Identification of a Human trans-3-Hydroxy-L-proline Dehydratase, the First Characterized Member of a Novel Family of Proline Racemase-like Enzymes

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Background: The activity of a subset of proline racemase-like proteins was unresolved. Several members of this family have been well characterized and are known to catalyze the racemization of free proline or trans-4-hydroxypoline. However, the majority of eukaryotic proline racemase-like proteins, including a human protein called C14orf149, lack a specific cysteine residue that is known to be critical for racemase activity. Instead, these proteins invariably contain a threonine residue at this position. The function of these enzymes has remained unresolved until now. In this study, we demonstrate that three of these enzymes have been resolved. C14orf149 is also the first human enzyme of proline racemase-like genes for which the enzymatic activity has been resolved. C14orf149 also serves to degrade trans-3-hydroxy-L-proline. Interestingly, a mutant C14orf149 exhibits epimerase activity.

Results: Cys-Thr type proline racemase-like enzymes, including human C14orf149, exhibit trans-3-hydroxy-L-proline dehydratase activity. C14orf149 catalyzes the dehydration of trans-3-hydroxy-L-proline to Δ1-pyrroline-2-carboxylate (Pyr2C). These are the first enzymes of this subclass of proline racemase-like genes for which the enzymatic activity has been resolved. C14orf149 is the first human enzyme that acts on trans-3-hydroxy-L-proline. Presumably, human C14orf149 serves to degrade trans-3-hydroxy-L-proline from the diet and originating from the degradation of proteins that contain this amino acid, such as collagen IV, which is an important structural component of basement membrane.

Conclusion: Human C14orf149 is a previously uncharacterized trans-3-hydroxy-L-proline dehydratase. These findings reveal a previously unknown pathway in human metabolism and will facilitate elucidation of the activity of related enzymes.

Significance: This result suggests that the enzymatic activity of these enzymes is dictated by a single residue. Presumably, human C14orf149 serves to degrade trans-3-hydroxy-L-proline from the diet and originating from the degradation of proteins that contain this amino acid, such as collagen IV, which is an important structural component of basement membrane.

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A family of eukaryotic proline racemase-like genes has recently been identified. Several members of this family have been well characterized and are known to catalyze the racemization of free proline or trans-4-hydroxypoline. However, the majority of eukaryotic proline racemase-like proteins, including a human protein called C14orf149, lack a specific cysteine residue that is known to be critical for racemase activity. Instead, these proteins invariably contain a threonine residue at this position. The function of these enzymes has remained unresolved until now. In this study, we demonstrate that three of these enzymes have been resolved. C14orf149 is also the first human enzyme of proline racemase-like genes for which the enzymatic activity has been resolved. C14orf149 also serves to degrade trans-3-hydroxy-L-proline. Interestingly, a mutant C14orf149 exhibits epimerase activity.

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Although prokaryotic proline racemase proteins have been known for a long time and have been well characterized, the first eukaryotic proline racemase was discovered only recently (1). It was initially discovered as an important factor for the mitogenic properties of Trypanosoma cruzi. Interestingly, the active site of the protein is required for its mitogenic properties (1, 2). Proline racemases have been evolutionarily preserved among trypanosomes and continue to attract considerable interest due to their potential as parasite-specific drug targets for the treatment of infections (3). The catalytic mechanism of these eukaryotic enzymes is essentially identical to that of the prokaryotic proline racemase proteins, which have been extensively studied (2, 4). Unlike alanine racemase and serine racemase, known proline racemases do not require pyridoxal-5-phosphate or any other cofactor. Instead, they operate by a two-base mechanism, in which two cysteine residues constitute the proton acceptor and donor that abstract and donate a proton from the proline α-carbon, respectively, thereby inverting its stereochemical configuration (2, 4). Interestingly, several studies (2, 5) have identified proline racemase-like genes that exhibit strong sequence similarity to proline racemases, but that appear to be missing a cysteine residue that is critical for the racemase activity. All known proteins of this type contain a threonine residue at this position instead. It has been confirmed that several of these proteins are devoid of racemase activity (5), as predicted, but their function has remained unresolved to date.

Most mammals contain a member of this subclass of proline racemase-like genes. The human gene is identified as C14orf149. Because considerable amounts of D-proline are present in human plasma, urine, and cerebrospinal fluid (6–8) and because D-serine and D-aspartate perform signaling functions in humans (9–11), it is conceivable that humans also synthesize and utilize D-proline. This prompted us to investigate the enzymatic activity of human C14orf149. To this end, we purified recombinant human C14orf149 and demonstrate that it is not a racemase but catalyzes the degradation of trans-3-hydroxy-L-proline (trans-3-L-Hyp) to Δ1-pyrroline-2-carboxylate (Pyr2C). In line with the finding that C14orf149 lacks one of the two cysteine residues that act as the proton acceptor and donor, the enzyme presumably operates by abstracting a proton from the trans-3-hydroxy-L-proline substrate, without donat-
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**Experimental Procedures**

**Materials**—[D7]-DL-Proline was purchased from Cambridge Isotope Laboratories (Andover, MA). UPLC grade solvents were from BioSolve (Valkenswaard, The Netherlands). Unless specified otherwise, all other chemicals came from Sigma-Aldrich (Zwijndrecht, The Netherlands).

**Cloning and Mutagenesis**—A construct containing the human C14orf149 gene was obtained by PCR from IMAGE clone 4864867 (Promega, Leiden, The Netherlands) using primers 5′-AAAAACTCTgAgCCgCAAgACCATggAgAg-3′ (forward) and 5′-AAAAAGATCCCAgTCACTTgAgAaAAA-TCCATCCC-3′ (reverse) and ligated into a pGEM-T vector (Promega). This vector was sequenced using the BigDye Terminator method starting from the T7 and SP6 sites in the pGEM-T vector. A documented SNP (rs11555240) was found to occur in the sequence, resulting in a single amino acid residue change (R9W). Sequencing of the IMAGE vector revealed that the SNP was already present in this vector. Because it is a documented SNP and the amino acid residue is not evolutionarily conserved, the assumption was made that it does not fundamentally alter the enzymatic activity of the enzyme. The C14orf149 construct was cut from the pGEM-T vector using XhoI and NotI restriction sites and ligated into a pGEX-6P1 vector (GE Healthcare, Diegem, Belgium), resulting in the vector pDA7. The T273C mutation was introduced in pDA7 by site-directed mutagenesis, using primers 5′-CAggTTgACAgAGtCTCCtGgCCCTCAggAgTAgACAgCC-3′ and 5′-ggCTgTCTC-ACCTTCTAgACggACTCCTTCAACCTTg-3′, yielding the vector pDA31. The M. musculus C14orf149 and S. kowalevskii LOC100374014 genes were excised from synthetic plasmids (GenScript, Piscataway, NJ), using EcoRI and Xhol restriction sites, and ligated into a pGEX-6P1 expression vector, resulting in pDA36 and pDA37, respectively.

**Expression and Purification**—The pDA7, pDA31, pDA36, and pDA37 plasmids were used to transform the Rosetta Escherichia coli strain (Merck, Schiphol-Rijk, The Netherlands). The cells were grown in liquid LB medium containing the appropriate antibiotics to an A600 of 0.5. The cells were then transferred to 1 liter of Terrific Broth with additional glycerol (per liter: 12 g of Tryptone, 24 g of yeast extract, and 8 ml of glycerol) and appropriate antibiotics, at a density of A660 = 0.1. The cells were allowed to grow to a density of A660 = 0.6 at 22 °C, at which point 0.1 mM isopropyl-1-thio-β-D-galactopyranoside was added. After 5 h of incubation at 22 °C, the cells were harvested by centrifugation and washed once with ice-cold PBS. The cells were lysed by sonication, and the lysate was cleared by centrifugation at 10,000 × g for 30 min. The protein was purified from the supernatant using glutathione-agarose 4B (GE Healthcare), according to the manufacturer's instructions. The S. kowalevskii LOC100374014 protein product was concentrated 10-fold by ultrafiltration using a 10-kDa cutoff filter (Millipore). The purified proteins were stored at −80 °C in a solution containing 50 mM Tris, pH 8.0, 15 mM reduced glutathione, and 20% w/v glycerol.

**Substrate Specificity Assay**—To check the substrate specificity, reaction mixtures containing 20 μg of the purified protein, 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, and 1 mM substrate in a total volume of 100 μl were incubated at 37 °C. 5-μl samples of the reaction mixtures were taken at regular time intervals and immediately added to a vial containing an ice-cold mixture of 25 μl 50 μM [D7]-DL-proline and 600 μl of acetonitrile to terminate the reaction and to deproteinize the sample. The terminated reactions were incubated on ice for 20 min and centrifuged (5 min at 13,000 rpm, at 4 °C). The supernatant was transferred to a glass vial and evaporated to dryness under N2 flow, at 40 °C. The residue was subjected to derivatization with a chiral reagent to enable the resolution of the different isomers. For this purpose, the residue was dissolved in 50 μl of water and mixed with 35 μl of 0.15 M sodium tetraborate and 50 μl of (S)-NIFU solution (2.5 mg/ml in acetonitrile). After 10 min of incubation at room temperature, 10 μl of 4 M HCl and 355 μl of water were added, and the samples were vacuum-filtered through a 0.2-μm filter and analyzed using UPLC-MS/MS as follows. A Waters Acquity UPLC instrument (Milford, MA) was fitted with a 100 × 2.1-mm BEH-C18 1.7 μm column. A step gradient profile was used, consisting of 83% solvent A for 4.5 min followed by 75% A for 3 min, where solvent A is 0.1% (v/v) formic acid, and solvent B is acetonitrile. The MS/MS instrument (Waters Xevo) was configured for positive electrospray ionization and operated in multiple reaction monitoring mode. The following multiple reaction monitoring settings for the different compounds were used: hydroxyproline (all isomers), 381.35 > 132.1, CV = 22.0 V, Ecoll = 14.0 V; GABA, 353.36 > 120.1, CV = 22.0 V, Ecoll = 22.0 V; Pyr2C, 114.05 > 96.0, CV = 22.0 V, Ecoll = 14.0 V; proline, 365.3 > 120.1, CV = 24.0 V, Ecoll = 22.0 V; [D7]-proline, 372.3 > 120.1, CV = 24.0 V, Ecoll = 22.0 V. Mass spectra obtained by direct infusion were recorded using a constant cone voltage of 24 V. The concentrations of the different compounds were calculated using a calibration curve of the different compounds prepared in water with [D7]-1-Pro as the internal standard.

**Colorimetric Enzyme Assay**—Reaction mixtures containing 25 mM Tris-HCl pH 8.0, 1 mg/ml purified enzyme, and 25 mM trans-3-hydroxy-L-proline in a total volume of 50 μl were incubated at 37 °C for 60 s. The reactions were terminated by the addition of 900 μl of ice-cold 3% trichloroacetic acid. Then, 50
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\[ \text{Cys} \] of a solution of 25 mg/ml 2-aminobenzaldehyde in ethanol was added to each terminated reaction, and the mixtures were incubated at 60 °C for 30 min. The absorbance was measured at 425 nm. For establishing the pH dependence, the Tris buffer was replaced with a mixture of 25 mM ammonium acetate and 25 mM potassium phosphate, the pH of which was adjusted using HCl and NaOH.

Preparation of Pyr2C—A reaction mixture containing 25 mM ammonium bicarbonate, pH 8.3, 1 mM D-proline, 0.25 mg of porcine d-amino acid oxidase, and 897 units of bovine catalase in a total volume of 100 µl was incubated for 60 min, at 37 °C. After this time, the reaction was terminated by the addition of 700 µl of ice-cold acetonitrile and incubated on ice for 30 min. The precipitate was removed by centrifugation (5 min, 20,000 × g), and the supernatant, containing Pyr2C, was used for experiments.

Tissue Distribution—A panel of glycerol-3-phosphate dehydrogenase-normalized cDNA samples prepared from human tissues was obtained from Clontech. PCR reactions were conducted with the following primers for C14orf149: 5′-ATGCTTATACCAAGGAAACCCACCC-3′ (forward) and 5′-ACTGTAAGGCAAATTGCCGTG-3′ (reverse). The primers included with the cDNA samples were used for glycerol-3-phosphate dehydrogenase.

Phylogenetic Analysis—The phylogenetic tree was estimated using maximum likelihood (phyML v3.0.1 (12)) using the BLOSUM62 substitution matrix and the nearest neighbor interchange algorithm. Protein sequences were initially aligned using Muscle (13) and then corrected manually.

RESULTS AND DISCUSSION

Inventory of Eukaryotic Proline Racemase-like Proteins—Several sequence motifs have been identified in proline racemase-like proteins that can be used to recognize proteins of this class in databases (5, 14). However, because it cannot be excluded that some homologs may have unusual sequence variations within these motifs, we made an inventory of eukaryotic proline racemase-like genes using pBLAST (15). The National Center for Biotechnology Information (NCBI) RefSeq database was searched using the T. cruzi proline racemase A (XP_811287.1) as the template, and the results were limited to eukaryotic organisms. Six sequences had low query coverage (<60%) and appeared to lack one of the two cysteine residues involved in the active site. These were assumed to be only partial sequences and were omitted. The resulting inventory of proline racemase-like proteins is listed in Table 1. These sequences were aligned (supplemental Fig. S2). A phylogenetic tree (Fig. 1) was derived from this alignment. The two cysteine residues that were previously found to be essential for the proline racemase activity are located at positions 145 and 364. The amino acids that are present at these two positions are included for each entry in Table 1. Interestingly, on the basis of these residues, three distinct types can be recognized: (i) proteins with a conserved pair of cysteine residues (Cys-Cys type); all known (hydroxy)proline racemases are of this type; (ii) proteins in which the Cys at position 145 is replaced with Ser (Ser-Cys type); and (iii) proteins in which the Cys at position 364 is replaced with Thr (Cys-Thr type). Only a minority of proline racemase-like proteins are of the Cys-Cys type known to catalyze racemization reactions: 19 out of 68 protein sequences (28%). 14 (21%) are of the Ser-Cys type, and 35 (51%) are of the Cys-Thr type. This suggests that the majority of the proline racemase-like proteins

| Accession | Organism | Cys-145 | Cys-364 |
|-----------|----------|---------|---------|
| XP_002487532.1 | Talaromyces stipitatus | Cys | Cys |
| XP_748142.1 | Aspergillus fumigatus | Thr | Cys |
| XP_659725.1 | Aspergillus nidulans | Thr | Cys |
| XP_001397089.1 | Aspergillus niger | Thr | Cys |
| XP_001397532.2 | Aspergillus niger | Ser | Cys |
| XP_003186930.1 | Aspergillus oryzae | Thr | Cys |
| XP_001287506.1 | Aspergillus oryzae | Cys | Cys |
| XP_001287065.1 | Aspergillus oryzae | Cys | Cys |
| XP_001289372.2 | Aspergillus oryzae | Cys | Cys |
| XP_001218441.1 | Aspergillus terreus | Cys | Thr |
| NPS_001029558.1 | Bos taurus | Cys | Cys |
| XP_002648080.1 | Branchistostoma floridana | Cys | Thr |
| XP_002648100.1 | Branchistostoma floridana | Cys | Cys |
| XP_002648111.1 | Branchistostoma floridana | Cys | Cys |
| XP_002648096.1 | Branchistostoma floridana | Cys | Cys |
| XP_002587801.1 | Branchistostoma floridana | Cys | Cys |
| XP_002807287.1 | Callithrix jacchus | Cys | Cys |
| XP_547837.1 | Canis familiaris | Cys | Cys |
| XP_002192543.1 | Ciona intestinalis | Cys | Cys |
| XP_002124652.1 | Ciona intestinalis | Cys | Cys |
| XP_694147.1 | Danio rerio | Cys | Cys |
| XP_001492119.2 | Equus caballus | Cys | Cys |
| XP_421428.2 | Gallus gallus | Cys | Cys |
| XP_381905.1 | Gibberella zeae | Cys | Cys |
| XP_388061.1 | Gibberella zeae | Cys | Cys |
| NP_653182.1 | Homo sapiens | Cys | Cys |
| XP_002160119.1 | Hydra magnipapillata | Cys | Cys |
| XP_001094065.1 | Macaca mulatta | Cys | Cys |
| XP_001377162.1 | Monodelphis domestica | Cys | Cys |
| NP_080314.1 | Mus musculus | Cys | Cys |
| XP_003042302.1 | Nectria haematococca | Cys | Cys |
| XP_003049610.1 | Nectria haematococca | Cys | Cys |
| XP_003052914.1 | Nectria haematococca | Cys | Cys |
| XP_003040311.1 | Nectria haematococca | Cys | Cys |
| NP_003042353.1 | Nectria haematococca | Cys | Cys |
| NP_003044531.1 | Nectria haematococca | Cys | Cys |
| NP_003043876.1 | Nectria haematococca | Cys | Cys |
| NP_003043680.1 | Nectria haematococca | Cys | Cys |
| NP_001627872.1 | Nemastella vectensis | Cys | Cys |
| NP_001622857.1 | Nemastella vectensis | Cys | Cys |
| NP_002166333.1 | Neosartorya fischeri | Cys | Cys |
| NP_002367893.1 | Nomascus leucogenys | Cys | Cys |
| NP_002723072.1 | Oryctolagus cuniculus | Cys | Cys |
| NP_00099803.1 | Pan troglodytes | Cys | Cys |
| NP_002556931.1 | Penicillium chrysogenum | Cys | Cys |
| NP_002556863.1 | Penicillium chrysogenum | Cys | Cys |
| NP_002488861.1 | Phaeosphaeria nodorum | Cys | Cys |
| NP_001153067.1 | Penicillium marneffei | Cys | Cys |
| NP_001729928.1 | Phaeosphaeria nodorum | Cys | Cys |
| NP_001125373.1 | Ponga abelia | Cys | Cys |
| NP_001101501.1 | Russula norvegica | Cys | Cys |
| NP_002734182.1 | Saccoglossus kowalevskii | Cys | Cys |
| NP_002734190.1 | Saccoglossus kowalevskii | Cys | Cys |
| NP_002734189.1 | Saccoglossus kowalevskii | Cys | Cys |
| NP_00790644.1 | Strongylentrotus purpuratus | Cys | Cys |
| NP_00798171.2 | Strongylentrotus purpuratus | Cys | Cys |
| NP_002200408.1 | Taeniopygia guttata | Cys | Cys |
| NP_002487532.1 | Talaromyces stipitatus | Cys | Cys |
| NP_00121871.1 | Trypanosoma cruzi | Cys | Cys |
| NP_003162.1 | Trypanosoma cruzi | Cys | Cys |
| NP_001008128.1 | Xenopus (Silurana) tropicalis | Cys | Cys |
in this inventory do not catalyze a racemization reaction. A few general patterns can be recognized in the phylogenetic tree (Fig. 1). Nearly all animals contain a single gene of the Cys-Thr type, except the marine animals, which contain both a Cys-Thr and a Cys-Cys protein. The kinetoplastid *T. cruzi* contains two members of the Cys-Cys type. Proteins of the Ser-Cys type appear to be unique to fungi. In addition, both Cys-Thr-type and Cys-Cys-type proteins occur in fungi, often in the same species. In this inventory, the only exceptions to these patterns are *Ciona intestinalis* (a sea squirt), which despite being a marine animal appears to contain only two proteins of the Cys-Cys variant, and *Hydra magnipapillata* (a hydra), which contains only a Cys-Thr-type protein.

Cloning and Recombinant Expression of Human C14orf149—Interestingly, like that of most animals, the human genome also contains a gene encoding a proline racemase-like protein of the Cys-Thr type. It is annotated as a “probable proline racemase” or “hydroxyproline-2-epimerase,” although the enzyme is of the Cys-Thr class and therefore lacks one of the cysteine residues known to be critical for the racemization/epimerization reaction. To investigate the enzymatic activity of this protein, we expressed a GST fusion construct of the human protein in *E. coli*. The protein was purified to >95% purity (Fig. 2). Under denaturating SDS-PAGE conditions, the protein migrates as a monomer of the expected molecular mass, 67 kDa (the GST tag contributes 29 kDa).
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Substrate of Human C14orf149—Goitia et al. (14) identified three residues that are involved in substrate recognition, allowing them to distinguish between enzymes that act on proline from those that act on hydroxyproline, at least in a limited set of prokaryotic and kinetoplastid proline racemase–like proteins. However, in the majority of the eukaryotic members of this family examined here, including human C14orf149, different amino acids are present at the corresponding positions, which makes predictions regarding the substrate of these enzymes difficult. Therefore, the activity of purified GST-C14orf149 was experimentally evaluated with a wide range of isomers of proline and hydroxyproline. Interestingly, only trans-3-hydroxy-L-proline was rapidly consumed upon incubation with GST-C14orf149, whereas no reaction was observed with any of the other potential substrates (Fig. 3A). A slight increase in the concentrations of these substrates was observed, which might be attributed to slight evaporation of the reaction mixtures. To test whether C14orf149 can also act on trans-3-hydroxy-L-proline when it is present in a peptide, three different fragments of collagen known to contain this amino acid were incubated with C14orf149. However, in this case, no reaction was observed (supplemental Fig. S1).

Product of trans-3-Hydroxy-L-proline Degradation by C14orf149—When free trans-3-hydroxy-L-proline was incubated with C14orf149, the expected product of the 2-epimerase reaction, cis-3-hydroxy-D-proline, was not formed (Fig. 3B), indicating that the enzyme does not catalyze an epimerase reaction. To investigate the nature of the product of trans-3-hydroxy-L-proline degradation, the reaction mixture was monitored by mass spectrometry. The reaction was terminated at different time points and infused into the MS instrument directly. MS scans were made in the range m/z = 2–650. In the reaction mixture containing trans-3-hydroxy-L-proline as the substrate, the formation of a new peak was observed at m/z = 114 (Fig. 4A). This peak did not appear in reactions containing any of the other proline or hydroxyproline isomers (not shown) or in control reactions performed in the absence of enzyme (not shown). Because the (S)-NIFE reagent used for the chiral derivatization contributes a mass of 250 Da to the product and its derivatization products generally fragment to yield an ion with m/z = 120.1, which was not observed, the product with m/z = 114 did not appear to have reacted with the (S)-NIFE reagent. This implied that it did not contain an available amino group. To gain further information with respect to the identity of this compound, its mass was determined with 10-ppm accuracy using an LTQ orbitrap XL mass spectrometer (Thermo scientific, Breda, The Netherlands). The m/z of the compound was found to be 114.05445, which is consistent with the ion [C₇H₇O₂N + H⁺] (expected m/z = 114.055504). These results led us to hypothesize that the product of the reaction could be Δ¹-pyrroline-5-carboxylate (Pyr5C) or Pyr2C, both known intermediates of proline and hydroxyproline metabolism (16). These two compounds can be distinguished on the basis of their reaction with hydrogen peroxide (17). Pyr2C reacts with H₂O₂ to yield γ-aminobutyric acid (GABA) in nearly quantitative yield, whereas the reaction with Pyr5C yields small amounts of glutamate, but no GABA. Therefore, a sample of the reaction mixture was incubated with hydrogen peroxide, and the formation of GABA and glutamate was measured by UPLC-MS/MS. As shown in Fig. 4B, the reaction with H₂O₂ exclusively yielded...
GABA, suggesting that the product of the degradation of trans-3-hydroxy-L-proline by C14orf149 is Pyr2C. To further confirm the identity of the product, authentic Pyr2C was prepared by the reaction of D-proline and D-amino acid oxidase (18, 19).

FIGURE 4. Identification of reaction product of trans-3-hydroxy-L-proline degradation by C14orf149. A, mass spectra of a reaction mixture containing C14orf149 and trans-3-hydroxy-L-proline, immediately after the addition of substrate and after 30 min of incubation. A newly formed peak at m/z = 114 is marked with an asterisk. Isomers of hydroxyproline have m/z = 381. The prominent peak at m/z = 224 is a side-product of the derivatization reaction. The inset shows the area around m/z = 114 in more detail. B, formation of GABA, but not glutamate, after incubation of the product with H2O2. Shown is a parent scan chromatogram from the reaction mixture after incubation with H2O2 showing the parents of m/z = 120.1. The expected retention times for GABA and glutamate are indicated.

FIGURE 5. Confirmation of enzymatic product as Δ1-pyrroline-2-carboxylate. A, fragmentation spectra of authentic Δ1-pyrroline-2-carboxylate and that of the m/z = 114 product of trans-3-hydroxy-L-proline degradation by C14orf149. The spectra were recorded at a collision energy of 15 eV and cone voltage of 24 V. B, chromatograms of authentic Δ1-pyrroline-2-carboxylate and of the product of trans-3-hydroxy-L-proline degradation by C14orf149. The chromatograms were recorded using the 114 > 96 multiple reaction monitoring transition. C, the proposed reaction mechanism for the dehydration of trans-3-hydroxy-L-proline catalyzed by C14orf149.
are highly similar, confirming the identity of the product. The chromatographic profiles of the authentic Pyr2C and the C14orf149 reaction mixtures were also compared, with the MS instrument set up to monitor the 114.05/H1102296.0 fragmentation reaction. As shown in Fig. 5B, the chromatographic elution profile of authentic Pyr2C matches that of the product of the C14orf149 reaction. The chromatogram exhibits two peaks with retention times of 0.56 and 4.31 min, which might be due to the spontaneous interconversion between Pyr2C and 5-aminooxy-pentanoic acid. A third peak is observed, which elutes at 0.91 min, but this peak is present even in blank samples containing only water and derivatization agent and therefore unrelated to Pyr2C or the product of the C14orf149 reaction. Taken together, these experiments suggest that human C14orf149 operates according to the mechanism depicted in Fig. 5C, catalyzing the dehydration of trans-3-hydroxy-L-proline to Δ1-pyrroline-2-carboxylate.

Other Cys-Thr-type Enzymes—To test whether other proline racemase-like proteins of the Cys-Thr type also catalyze a dehydratase reaction, we investigated the enzymatic activity of M. musculus C14orf149 (NCBI accession ID NP_080314.1) and S. kowalevskii LOC100374014 (XP_002734182.1). The M. musculus protein is orthologous to the human protein, whereas the S. kowalevskii protein is only distantly related (Fig. 1). GST fusion proteins were expressed in E. coli, purified, and incubated with different isomers of proline and hydroxyproline. As observed for the human enzyme, only trans-3-hydroxy-L-proline was consumed by these enzymes (Fig. 6A), demonstrating that they can utilize the same substrate. The expected product of the 2-epimerization reaction, cis-3-hydroxy-D-proline, was again not formed in these reactions (Fig. 6B). Instead, as found for human C14orf149, the product formed was Pyr2C, as evidenced by the appearance of GABA upon incubation with H2O2. The expected retention times for these amino acids are indicated.

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for human C14orf149, the product formed was Pyr2C, as evidenced by the appearance of GABA upon incubation of the reaction mixture with H2O2 (Fig. 6C).

Enzymatic Parameters of Human C14orf149—Accurate quantification of Pyr2C using UPLC-MS/MS was hindered by its elution in two asymmetric peaks. Therefore, a colorimetric assay was developed to enable accurate kinetic measurements. The assay is based on the reaction of o-aminobenzaldehyde with Pyr2C (18, 19), which yields a colored reaction product (\(\lambda_{\text{max}} = 425 \text{~nm}, e = 1045 \text{~mol}^{-1} \text{~cm}^{-1} \text{~in 3% trichloroacetic acid solution} \)). Using this assay, the kinetic parameters of C14orf149 were established. Fig. 7A shows the pH dependence of the enzyme. The Michaelis-Menten parameters of the enzyme were obtained by measuring the initial reaction rate at different substrate concentrations. A Lineweaver-Burk plot calculated from these data is shown in Fig. 7B. From these results, the \(K_m\) and \(V_{\text{max}}\) were calculated and found to be 7.23 mm and 39.5 μmol/min/mg, respectively.
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Tissue Distribution of Human C14orf149—The distribution of C14orf149 transcripts was examined in a panel of cDNA samples from 16 different human tissues (Fig. 8) using PCR. A product of the expected size was observed in all tissue samples, suggesting that human C14orf149 is ubiquitously expressed.

Racemase Activity after Introduction of a T273C Mutation—We hypothesized that the conversion of trans-3-hydroxy-L-proline to Δ1-pyrroline-2-carboxylate occurs by abstraction of a hydrogen from the α-carbon of the substrate followed by spontaneous hydrolysis and tautomerization of the intermediate product (Fig. 5C) (20). It is then conceivable that upon reintroducing the second cysteine in the enzyme, lost in the evolutionary process, the enzyme might regain racemase activity. Therefore, we constructed a T273C mutant of human C14orf149 and incubated the purified, N-terminally GST-tagged mutant enzyme with trans-3-hydroxy-L-proline. As shown in Fig. 9A, the T273C mutant indeed yields the isomer expected from racemization, cis-3-hydroxy-D-proline. No Pyr2C could be detected in the reaction mixture, either by UPLC-MS/MS or spectrophotometrically (after the addition of o-aminobenzoic acid), indicating that the mutant enzyme has lost its original dehydratase activity. The substrate is consumed by the mutant enzyme at a much lower rate (3.48 μmol/mg/min, or 8.8% of the wild type). Surprisingly, the mutant enzyme also catalyzed the racemization of L-proline to D-proline (Fig. 9B), albeit at a very low rate of 0.57 μmol/mg/min.

Conclusion—On the basis of two specific residues in the active site, located at positions 145 and 364 in our sequence alignment, three types of eukaryotic proline racemase-like genes can be discerned. The first type contains cysteine residues at both the 145 position and the 364 position (Cys-Cys type). All characterized members of this type are racemases that catalyze dehydration reactions. To the best of our knowledge, this is the first study describing the enzymatic activity of proline racemase-like proteins of this type. It is also the first identification of a human protein that acts on trans-3-hydroxy-L-proline. It appears likely that C14orf149 serves to catalyze the racemization of L-proline to D-proline, and the shown area of the L-proline peak is therefore not proportional to its concentration.

Both trans-4-hydroxy-L-proline and trans-3-hydroxy-L-proline result from collagen breakdown, but they are degraded via different pathways. Pyr2C reductase (Pyr2C reductase) activity has been demonstrated in mammals (28, 29), but the gene encoding the enzyme has not yet been identified.
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this protein is critical for its conformation and function (24). In this context, it is of interest to note that two independent studies (25, 26) have linked the genomic locus containing C14orf149 (14q23.1) to the occurrence of aneurysms. Possibly, alterations (25, 26) have linked the genomic locus containing this context, it is of interest to note that two independent stud-

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