. However, under the experimental conditions used, we were unable to detect consistent differences in the abundance of calnexin associated with either the wild-type or mutant forms of the rapidly migrating PHEX protein.

Our pulse-chase experiments demonstrate that the C85R, G579R and S711R PHEX proteins undergo degradation in the ER and that S711R is the least stable. The divergence of mutant proteins from the biosynthetic to the degradative pathway has also been demonstrated for the CFTR mutant ΔF508 (31). In that case, it was proposed that, during translation, the core-glycosylated form of wild-type CFTR is sorted

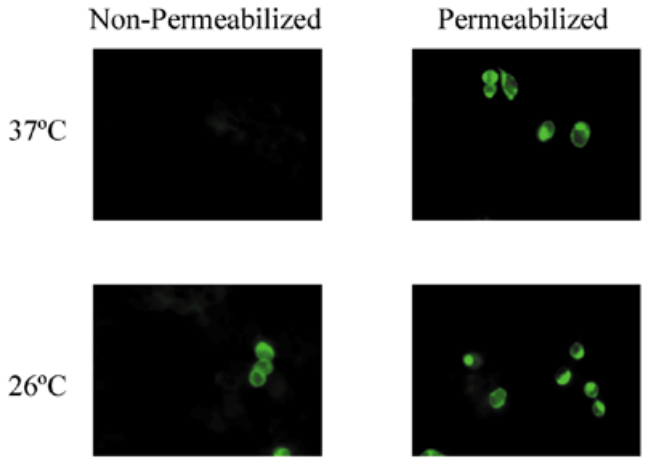

Figure 9. Effect of temperature on cell surface localization of the S711R mutant PHEX protein. HEK(293) cells expressing the S711R mutant PHEX protein were grown on coverslips at 37°C (top) or 26°C (bottom) and incubated in the absence (left, non-permeabilized) or presence (right, permeabilized) of 0.5% Triton X-100. The cells were fixed and incubated sequentially with anti-PHEX antibody and an FITC-conjugated mouse IgG, as described in Materials and Methods. Slides were visualized on a Zeiss fluorescent microscope.

within the ER into an 'export-competent' pool which is resistant to degradation, and that the principal consequence of the ΔF508 mutation is a decrease in the efficiency of this sorting process (31). We suggest that a similar mechanism pertains to the disease-causing PHEX mutations examined in the present study.

There are several genetic disorders in which mutations result in protein misfolding and retention in the ER. These include missense mutations in CFTR which are responsible for cystic fibrosis (28), and in aquaporin-2 (29) and the V2 vasopressin receptor (32) which result in the two major forms of hereditary nephrogenic diabetes insipidus. In all three cases, rescue of the ER-sequestered mutant proteins was achieved using either chemical (28,29) or specific pharmacological (32) chaperones. Partial cell surface expression of the mutant protein and partial restoration of protein function was achieved by incubating cells expressing the ΔF508 CFTR (28) and aquaporin-2 (29) mutants with glycerol. The latter is known to stabilize protein conformation (33) and to increase the rate of *in vivo* protein refolding (34). However, not all mutations leading to ER-sequestered CFTR were corrected by the chemical chaperone (29), indicating that glycerol did not lead to generalized disruption of ER quality control processes. Rather, the corrective effects of glycerol appeared to correlate with the degree of temperature sensitivity of specific CFTR mutants (29). In addition, incubation of the ΔF508 CFTR mutant at 26°C rescued the protein out of the ER to the plasma membrane, where it exhibited functional activity (28). In the present study, we were unable to elicit cell surface expression of the C85R, G579R and S711R mutant PHEX proteins by incubating the respective HEK(293)-producing cells with glycerol under the conditions used in the reports described above (28,29). However, incubation at 26°C was able to partially rescue the S711R PHEX protein out of the ER. We suggest that these findings may reflect the degree of misfolding of the mutant PHEX proteins and that, in the case of S711R, the ER-retention phenotype is leaky, as demonstrated for ΔF508 CFTR (31).

It is of interest that misfolding and ER sequestration of mutant V2 vasopressin receptors were partially corrected by incubating cells producing the mutant proteins with selective non-peptidic and permeable V2 receptor antagonists (32), indicating that small ligands can act as pharmacological chaperones. In this regard, it may be possible to increase cell surface expression of ER-sequestered PHEX mutants by the addition of permeable inhibitors of PHEX catalytic activity. Clearly, further work is necessary to identify PHEX substrates and such inhibitors.

In summary, this study represents the first characterization of wild-type and mutant PHEX protein expression, glycosylation and cellular targeting. We demonstrate that, in contrast to wild-type PHEX which is expressed at the plasma membrane, the C85R, G579R and S711R mutants remain trapped in the ER and are not terminally glycosylated. We thus provide a mechanism for loss of PHEX function in XLH patients expressing these missense mutations. Further work is necessary to determine whether these mutations also interfere with catalytic activity.

MATERIALS AND METHODS

Construction of cDNAs encoding PHEX mutants

PHEX missense mutations, 253T→C (C85R), 1735G→C (G579R) and 2133A→T (S711R), identified in XLH patients, and 1742T→A (E581V), investigated in NEP (26) and PHEX (22), were generated by PCR-mutagenesis (35) and confirmed by sequence analysis with the *Taq* sequencing dye terminator loaded on an Applied Biosystems 373A system (Université Laval, Quebec City, Quebec). Wild-type and mutant cDNAs were subcloned in plasmid pCDNA3 (Invitrogen, Carlsbad, CA), with the RSV promoter, for transfection in mammalian cells.

Cell transfection

HEK(293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (BRL Life Technologies, Burlington, Ontario) containing 10% fetal bovine serum at 37°C in 5% CO_2 and 95% air. For each P100 mm plate containing 1×10^6 cells, transfection was accomplished with 10 μg of cDNA using the calcium-phosphate co-precipitation method (36). The cells were treated with sodium butyrate (final concentration of 10 mM) 24 h after transfection. Where indicated, cells were grown in the same medium containing 1 M glycerol for 48 h.

SDS–PAGE and immunoblot analyses

Whole cell lysates were prepared in 6× Laemmli sample buffer (37) and boiled for 3 min. Separation of proteins was performed on SDS–PAGE (10% acrylamide) and proteins were transferred to nitrocellulose membranes for 1 h. Incubation with a mouse monoclonal anti-PHEX antibody 15D7 (BioMep, Montreal, Quebec) at a 1:200 dilution was performed as described previously (15) and was followed by incubation with peroxidase-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA) at a 1:3000 dilution. Immune complexes were visualized by chemiluminescence

with the ECL kit (Amersham Phamacia Biotech, Piscataway, NJ).

Endoglycosidase digestion

Recombinant PHEX proteins in whole cell extracts were boiled for 10 min in 10× denaturation buffer (5% SDS, 10% β-mercaptoethanol) and incubated for 1 h at 37°C with either PNGase F or endo H, according to the manufacturer's recommendations (New England Biolabs, Missisauga, Ontario). Digestion products were fractionated on SDS–PAGE and subjected to immunoblot analyses as described above.

Cell surface biotinylation

Cells were plated at a density of 2×10^5 cells per well, in a 24-well plate. Cells expressing PHEX proteins were washed with PBS and then labeled with a membrane impermeant biotin analog, sulfo-NHS-LC-biotin (Pierce, Rockford, IL) (2.0 mg/ml) for 25 min as described previously (38). Biotin-labeled proteins were separated from unlabeled proteins by overnight incubation of cell lysates with NeutrAvidin resin (Pierce) at 4°C with end-over-end mixing. The resin was pelleted by centrifugation, and both the pellet and supernatant fractions were electrophoresed on SDS–PAGE and analyzed by western blotting. The membrane was first probed with the mouse monoclonal anti-PHEX antibody. The membranes were then stripped and probed with a rabbit anti-actin antibody (Sigma, St Louis, MO).

Immunofluorescence

Transfected HEK(293) cells were grown on glass coverslips and fixed in PBS containing 4% paraformaldehyde. Where indicated, cells were permeabilized with 0.5% Triton X-100. Immunostaining was accomplished with anti-PHEX (1:10 dilution), anti-calnexin (1:40 dilution) (BD Biosciences, Missisauga, Ontario), or anti-Na^+/K^+-ATPase (1:40 dilution) (Upstate Biotechnology, Lake Placid, NY) antibodies for 1 h at room temperature. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:50 dilution) (Zymed Laboratories, San Francisco, CA) was used for fluorescence labeling at room temperature for 1 h. After several washes, the coverslips were mounted on slides and the cells viewed with a Zeiss fluorescent microscope.

Co-immunoprecipitation of PHEX with calnexin

HEK(293) cells expressing PHEX proteins were solubilized in 1% N-octylglucoside-PBS and centrifuged. The supernatant was collected and incubated with either rabbit polyclonal anti-PHEX or anti-calnexin antibodies (kindly provided by Dr J.J. Bergeron, McGill University), for 16 h at 4°C and mixed by end-over-end rotation. Immunoprecipitation was also performed with pre-immune serum as described above. Protein A–Sepharose beads (Amersham Pharmacia Biotech) (25 µl of a 10% suspension) were added and the incubation allowed to proceed for 2 h at 4°C. Beads were collected by centrifugation, washed three times and resuspended in Laemmli buffer (37). The mixture was boiled for 3 min, centrifuged and the supernatant loaded on SDS–PAGE. Immunoprecipitated proteins were visualized by western blotting with the monoclonal anti-PHEX antibody.

Pulse-chase studies

PHEX cDNA transfected HEK(293) cells were grown on 60 mm dishes, washed in DMEM lacking methionine and incubated in the same medium for a period of 20 min at 37°C. The cells were then labeled for 30 min at 37°C by the addition of [^{35}S]methionine (200 µCi/dish) (ICN, Irvine, CA). After the radioactive pulse, the cells were rinsed twice in the same medium containing an excess of non-radioactive methionine (0.15 mg/ml) and were chased for various periods of time in this medium (t = 0, 30, 60, 120 and 180 min). The cells were then washed and scraped in cold PBS. The PBS was removed by centrifugation and the cells were solubilized with 200 µl of 1% N-octylglucoside. Radiolabeled PHEX proteins were immunoprecipitated as described above, and analyzed by SDS–PAGE and fluorography.

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REFERENCES

1. Rasmussen, H. and Tenenhouse, H.S. (1995) Mendelian hypophosphatemias. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease, 7th edn*. McGraw Hill Book Co., New York, NY, pp. 3717–3745.
2. Tenenhouse, H.S. and Econs, M.J. (2001) Mendelian hypophosphatemias. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*, *8th edn*. McGraw Hill Book Co., New York, NY, pp. 5039–5067.
3. The HYP Consortium (1995) A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. *Nat. Genet.*, **11**, 130–136.
4. Beck, L., Soumounou, Y., Martel, J., Krishnamurthy, G., Gauthier, C., Goodyer, C. and Tenenhouse, H.S. (1997) *Pex/PEX* tissue distribution and evidence for a deletion in the 3′ region of the *Pex* gene in X-linked hypophosphatemic mice. *J. Clin. Invest.*, **99**, 1200–1209.
5. Strom, T.M., Francis, F., Lorenz, B., Boeddrich, A., Econs, M., Lehrach, H. and Meitinger, T. (1997) *Pex* gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia. *Hum. Mol. Genet.*, **6**, 165–171.
6. Tenenhouse, H.S. (1999) X-linked hypophosphatemia: a homologous disorder in humans and mice. *Nephrol. Dial. Transplant.*, **14**, 333–341.
7. Francis, F., Strom, T.M., Hennig, S., Boddrich, A., Lorenz, B., Brandau, O., Mohnike, K.L., Cagnoli, M., Steffens, C. and Klages, S. (1997) Genomic organization of the human PEX gene mutated in X-linked hypophosphatemia. *Genome Res.*, **7**, 573–585.
8. Roques, B.P., Noble, F., Daugé, V., Fournié-Zaluski, M.-C. and Beaumont, A. (1993) Neutral endopeptidase 24.11: Structure, inhibition, and experimental and clinical pharmacology. *Pharmacol. Rev.*, **45**, 87–146.
9. Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D. and Yanagisawa, M. (1994) ECE-1: A membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell*, **78**, 473–485.
10. Emoto, N. and Yanagisawa, M. (1995) Endothelin-converting enzyme-2 is a membrane bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J. Biol. Chem.*, **270**, 15262–15268.
11. Turner, A.J. and Tanzawa, K. (1997) Mammalian membrane metallopeptidases: NEP, ECE, KELL and PEX. *FASEB J.*, **11**, 355–364.

12. Du, L., Desbarats, M., Viel, J., Glorieux, F.H., Cawthorn, C. and Ecarot, B. (1996) cDNA cloning of the murine *Pex* gene implicated in X-linked hypophosphatemia and evidence for expression in bone. *Genomics*, **36**, 22–28.
13. Ruchon, A.F., Marcinkiewicz, M., Siegfried, G., Tenenhouse, H.S., DesGroseillers, L., Crine, P. and Boileau, G. (1998) Pex mRNA is localized in developing mouse osteoblasts and odontoblasts. *J. Histochem. Cytochem.*, **46**, 459–468.
14. Guo, R. and Quarles, D. (1997) Cloning and sequencing of human PEX from a bone cDNA library: Evidence for its developmental stage-specific regulation in osteoblasts. *J. Bone Miner. Res.*, **12**, 1009–1017.
15. Ruchon, A.F., Tenenhouse, H.S., Marcinkiewicz, M., Siegfried, G., Aubin, J.E., DesGroseillers, L., Crine, P. and Boileau, G. (2000) Developmental expression and tissue distribution of Phex protein: Effect of the Hyp mutation and relationship to bone markers. *J. Bone Miner. Res.*, **15**, 1440–1450.
16. Ecarot, B., Glorieux, F.H., Desbarats, M., Travers, R. and Labelle, L. (1992) Defective bone formation by Hyp mouse bone cells transplanted into normal mice: Evidence in favor of an intrinsic osteoblast defect. *J. Bone Miner. Res.*, **7**, 215–220.
17. Meyer, R.A.,Jr, Tenenhouse, H.S., Meyer, M.H. and Klugerman, A.H. (1989) The renal phosphate transport defect in normal mice parabiosed to X-linked hypophosphatemic mice persists after parathyroidectomy. *J. Bone Miner. Res.*, **4**, 523–532.
18. Nesbitt, T., Coffman, T.M., Griffiths, R. and Drezner, M.K. (1992) Cross-transplantation of kidneys in normal and Hyp mice: Evidence that the Hyp phenotype is unrelated to an intrinsic renal defect. *J. Clin. Invest.*, **89**, 1453–1459.
19. Lipman, M.L., Panda, D., Bennett, H.P.J., Henderson, J.E., Shane, E., Shen, Y., Goltzman, D. and Karaplis, A.C. (1998) Cloning of human Pex cDNA. Expression, subcellular localization, and endopeptidase activity. *J. Biol. Chem.*, **273**, 13729–13737.
20. Lyles, K.W., Burkes, E.J., McNamara, C.R., Harrelson, J.M., Pickett, J.P. and Drezner, M.K. (1985) The concurrence of hypoparathyroidism provides new insights to the pathophysiology of X-linked hypophosphatemia. *J. Clin. Endocrinol. Metab.*, **60**, 711–717.
21. Cowgill, L.D., Goldfarb, S., Lau, K., Slatopolsky, E. and Agus, Z.S. (1979) Evidence for an intrinsic renal tubular defect in mice with genetic hypophosphatemic rickets. *J. Clin. Invest.*, **63**, 1203–1210.
22. Boileau, G., Tenenhouse, H.S., DesGroseillers, L. and Crine, P. (2001) Characterization of PHEX endopeptidase catalytic activity: identification of parathyroid-hormone-related peptide 107–139 as a substrate and osteocalcin, PPi and phosphate as inhibitors. *Biochem. J.*, **355**, 707–713.
23. Sabbagh, Y., Jones, A.O. and Tenenhouse, H.S. (2000) PHEXdb, a locus-specific database for mutations causing X-linked hypophosphatemia. *Hum. Mutat.*, **16**, 1–6.
24. Rowe, P.S.N., Oudet, C.L., Francis, F., Sinding, C., Pannetier, S., Econs, M.J., Strom, T.M., Meitinger, T., Garabedian, M., David, A. *et al.* (1997) Distribution of mutations in the PEX gene in families with X-linked hypophosphatemic rickets (HYP). *Hum. Mol. Genet.*, **6**, 539–549.
25. Dion, N., Le Moual, H., Crine, P. and Boileau, G. (1993) Kinetic evidence that His-711 of neutral endopeptidase 24.11 is involved in stabilization of the transition state. *FEBS Lett.*, **318**, 301–304.
26. Devault, A., Nault, C., Zollinger, M., Fournie-Zaluski, M., Roques, B.R., Crine, P. and Boileau, G. (1988) Expression of neutral endopeptidase (Enkephalinase) in heterologous COS-1 cells. Characterization of the recombinant enzyme and evidence for a glutamic acid residue at the active site. *J. Biol. Chem.*, **263**, 4033–4040.
27. Ou, W.J., Cameron, P.H., Thomas, D.Y. and Bergeron, J.J. (1993) Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature*, **364**, 771–776.
28. Sato, S., Ward, C.L., Krouse, M.E., Wine, J.J. and Kopito, R.R. (1996) Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. *J. Biol. Chem.*, **271**, 635–638.
29. Tamarappoo, B.K. and Verkman, A.S. (1998) Defective aquaporin-2 trafficking in nephrogenic diabetes insipidus and correction by chemical chaperones. *J. Clin. Invest.*, **101**, 2257–2267.
30. Sousa, M.C., Ferrero-Garcia, M.A. and Parodi, A.J. (1992) Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *Biochemistry*, **31**, 97–105.
31. Ward, C.L. and Kopito, R.R. (1994) Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J. Biol. Chem.*, **269**, 25710–25718.
32. Morello, J.P., Salahpour, A., Laperriere, A., Bernier, V., Arthus, M.F., Lonergan, M., Petaja-Repo, U., Angers, S., Morin, D., Bichet, D.G. and Bouvier, M. (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J. Clin. Invest.*, **105**, 887–895.
33. Gekko, K. and Timasheff, S.N. (1981) Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry*, **20**, 4677–4686.
34. Sawano, H., Koumoto, Y., Ohta, K., Sasaki, Y., Segawa, S. and Tachibana, H. (1992) Efficient *in vitro* folding of the three-disulfide derivatives of hen lysozyme in the presence of glycerol. *FEBS Lett.*, **303**, 11–14.
35. Yang, X.-F., Fourier, H., Dion, N., Crine, P. and Boileau, G. (1994) Site-directed mutagenesis and transfection methods in the study of prohormone processing. *Neuroprotocols*, **5**, 157–168.
36. Sambrook, J., Fritsch, E.F.and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor, NY.
37. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
38. Daniels, G.M. and Amara, S.G. (1998) Selective labeling of neurotransmitter transporters at the cell surface. *Methods Enzymol.*, **296**, 307–318.