厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

遺伝性鉄芽球性貧血の診断分類と治療法の確立

ALAS2 遺伝子変異の評価法に関する研究

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研究要旨：赤芽球特異的アミノレブリン酸合成酵素(ALAS2)の遺伝子変異は X 染色体連鎖鉄芽球性貧血(XLSA)の原因となる事が以前より知られていたが、近年、ALAS2 遺伝子の機能獲得型の変異は X 染色体連鎖プロトポルフィリン症（XLPP）の原因となる事が報告された。今のところ機能獲得型の変異についての報告は多くはないが、今後 ALAS2 遺伝子に変異が同定された場合、その変異が機能喪失型であるのか、機能獲得型の変異であるのを明らかにする事は鑑別診断の上からも重要である。本研究では変異タンパク質を組換タンパク質として大腸菌を用いて発現させ、Tag-free の状態で精製した後に in vitro における酵素活性を測定する方法を確立した。また、in vitro の酵素活性の測定のみでは判定できない場合には、ヒト由来の培養細胞を用いて、in vivo における酵素活性を測定する方法を確立した。

A. 研究目的

アミノレブリン酸合成酵素(5-aminolevulinate synthase; ALAS)はヘム生合成系の初発反応を触媒する酵素で、ヒトでは全ての細胞で発現する非特異的 ALAS(ALAS1 または ALAS-N)と赤芽球にのみ特異的に発現し赤血球にけるヘモグロビン合成に必要なヘムを供給する役割を持つ赤芽球特異的 ALAS(ALAS2 または ALAS-E) の２種類のアイソザイムが存在する。ヒトの ALAS1 遺伝子は三番染色体に、また ALAS2 遺伝子は X 染色体上に map されており、それぞれのアイソザイムは異なる発現調節を受ける事が知られている。疾患に関連しては、ALAS1 遺伝子の遺伝的変異により発症する疾患は報告されていないが、ALAS2 遺伝子の異常は X 染色体連鎖鉄芽球性貧血 (X-linked sideroblastic anemia)の原因となる事が知られていた。しかしながら、近年、ALAS2 遺伝子の変異が伴性優性の遺伝形式をとる骨髄性プロトポルフィ

リン症 (X-linked dominant protoporphyria; XLDP)の原因となる事が報告された。XLSA は ALAS2 の機能喪失型の変異、XLDP は ALAS2 の機能獲得型の変異により発症すると考えられるが、変異の部位のみからは、それを区別する事はできない。従って、本研究では ALAS2 遺伝子の変異が機能喪失型の変異であるのか、あるいは機能獲得型の変異であるのかを明らかにする方法を確立する事を目的とした。

B. 研究方法

1. in vitro 酵素活性の測定

まず、ALAS2 タンパク質を組換えタンパク質として大腸菌で発現させ、精製して酵素活性を測定した。機能獲得型の変異はカルボキシル末端(C 末端)に近い領域で同定されている事、更に同じ領域では機能喪失型の変異も同定されている事から、組換えタンパク質も発現や精製の過程で C 末端が保存されている必要があると考え、そのような条件を満たすシステムとして New England BioLab 社の Impact system を使用した。本システムでは目的タンパク質の C 末端側に Intein-tag と Chitin binding domailn (CBD)を融合タンパク質として発現させ、CBD が結合する Chitin カラムを用いて精製した後に、dithiothreitol(DTT)を用いて Intein の自己切断機能を誘導する事により、目的タンパク質の C 末端側のアミノ酸配列に影響を与える事なく tag を有しない組換えタンパク質を得る事が可能となる。実際には、野生型 ALAS2 cDNA や、患者で同定された ALAS2 変異を点変異導入法により挿入した変異型 cDNA を pTBX ベクターに組み込み、それらのベクターを用いて大腸菌株 BL21(DE3)を形質転換した後に成熟型 ALAS2-Intein-CBD 融合タンパク質の発現を誘導する。次に超音波装置を用いて大腸菌を破砕し、可溶化した組換えタンパク質を Chitin ビーズを用いて精製し、DTT により Intein の自己切断活性を誘導し成熟型 ALAS2 の C 末端を Intein の融合部から切り離すことにより Tag-free の組換え成熟型 ALAS2 タンパク質を得る。それらを用いて in vitro における ALAS 酵素活性を測定した。

2. in vivo 酵素活性の測定

次に、in vivo おける酵素活性を評価する実験系の構築を、Invitrogen 社の Flp-In T-Rex system を用いて試みた。それぞれの変異体の酵素活性を比較するためには、異なる細胞内で同程度に野生型あるいは変異型 ALAS2 を発現させる必要があるが、このシステムを用いると、HEK293 細胞の genome DNA の中の遺伝子発現が抑制されない特定の領域に目的遺伝子を組み込む事ができ (Flp-In system)、また、tetracyclin (Tet)により制御可能なプロモーターを利用して目的遺伝子

の発現量を制御する事が可能となる(T-Rex system)。従って、それぞれの変異遺伝子の mRNA の発現量を調節して同程度に揃える事ができ、転写レベルでの差を考慮する事なく、それぞれの酵素の in vivo における活性を比較する事が可能になる。ALAS2 タンパク質を HEK293 細胞で強制発現させると、酵素活性に応じてポルフィリン体が蓄積するが、蓄積したポルフィリン体は紫外線照射下で特徴的なピンク色の蛍光を発する。その蛍光の強度を比較する事により in vivo における酵素活性の比較とした。また、これらの細胞を用いて野生型や変異型の ALAS2 酵素を発現させた後にシクロヘキシミド(CHX)を培養液中に添加して翻訳を抑制し、その後系時的に試料を調製して ALAS2 タンパク質の量がどの様に変化するかを Western blot 法で検出する事により、ALAS2 タンパク質の細胞内におけるおおよその半減期も測定した。

(倫理面への配慮)

本研究における鉄芽球性貧血患者とその家族からの検体の採取にあたっては、東北大学医学部倫理委員会の審査を受け許可されたプロトコールに基づき、本人または親権者の同意を書面で得た後に実施された。

C. 研究結果

1. 大腸菌を用いた組換えタンパク質の精製

野生型および変異型の ALAS2 タンパク質を発現させるためのベクター(野生型酵素の発現ベクターは pTBX-ALAS2) を用いて BL21(DE3)大腸菌を形質転換し、IPTG を用いて発現誘導をした後に、大腸菌を溶菌バッファーの中で超音波を用いて破砕し、遠心して得られた上清（可溶性画分）を Chitin beads を用いて精製し、DTT により Intein の自己切断機能を活性化する事により CBD-Intein tag を取り除いた。精製した組換えタンパク質の純度を、SDS-PAGE 後のアクリルアミドゲルをタンパク染色する事により検討したところ、予想される部位にほぼ単一のバンドが検出された。その試料の酵素活性を測定したところ、生体から抽出された天然型 ALAS タンパク質と同等の酵素活性が得られる事を確認した。同様にして得られた変異型 ALAS2 タンパク質の酵素活性を測定したところ、R170C, R170L, M567I 変異を有する組換えタンパク質の酵素活性は野生型の約 1/3 以下に低下していたが、V562A の変異を有する酵素の活性は野生型に比べて同等以上に亢進していた。

2. in vivo における酵素活性の測定。

次に、M567I と V562A 変異酵素の細胞内における半減期を野生型と比較したところ、M567I 変異酵素

の半減期は野生型と比べて延長していたのに対し、V562A 変異酵素の半減期は野生型と比較して短縮していた。次にポルフィリン体の発する蛍光を指標として in vivo における酵素活性を定性的に比較したところ、V562A 変異と M567I 変異のいずれの変異体の酵素活性も野生型に比較して低下していた。特に M567I の in vivo における酵素活性の低下は顕著であった。

D. 考察

以前からの検討結果も含めると、X 染色体連鎖鉄芽球性貧血患者で同定された ALAS2 遺伝子変異のうち、多くの変異は in vitro の酵素活性が低下することが明らかになった。また、そのような変異体は in vivo における半減期が野生型に比較して延長するような場合でも、in vivo における酵素活性はほとんど常に野生型より低下していると考えてよい事が明らかになった。このことは、タンパク質寿命の延長によっても酵素活性の低下を補填する事はできない事を示唆している。すなわち、in vitro における酵素活性が低下することが検出できた場合には、その変異は機能喪失型の変異と考えてよいものと思われる。

一方、in vitro における酵素活性が野生型と同等以上である場合でも、in vivo におけるタンパク質の半減期が短縮し細胞内における酵素活性は低下する場合がある事が明らかになった。従って、in vitro の酵素活性が亢進している場合には、in vivo における酵素活性を野生型と比較する事で、当該変異が機能喪失型であるのか、真に機能獲得型であるのかを判定することが重要であると考えられた。

E．結論

近年、ALAS2 遺伝子の機能獲得型の変異により赤芽球性ポルフィリン症を発症する事が報告された。従って、ALAS2 遺伝子の変異を同定した場合、その変異が ALAS2 タンパク質の機能を喪失させるのか、あるいは機能を亢進させるのかを明らかにする事は、疾患の診断の上からも重要である。本研究により、ALAS2 変異が機能喪失型の変異であるのか、あるいは機能獲得型の変異であるのかを系統立てて明らかにする方法を確立した。

F. 研究発表

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2. 第 73 回日本血液学会総会（平成23年10月14-16日／名古屋国際会議場／名古屋市）Rie Ohba, Kazumichi Furuyama, Atsushi Manaba, Etsuro Ito, Seiji Kojima, Keiya Ozawa, Hideo Harigae「Characteristics of sideroblastic anemia in Japan-from the analysis of multicenter study」(口演)
3. 第53回アメリカ血液学会総会（平成23年12月10-13日／サンディエゴコンベンションセンター／アメリカ合衆国サンディエゴ市）Kiriko Kaneko, Akiko Shibasaki, Hiroshi Nishiyama, Takuo Hirose, Koji Ohba, Kazuhito Totsune, Kazumichi Furuyama and Kazuhiro Takahashi「Expression of (pro)renin receptor in human erythroid cell lines: the effect of interferon-gamma」（ポスター）

G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   該当無し

2. 実用新案登録
   該当無し

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

遺伝性鉄芽球性貧血の診断分類と治療法の確立

小児科領域の調査研究
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研究要旨： 日本小児血液学会は平成21年2月より再生不良性貧血(AA)、骨髄異形性症候群(MDS)および先天性造血不全症候群(CBFS)を対象とした中央診断を開始した。レビュー開始から平成23年8月までに500例がレビューされた。レビュー結果より鉄芽球性貧血の症例が3例みつかった。遺伝子解析は未施行であるが、そのうちの1例はビタミン B6投与により貧血の改善がみられた。中央診断を行うことにより極めて希少とされる遺伝性鉄芽球性貧血の症例の発掘につながるものと考えられる。

A. 研究目的

鉄芽球性貧血は、骨髄に環状鉄芽球という特徴的な赤芽球の出現を特徴としている。この環状鉄芽球は赤芽球のミトコンドリアと呼ばれる器官への鉄の異常蓄積により形成される。鉄芽球性貧血は、遺伝性鉄芽球性貧血と後天性鉄芽球性貧血に大別されるが、遺伝性鉄芽球性貧血は患者数が少ないために、調査研究がほとんど行われたことがなく、その疫学や病態は現在でもわかっていない。遺伝性鉄芽球性貧血は、X 連鎖性鉄芽球性貧血（XLSA）と呼ばれているが、発症家系が約 40 家系と少なく、希少疾患であるため、その発症頻度や病態についての詳細な調査・解析はなされていなかった。XLSA として正確に診断されるようになれば、ビタミン B6 の投与により貧血が改善する可能性があり、正確な診断は極めて重要である。

E. 研究方法

2009 年 2 月より、日本小児血液学会においては再生不良性貧血(AA)、骨髄異形性症候群(MDS) および先天性骨髄不全症候群(CBFS)を対象とした中央診断を開始しており、XLSA も施設での診断のみでなく、中央診断されるようになった。中央診断事務局を名古屋大学小児科に設置した。AA、MDS、あるいは CBFS が疑われる症例が発生した場合は、各施設から事務局に連絡をもらい、登録番号を発行した。レビューは骨髄および末梢血塗抹標本を 2 施設（名古屋大学小児科、聖路加国際病院小児科）で、骨髄病理標

本を1施設（名古屋第一赤十字病院病理部）で行った。

（倫理面への配慮）

中央診断およびそれに伴う検査については、名古屋大学医学部倫理委員会の承認を得、患者及び患者保護者の同意を取得した後に行った。

F. 研究結果

平成21年2月から平成23年8月までに500例がレビューされ、鉄芽球性貧血が3例含まれていた。遺伝子解析は未施行である。このうちの1例は生後10か月で小球性低色素性貧血を発症した。当初鉄欠乏性貧血として、鉄剤の投与を行われたが、貧血は改善せず、骨髄検査を行うこととなった。骨髄は低形成で、赤芽球の異形成は乏しかったが、鉄染色にて環状鉄芽球がわずかに認められた。遺伝性鉄芽球性貧血を疑い、ビタミン B6を投与したところ、貧血の改善がみられた。遺伝子解析は今後行っていく予定である。

G. 考察

遺伝性鉄芽球性貧血の本邦での報告例は非常に少なく、まだ病態について明らかになっていない。今回見つかった例のように他の貧血と診断されている例もあるものと考えられる。また形態学的診断も困難を極め、中央診断が重要と考えられる。今後は症例の集積を行い、あわせて遺伝子診断も確定診断の上から非常に重要と考えられる。

H. 結論

中央診断を行うことにより、鉄芽球性貧血が3例見つかった。そのうちの1例はビタミン B6に反応し、遺伝性鉄芽球性貧血が疑われた。今後も中央診断を継続していく中に、鉄芽球性貧血が新規診断され症例が蓄積されていくものと考えられる。

F. 研究発表

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G. 知的財産権の出願・登録状況

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌【欧文】

著者名	論文タイトル名	雑誌名	巻・号・ページ・出版年
Kadirvel S, Furuyama K, Harigae H, Kaneko K, Tamai Y, Ishida Y, Shibahara S.	The carboxyl-terminal region of erythroid-specific 5-aminolevulinate synthase acts as an intrinsic modifier for its catalytic activity and protein stability.	Experimental Hematology	40(6):477-486.2012
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Ⅳ. 研究成果の刊行物、別冊

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The carboxyl-terminal region of erythroid-specific 5-aminolevulinate synthase acts as an intrinsic modifier for its catalytic activity and protein stability

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Erythroid-specific 5-aminolevulinate synthase (ALAS2) is essential for hemoglobin production, and a loss-of-function mutation of ALAS2 gene causes X-linked sideroblastic anemia. Human ALAS2 protein consists of 587 amino acids and its carboxyl(C)-terminal region of 33 amino acids is conserved in higher eukaryotes, but is not present in prokaryotic ALAS. We explored the role of this C-terminal region in the pathogenesis of X-linked sideroblastic anemia. In vitro enzymatic activity was measured using bacterially expressed recombinant proteins. In vivo catalytic activity was evaluated by comparing the accumulation of porphyrins in eukaryotic cells stably expressing each mutant ALAS2 tagged with FLAG, and the half-life of each FLAG-tagged ALAS2 protein was determined by Western blot analysis. Two novel mutations (Val562Ala and Met567Ile) were identified in patients with X-linked sideroblastic anemia. Val562Ala showed the higher catalytic activity in vitro, but a shorter half-life in vivo compared to those of wild-type ALAS2 (WT). In contrast, the in vitro activity of Met567Ile mutant was about 25% of WT, while its half-life was longer than that of WT. However, in vivo catalytic activity of each mutant was lower than that of WT. In addition, the deletion of 33 amino acids at C-terminal end resulted in higher catalytic activity both in vitro and in vivo with the longer half-life compared to WT. In conclusion, the C-terminal region of ALAS2 protein may function as an intrinsic modifier that suppresses catalytic activity and increases the degradation of its protein, each function of which is enhanced by the Met567Ile mutation and the Val562Ala mutation, respectively. © **2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

5-Aminolevulinate synthase (ALAS) is the first and rate-limiting enzyme in the heme biosynthetic pathway [1]. There are two isozymes of ALAS in higher eukaryotes, ALAS1 and ALAS2. ALAS1 (alternatively, ALAS-N) is expressed ubiquitously in all types of nucleated cells, and expression of ALAS2 (or ALAS-E) is restricted in erythroid cells and essential for hemoglobin production during erythroid differentiation [1]. Both ALAS1 and ALAS2, which are encoded by the distinct nuclear genes, function in mitochondria [2,3], and the amino-terminal region of each isozyme acts as a targeting signal for mitochondrial translocation [4–6]. The remaining regions of ALAS protein consist of a core catalytic region and a carboxyl terminal (C-terminal) region, and the catalytic region is conserved among several species [7]. In addition, the C-terminal region of 33 amino acids (positions 555–587), which is encoded by the 11th exon of the human ALAS2 gene, is well conserved in higher eukaryotes, but the equivalent region is not present in bacterial ALAS [7]. It is conceivable that the C-terminal region of mammalian ALAS2 protein might have an important regulatory role in heme biosynthesis.

The human ALAS2 gene that is mapped on X chromosome consists of 11 exons [8], and a genetic mutation of the ALAS2 gene causes X-linked sideroblastic anemia (XLSA) [9,10] or X-linked dominant protoporphyria [11]. To the best of our knowledge, >50 different mutations of

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the ALAS2 gene have been identified in about 100 pedigrees with XLSA [12–14]. Reported mutations in patients with XLSA are distributed from the 5th exon to the 11th exon of the human ALAS2 gene, but only four mutations were detected in 11th exon [14–17]. In the case of X-linked dominant protoporphyria, two different frame-shift mutations have been identified in the 11th exon of the ALAS2 gene in two independent probands [11]. These frame-shift mutations cause deletions of 19 and 21 amino acids at the C-terminal end of ALAS2, both of which are accompanied by replacement of the C-terminal end with one unrelated amino acid and an unrelated peptide of 23 amino acids, respectively. Using recombinant proteins expressed in *Escherichia coli*, those authors provided evidence that deletion of 19 or 21 amino acids at C-terminal end increased the catalytic activity of ALAS2, suggesting that the C-terminal region can inhibit the enzymatic activity of ALAS2 [11]. Recently, it was also reported that the substitution (Tyr586Phe) at the penultimate amino acid of the C-terminal of ALAS2 increased its catalytic activity in vitro, which might be related to the severe phenotype of congenital erythropoietic porphyria [18]. Interestingly, such gain-of-function mutations of the ALAS2 gene were solely identified within the C-terminal region of ALAS2 protein. However, it is still unclear how the C-terminal region of ALAS2 is involved in the regulation of ALAS2 function in vivo.

Here, we report novel missense mutations in the 11th exon of the ALAS2 gene in independent probands with XLSA. Based on in vitro and in vivo functional studies of these mutants, as well as a C-terminal deletion mutant, we provide evidence that the C-terminal region of human ALAS2 protein reduces its catalytic activity and protein stability in mitochondria.

Case reports

Case 1

Japanese male proband presented with microcytic hypochromic anemia (hemoglobin: 8.1 g/dL; mean corpuscular volume: 57.7 fL) at age 14 years. Serum ferritin, serum iron, and total iron binding capacity were 222.7 ng/mL, 242 μg/dL, and 279 μg/dL, respectively. Proband's mother and maternal uncles had mild anemia, but they did not receive any medication for anemia.

Bone marrow examination of the patient showed erythroid hyperplasia (myeroid to erythroid ratio [M:E] = 0.45), with ringed sideroblasts comprising >10% of nucleated cells. Pyridoxine treatment (80 mg/d) was started, and the hemoglobin concentration gradually increased from 7.3 g/dL to 12.0 g/dL after 14 months.

Case 2

Japanese male proband was admitted to the hospital at age 36 years because of microcytic hypochromic anemia (hemoglobin: 6.5 g/dL; mean corpuscular volume: 64.4 fL) with systemic iron overload (ferritin: 2581.4 ng/mL). Anemia was pointed out before he was school age, but he did not receive any medication for anemia. Prussian blue staining of bone marrow cells revealed the presence of ring sideroblasts in the proband, and the diagnosis of sideroblastic anemia was established. Pyridoxine treatment (60 mg/d) was started when hemoglobin was 5.4 g/dL, then anemia was improved after 1 month to 9.9 g/dL hemoglobin. Although pyridoxine treatment was continued for an additional 4 months, the hemoglobin level did not exceed 10 g/dL.

Materials and methods

Reagents

Chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), Nacalai Tesque (Kyoto, Japan), or Wako Pure Chemicals (Osaka, Japan). Restriction enzymes and modifying enzymes used for construction of each plasmid were purchased from New England Biolabs (Ipswich, MA, USA), unless otherwise noted. ExTaq DNA polymerase and PrimeStar Max DNA polymerase were purchased from Takara Bio Inc. (Shiga, Japan) and were used for polymerase chain reaction (PCR) and site-directed mutagenesis, respectively. Protein concentration was measured with Bio-Rad Protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) or Pierce 660 nm Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and Western blot analysis were performed as described previously [19]. Prestained XL-ladder broad range (APRO Science, Tokushima, Japan) was loaded as a size marker for SDS-PAGE and Western blot analysis.

Identification of ALAS2 mutations

Genetic analyses performed in this project had been approved by the ethical committee of Tohoku University School of Medicine. Blood samples were drawn from the probands and the family members after informed consent. Genomic DNA was then extracted from them using QIAamp DNA Blood Midi Kit (Qiagen GmbH, Hilden, Germany). All exons including exon-intron boundaries, the proximal promoter region, and the erythroid enhancer in intron 8 of ALAS2 gene were amplified using ExTaq DNA polymerase. Sequences of primers and the condition for PCR were reported previously [20], except for an antisense primer for exon 5 and a primer pair for the erythroid-specific enhancer region in intron 8. The sequence of antisense primer for exon 5 used is (5′-TCATCTCCTCTGGCCACTGC-3′). For the amplification of the erythroid-specific enhancer in intron 8, the following primers were used: sense, 5′-GGTACCACTCGCATCCCACTGCA GAG-3′and antisense, 5′-GGTACCACACAGCCAAAGGCCTT GCC-3′. Each amplified DNA fragment was electrophoresed on 1% agarose gel in TAE buffer and stained with ethidium bromide. DNA fragment was excised from the gel for purification using QIAquick Gel Extraction Kit (Qiagen GmbH). Purified DNA fragment was directly sequenced using BigDye terminator v1.1 cycle sequencing kit and ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The same primers were used for PCR and direct sequencing analysis. Sequencing results were

analyzed using Lasergene software (DNASTAR Inc., Madison, WI, USA), and the mutation of ALAS2 gene was confirmed by repeated amplification and direct sequencing.

Expression and purification of recombinant ALAS2 proteins

Complementary DNA for human mature ALAS2 that lacks the amino-terminal region was amplified with PrimeStar Max DNA polymerase (Takara Bio Inc.) using the following primers (sense, 5′-GGTGGTCATATGATCCACCTTAAGGCAACAAAGG-3′; antisense, 5′-GGCATAGGTGGTGACATACTG-3′), each of which was phosphorylated at its 5′ end beforehand. Amplified complementary DNA (cDNA) was digested with NdeI restriction enzyme, and was cloned between NdeI site and blunt ended SapI site of pTXB1 expression vector (New England Biolabs). Resulting plasmid, named as pTXB1-AEm, expresses human mature ALAS2 in *E. coli* as a fusion protein with Intein tag and Chitin binding domain at its C-terminal end. Using pTXB1-AEm as a template, each mutation or deletion was introduced using PrimeStar Max site-directed mutagenesis kit (Takara Inc.). The sequences of primers used for mutagenesis are available upon request. After the amplification of cDNA or mutagenesis, the sequence of mature ALAS2 cDNA and the junction sequence for fusion protein was confirmed by DNA sequencing before use. These expression vectors were used for transformation of the *E. coli* strain, BL21(DE3). Expression and purification of recombinant proteins were performed according to manufacturer's instruction for Impact System (New England Biolabs), with minor modifications. Briefly, expression of recombinant proteins was induced in *E. coli* with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at 25°C for overnight. The isopropyl β-D-1-thiogalactopyranoside–treated cells were collected by centrifugation and resuspended with lysis buffer (20 mM Tris-HCl [pH 8.5], 300 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL antipain, 1 μg/mL pepstatin, and 1 μg/mL leupeptin). After sonication and centrifugation, cleared cell lysates were incubated with Chitin beads for 1 hour at 4°C, and then washed with wash buffer (20 mM Tris-HCl [pH 8.5], 500 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). To obtain a tag-free recombinant mature ALAS2 protein, on-column cleavage was induced with 50 mM dithiothreitol in wash buffer at room temperature for 16 hours. After the elution from the column, each recombinant protein was dialyzed against wash buffer before use. Purity of each recombinant protein was examined using SDS-PAGE, followed by staining with Quick-CBB PLUS (Wako Pure Chemical). Enzymatic activity of each recombinant protein was measured according to the protocol described previously [21]. Student's *t* test was performed for statistical analysis.

Expression of wild-type or mutant ALAS2 protein in eukaryotic cells

The plasmid "pGEM-AET," which carries cDNA for full-length ALAS2 tagged with FLAG at its C-terminal, was described previously [22]. Site-directed mutagenesis was performed by PrimeStar Max mutagenesis kit (Takara Inc.) using pGEM-AET as a template to obtain cDNA encoding each FLAG-tagged mutant. In addition, cDNA encoding FLAG-tagged luciferase protein was constructed by replacing ALAS2 cDNA in pGEM-AET with amplified luciferase cDNA derived from pGL3 basic (Promega Corporation, Madison, WI, USA).

For establishing the stable transformants in which expression of FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein is inducible with tetracycline, cDNA for each protein was cloned into pcDNA5/FRT/TO vector (Invitrogen Corporation, Carlsbad, CA, USA). The resulting cDNA construct was then co-introduced with pOG44 vector into Flp-In T-REx 293 cells (Invitrogen), derived from human embryonic kidney cells (HEK293). After transfection, cells were incubated with 100 μg/mL Hygromycin B (Wako Pure Chemicals) and 15 μg/mL Blasticidin (Invitrogen). At least three independent clones, which were resistant to Hygromycin B and sensitive to Zeocin (Invitrogen), were selected and expanded for subsequent experiments. This phenotype of a given clone confirmed the integration of each cDNA expression cassette into the expected site in the genome of Flp-In T-REx 293 cell line.

For the determination of protein stability, expression of wild-type ALAS2 or mutant ALAS2 was induced by the addition of tetracycline into the culture medium (final concentration of 1 μg/mL) for 48 or 72 hours, and then the culture medium was replaced with fresh complete medium containing tetracycline with or without 10 μM cycloheximide. At 0, 3, 6, 9, and 12 hours after incubation, cells were harvested and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% TritonX-100, 1 mM sodium fluoride, 0.4 mM Na_3VO_4, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). Cell lysates were centrifuged at 13,200*g* for 10 minutes at 4°C, and the supernatants were used for SDS-PAGE. Expression of FLAG-tagged ALAS2 protein was detected by Western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) as a first antibody. For normalization of loaded samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with anti-GAPDH monoclonal antibody (MAB374; Millipore Corporation, Billerica, MA, USA) as a first antibody. For a second antibody, horseradish peroxidase–conjugated anti-mouse IgG (NA931Vl GE Healthcare, UK Limited, Buckinghamshire, UK) was used. Intensity of each band was measured using ImageJ software (available at http://rsb.info.nih.gov/ij/). The intensity of each band for FLAG-tagged ALAS2 was normalized with that of GAPDH, and the normalized intensity of FLAG-tagged ALAS2 at each time point was compared with that of the sample harvested at 0 hour. We repeated this series of experiments three times for each clone, and an average of these results was used for determination of the half-life of each protein.

The catalytic activity of each mutant protein was also evaluated by comparing the accumulation of porphyrins in Flp-In T-Rex 293 cells that expressed wild-type or mutant ALAS2 cDNA in an inducible manner. For this assay, cells of low-passage numbers (between passage 5 and passage 15) were used for obtaining reproducible results. To induce expression of wild-type ALAS2 or mutant ALAS2 protein in isolated cell lines, cells were treated for 60 hours with tetracycline at a suitable concentration (12.5–50 ng/mL), depending on cell lines. Then, cells were washed with phosphate-buffered saline twice and collected in the sample tube. Flp-In T-REx 293 cells, which express FLAG-tagged luciferase protein in an inducible manner, were also treated with tetracycline as a negative control. Cells were separately collected for Western blot analysis and RNA preparation. Real-time PCR analysis was performed as described previously [23]. Remaining cells were collected by centrifugation and then packed cells were exposed to ultraviolet light for detection of porphyrins.

Table 1. Summarized features of recombinant ALAS2 proteins

Recombinant protein	In vitro enzymatic activity (nmol ALA/mg protein/h)			Half-life in HEK293 cells (h)	Porphyrin accumulation in HEK293 cells
	Without PLP (% of wild-type)	With 200 μM PLP (% of wild-type)	Ratio with/without PLP		
Wild-type	14,824 ± 754 (100%)	27,627 ± 713 (100%)	1.86	7.8	+++
Val562Ala	22,324 ± 1,555 (150.6%)	32,300 ± 709 (116.9%)	1.44	5.3	++
Met567Ile	5,653 ± 897 (38.1%)	6,975 ± 299 (25.2%)	1.23	>12	±
Ser568Gly*	(19.5%)*	(31.6%)*	2.51*	>12	±
delC33	15,769 ± 382 (106.4%)	53,066 ± 1,843 (192.1%)	3.37	>12	++++

*Data with GST-fused Ser568Gly protein taken from reference [15].

Results

Identification of novel mutations of ALAS2 gene

Analyzing the genomic DNA extracted from the proband of case 1, we identified the c.T1685C mutation in the 11th exon of ALAS2 gene (Supplementary Figure E1A, upper panel; online only, available at www.exphem.org). This transition results in an amino acid substitution at the 562nd residue of ALAS2 protein from valine to alanine (Val562Ala). The same mutation was identified in one allele of the proband's mother (Supplementary Figure E1A, middle panel; online only, available at www.exphem.org), and the proband's father does not carry this mutation (Supplementary Figure E1A, lower panel; online only, available at www.exphem.org), indicating the X-linked inheritance of this mutation. For the proband of case 2, the c.G1701C transversion was identified in exon 11 of ALAS2 gene (Supplementary Figure E1B; online only, available at www.exphem.org), the mutation of which results in an amino acid substitution at the 567th residue from methionine to isoleucine (Met567Ile).

To exclude the possibility that these mutations represent single nucleotide polymorphisms, we examined the 11th exon of ALAS2 gene in 96 Japanese healthy volunteers (57 male and 39 female subjects, with the total allele number of 135) using PCR followed by direct sequencing. As a result, no base change was found in the 11th exon of ALAS2 gene in these subjects, suggesting that the mutation found in each proband might not represent single nucleotide polymorphism. It is therefore conceivable that either the c.T1685C or c.G1701C mutation might be responsible for XLSA.

Enzymatic activities of mutant ALAS2 proteins in vitro

Wild-type ALAS2 or each mutant ALAS2 protein was expressed in *E. coli* and purified as a tag-free protein. The combination of pTXB1 expression vector and IMPACT system allowed us to obtain a tag-free/C-terminal intact recombinant protein. Indeed, modified Coomassie Brilliant Blue staining of the gel after SDS-PAGE revealed that the purity of each prepared protein was >95% (data not shown). These recombinant proteins were suitable for determination of the catalytic activity of each mutant protein that carries the amino acid substitution near the C-terminal end.

We measured the catalytic activity of each recombinant ALAS2 protein with or without pyridoxal 5-phosphate (PLP). Data are summarized in Table 1. Unexpectedly, the catalytic activity of Val562Ala protein was significantly higher than that of wild-type protein ($p = 0.0046$), when the activity was measured without PLP in the assay mixture. In addition, in the presence of 200 μM PLP, Val562Ala mutant showed significantly higher activity than that of wild-type ALAS2 ($p = 0.0087$). In contrast, the catalytic activity of Met567Ile protein was lower than that of wild-type protein, irrespective of without PLP ($p = 0.0011$) or with PLP ($p = 0.0003$). It is also noteworthy that the PLP-associated increases in enzymatic activities were 86%, 44%, and 23% for wild-type, Val562Ala, and Met567Ile proteins, respectively, suggesting that Val562Ala and Met567Ile mutations decreased the responsiveness to PLP (Table 1). The lowest PLP responsiveness of Met567Ile mutant protein might account for the clinical course of the proband in case 2; that is, the anemia of this proband was improved only marginally, despite pyridoxine treatment.

Because we previously reported on the Ser568Gly mutation [15], which is also located in the C-terminal region of human ALAS2 protein, the reported data for the Ser568Gly mutation were included as a reference in Table 1. In vitro enzymatic activity of glutathione *S*-transferase (GST)–fused Ser568Gly was significantly lower than that of the GST–fused wild-type ALAS2 with or without PLP [15]. Therefore, the functional consequence of amino acid substitution at Ser568 was similar to that of Met567Ile (Table 1). In addition, the degree of PLP-mediated increase in Ser568Gly activity, indicated as "ratio with/without PLP" in Table 1, was larger than that with wild-type protein, although the possibility remains that the GST tag might have influenced the PLP responsiveness of a recombinant ALAS2 protein. We, therefore, included Ser568Gly mutant in subsequent analyses.

The higher catalytic activity of Val562Ala protein prompted us to examine the function of the C-terminal region of ALAS2 protein. We measured the enzymatic

activity of the deletion mutant that lacks the 33 amino acids at the C-terminal end (positions 555–587) of human ALAS2 (delC33 mutant), the region of which was conserved among mammalian ALAS2 proteins, including Val562. As shown in Table 1, the enzymatic activity of the delC33 mutant was higher by two times in the presence of PLP than that of wild-type ALAS2 ($p = 0.002$), whereas they showed similar enzymatic activity in the absence of PLP. These results suggest that the 33 amino acids at the C-terminal end of human ALAS2 protein might repress the enzymatic activity, probably by interfering with the access of PLP cofactor to the catalytic site.

Stability of mutant ALAS2 proteins in vivo

We were interested in studying how the Val562Ala mutation is associated with XLSA, despite higher enzymatic activity. We examined the stability of the Val562Ala mutant protein and other C-terminal mutant proteins in vivo. When human ALAS2 protein is expressed as a FLAG-tagged protein in eukaryotic cells, the precursor and mature proteins should be detected as 65.7-kDa and 60.5-kDa proteins, respectively. As shown in Figure 1B (upper panel) and Figure 2 (middle panel), FLAG-tagged wild-type ALAS2 and mutant ALAS2 proteins, except for delC33 mutant, were detected as bands at about 60 kDa, an expected size of the mature protein. These results suggest that the leader peptide at the N-terminal end was cleaved after translocation of the precursor protein into mitochondria [4]. In fact, the precursor protein was detected at an expected size, when HeLa cells were transfected with FLAG-ALAS2 expression vector, and then incubated with hemin (Supplementary Figure E2; online only, available at www.exphem.org), which is known to inhibit mitochondrial translocation of ALAS precursor protein into mitochondria [4]. Based on our experiments (Fig. 1A–C), the half-lives of wild-type and Val562Ala mature proteins in mitochondria were calculated as 7.8 hours and 5.3 hours, respectively. The half-life of the Val562Ala mutant protein (Fig. 1C) is shorter than that of wild-type ALAS2 protein (Fig. 1B). In contrast, the half-life of Met567Ile (Fig. 1D) or Ser568Gly (Fig. 1E) mutant was not measurable by our experiments because the 50% reduction of the protein level was not observed within 12 hours for these mutants. Thus, the half-lives of Met567Ile and Ser568Gly mutants were longer than 12 hours. Importantly, the amino acid substitutions in the C-terminal region influenced the stability of the mature ALAS2 protein in mitochondria in different manners. Namely, Val562Ala mutation results in destabilization of the mature protein, and either Met567Ile or Ser568Gly mutation stabilizes the mature protein in mitochondria.

In addition, we measured the half-life of delC33 mutant in HEK293-derived cells (Fig. 1F), showing that the 50% reduction was not observed within 12 hours, which was similar to Met567Ile and Ser568Gly mutants. These results suggested that the 33 amino acids at C-terminal region of ALAS2 protein suppressed the catalytic activity in vitro, as well as protein stability in mitochondria. Our data also indicate that Val562Ala mutation might enhance the destabilization function of the C-terminal region, whereas Met567Ile and Ser568Gly mutations might enhance the suppressive function for enzymatic activity.

Enzymatic activity of each ALAS2 mutant protein in vivo

Val562Ala mutant showed higher enzymatic activity in vitro (Table 1), but it was less stable in mitochondria (Fig. 1A) compared with wild-type ALAS2. On the other hand, Met567Ile and Ser568Gly mutants showed lower enzymatic activities in vitro (Table 1), with prolonged half-lives in mitochondria (Fig. 1A). We, therefore, determined the catalytic activity of each mutant protein in vivo. For this purpose, we compared the accumulation of porphyrins in HEK293-derived cells that expressed wild-type protein or a mutant protein, as we described previously [20]; that is, the accumulation of porphyrins was evaluated by comparing the intensity of the fluorescence under ultraviolet light (Fig. 2, upper panel). The accumulation of porphyrins was detected in cells expressing wild-type ALAS2, but not in cells expressing tagged luciferase. These results indicate that FLAG-tagged ALAS2 is catalytically active in mitochondria. In contrast, the accumulation of porphyrins was decreased in cells expressing Val562Ala, Met567Ile, or Ser568Gly protein, compared to cells expressing wild-type ALAS2. Among these three missense mutations, Val562Ala mutant showed higher catalytic activity than did Met567Ile or Ser568Gly mutant (Fig. 2, upper panel). In addition, the highest porphyrin accumulation was observed in cells expressing delC33. Of note, the expression level of Val562Ala mutant protein was much lower than that of any other mutant or wild-type ALAS2, as judged by Western blot analysis (Fig. 2, middle panel), although there was no significant difference in relative expression level of each mutant ALAS2 messenger RNA (Fig. 2, lower panel). These results suggest that Val562Ala mutant protein is catalytically hyperactive but unstable in mitochondria, which is consistent in part with the higher enzymatic activity detected in vitro (Table 1) and with the short-half life in vivo (Fig. 1A).

In conclusion, Val562Ala, Met567Ile, or Ser568Gly ALAS2 has lower enzymatic activity in mitochondria compared with the activity of wild-type ALAS2. Therefore, these three mutations are categorized as a loss-of-function mutation and are responsible for sideroblastic anemia.

Discussion

It is well known that a loss-of-function mutation of the ALAS2 gene causes XLSA. In addition to the ALAS2 gene, other genes (e.g., SLC25A38 [24], GLRX5 [25], ABCB7 [26], PUS1 [27], SLC19A2 [28], and mitochondrial DNA [29]) were reported to be responsible for

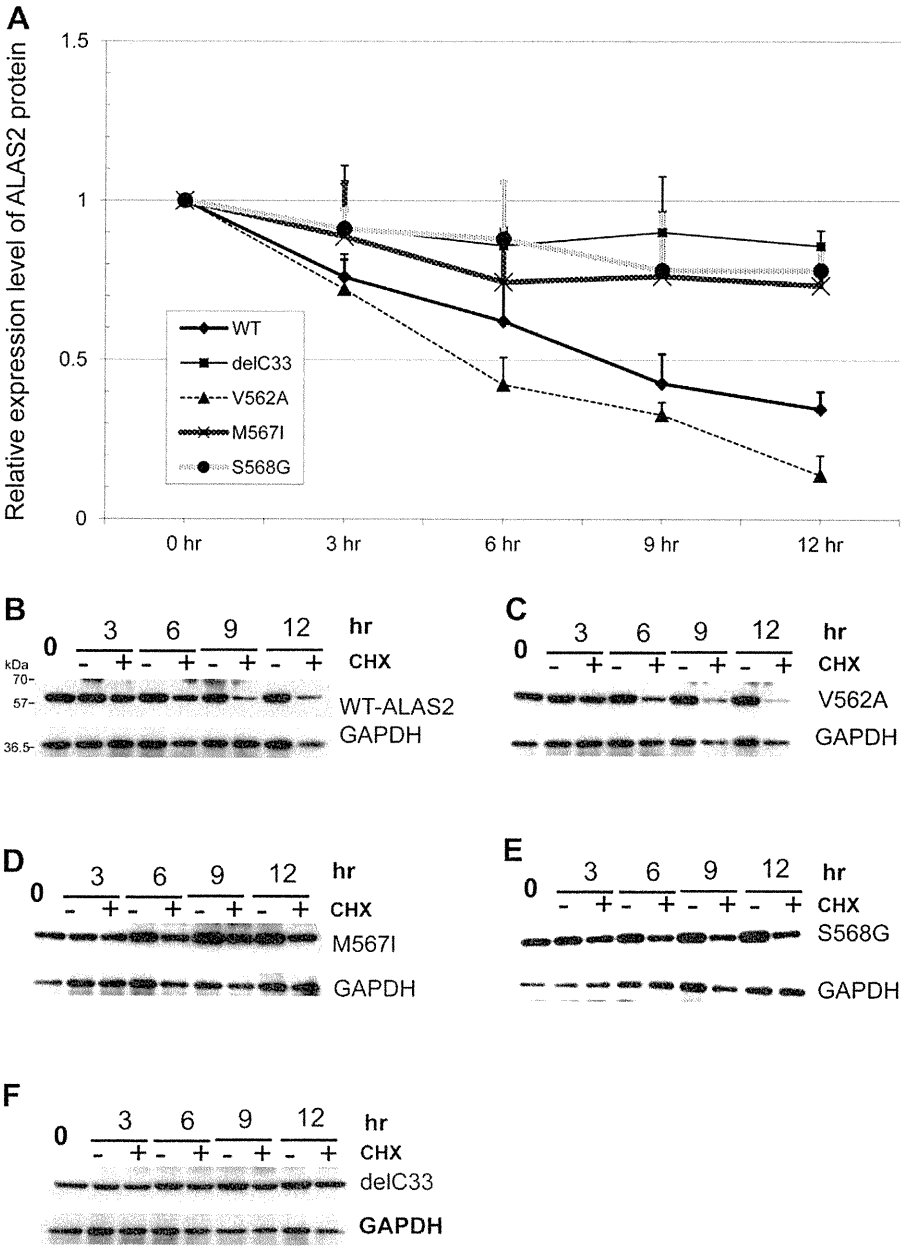

Figure 1. Effect of cycloheximide on FLAG-tagged ALAS2 protein level in eukaryotic cells. Expression of each FLAG-tagged protein was induced with tetracycline (1 μg/mL) in HEK293-derived cells for 48 hours, and then cells were treated with 10 μg/mL cycloheximide (CHX) for the indicated hours. Cells were collected and lysed in RIPA buffer, and FLAG-tagged proteins were detected by Western blot analysis (**B**–**F**). The intensity of the FLAG-tagged protein was normalized with the intensity of GAPDH for each time point. In (**A**), the relative intensity representing FLAG-tagged protein at 0 hours was considered to be 100%. The half-life of each protein was calculated on the basis of 50% reduction of each protein expression from the relative expression curves obtained from the samples with CHX. Average value of three independent experiments was used for preparing (**A**). Representative data of each ALAS2 protein are shown (**B**–**F**): (**B**) wild-type (WT) ALAS2; (**C**) Val562Ala; (**D**) Met567Ile; (**E**) Ser568Gly; and (**F**) delC33.

hereditary or congenital sideroblastic anemia. Among these candidate genes, mutations in ALAS2 gene are most frequently identified in patients with sideroblastic anemia [30], but characterization of each mutant ALAS2 protein was not fully performed. To the best of our knowledge, 24 of 56 mutations of the ALAS2 gene were characterized in vitro using recombinant proteins with or without a peptide-tag [9,10,14,15,20,21,31–36]. In the 11th exon of the ALAS2 gene, Ser568Gly [15], Arg559His [17], Arg560His [16], and Arg572His [14] mutations have been reported; however, only Ser568Gly and Arg572His mutants were characterized using recombinant proteins. Concerning the Ser568Gly mutation [15], we confirmed that Ser568Gly mutation resulted in decreased enzymatic activity in vitro (about 30% of wild-type with PLP in the assay mixture), as shown in Table 1. In contrast, Ducamp et al. [14] were

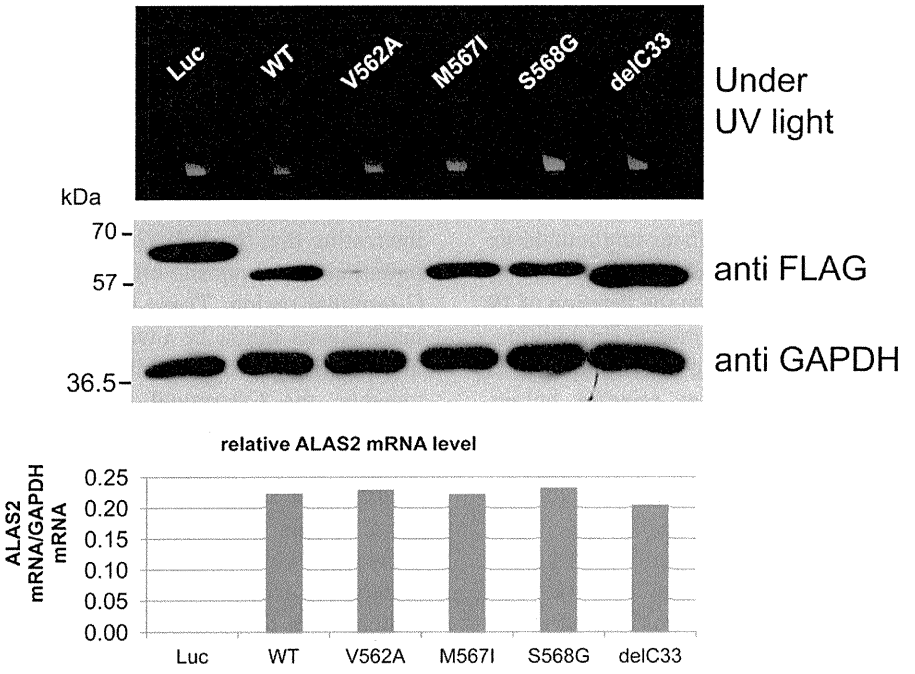

Figure 2. Evaluation of porphyrin production in cells expressing each ALAS2 mutant protein. Each FLAG-tagged ALAS2 protein or FLAG-tagged luciferase protein as a control was expressed in Flp-In T-REx 293 cells. Accumulation of porphyrins in each cell line was visualized by ultraviolet light exposure (upper panel). Expression levels of each FLAG-tagged protein and GAPDH (loading control) were detected by Western blot analysis (middle panels). Expression level of ALAS2 messenger RNA (mRNA) was measured by real-time PCR, and it was normalized with the expression level of GAPDH mRNA. Note that the data confirm the similar mRNA level of each ALAS2 protein (lower panel).

unable to determine the defect of the Arg572His mutant using an in vitro system because the mutant protein showed the enzymatic activity similar to that of wild-type ALAS2.

Measurement of enzymatic activity of every recombinant protein in vitro is one of the most useful techniques to characterize a mutant ALAS2 protein. Met567Ile mutant showed lower enzymatic activity than did wild-type protein (see Fig. 2), suggesting that this mutation causes sideroblastic anemia. In contrast, we were unable to uncover the pathogenesis of Val562Ala mutant protein using this in vitro assay system, indicating a limitation of the in vitro assay system with a bacterially expressed recombinant protein. In fact, using the in vivo system (Fig. 2), we have successfully demonstrated that the Val562Ala mutant protein showed lower porphyrin accumulation due to enzyme instability compared to wild-type ALAS2. In addition, the half-life of mature Val562Ala protein (5.3 hours) was shorter than that of wild-type ALAS2 (7.8 hours) (Fig. 1), suggesting that Val562Ala mutation altered the protein stability in mitochondria. These in vivo methods seem to be useful to characterize a mutant protein that does not show decreased enzyme activity in the in vitro assay system.

It is of particular interest that the Val562Ala and Met567Ile mutants exerted opposite effects on the enzymatic activity in vitro (Table 1) and on the protein stability in mitochondria (Fig. 1). In this connection, the deletion of 33 amino acids at C-terminal end of ALAS2 protein, the region of which contains Val562 and Met567 residues, resulted in higher enzymatic activity in vitro and in vivo (Table 1) and stable protein with a longer half-life in mitochondria (Fig. 1). The C-terminal region has a suppressive function on enzymatic activity, as well as protein stability in mitochondria. Because this region is conserved in eukaryotic ALAS2 but is absent in prokaryotic ALAS, the suppressor domain might be involved in the functional regulation of ALAS2 in mitochondria. In fact, in the C-terminal region, two frame-shift mutations of the ALAS2 gene were reported to cause X-linked dominant protoporphyria [11], and six (including present two cases) missense mutations were identified in patients with XLSA. In addition, it was recently reported that the Tyr586Phe mutation of ALAS2 protein increased the enzymatic activity, which can contribute to the severe clinical phenotype of the patient with congenital erythropoietic porphyria [18]. These results suggest that the C-terminal region of ALAS2 functions as an intrinsic suppressor for protoporpyrin production in erythroid cells.

It is still unclear how this C-terminal region suppresses the enzymatic activity of ALAS2 protein in mitochondria. It has been reported that certain amino acids are essential for catalytic activity of mouse Alas2 [18,37–45]. However, only limited information is available concerning the role of the C-terminal region in the catalytic activity of ALAS2. To-Figueras et al. [18] performed the stoichiometric analysis of the mature ALAS2 protein to characterize Tyr586-Phe mutant, which was reported as a gain-of-function

mutation at the penultimate C-terminal amino acid of ALAS2 protein. Steady-state kinetic analyses revealed that Tyr586Phe mutant showed higher catalytic activity with greater catalytic efficiency for glycine and succinyl-CoA than those of wild-type ALAS2. In addition, these authors provided evidence that the Tyr586Phe mutant enzyme was able to form and release ALA more rapidly than wild-type enzyme. Similar mechanisms might underlie the increased activity of every C-terminal deletion mutant, including the mutant ALAS2 protein with the deletion of 19 or 21 amino acids [11] and the delC33 mutant. In addition, the delC33 mutant expressed enzymatic activity similar to wild-type ALAS2 without PLP in assay mixture, but its enzymatic activity was increased about twofold compared to the wild-type with PLP (Table 1). These results suggest that this region might be involved in efficient use of PLP or accessibility of PLP to the catalytic site.

Crystal structure analysis of homodimeric ALAS from Rhodobactor capsulatus ($ALAS_{RC}$) revealed that $ALAS_{RC}$ showed open or closed structure, which was related to the conformational change of the active site loop [17]. This active site loop consists of evolutionally conserved structure at the C-terminal region of $ALAS_{RC}$, and seems to cover the catalytic site, which is located at the homodimer interface of ALAS protein. It should be noted that $ALAS_{Rc}$ does not contain the C-terminal region equivalent to that of mammalian ALAS2 [17]. The open conformation was observed only in the substrate-free $ALAS_{RC}$ protein, and the closed conformation was observed in $ALAS_{RC}$ protein that bound glycine and succinyl-CoA. To clarify the functional consequence of the conformational change of this active site loop, Lendrihas et al. introduced a mutation into nonconserved amino acid at this active site loop in mouse Alas2 protein and obtained several hyperactive variants [46]. Pre–steady-state kinetic analysis revealed that release of ALA from the catalytic site of the enzyme, which is coincident with opening of the active site loop [45], was accelerated in these hyperactive variants. Because the release of ALA from catalytic site is the rate-limiting step of enzymatic reaction of ALAS [47], these results suggest that the dynamic conformational change of this active site loop might control the rate of the reaction. Importantly, the accelerated release of ALA from the enzyme was also proposed in Tyr586Phe mutant [18]. It is therefore possible that the C-terminal domain of human ALAS2 protein is involved in the regulation of the conformational change of the active site loop.

In the present study, we determined the stability of ALAS2 protein in vivo, although the protein was tagged with a small peptide and expressed in HEK293-derived cells. Based on our assay condition, the half-life of human ALAS2 mature protein is 7.8 hours; however, it is not clear whether this result is comparable with that of the native ALAS2 protein in erythroid mitochondria, which has never been reported. On the other hand, this assay revealed that the stability of the Val562Ala mutant protein was decreased in mitochondria (Fig. 1), although the in vitro assay using purified recombinant protein failed to detect the unstable property of this mutant. In addition, our in vivo assay system clearly showed that the C-terminal region of 33 amino acids of human ALAS2 protein suppressed the enzymatic activity and decreased the protein stability. It is also interesting that the Val562Ala mutation and the Met567Ile mutation have opposite effects on the two functions of the C-terminal region. These results suggest that independent mechanisms might be involved in the reduction of enzymatic activity and destabilization in mitochondria. Taken together, the C-terminal region of ALAS2 protein can decrease catalytic activity by altering the open or closed structure of the catalytic site, while the post-translational modification of the C-terminal region, which is induced by a certain intracellular condition (e.g., increased or decreased oxidative stress) or by the association with other molecules, can enhance the disappearance of ALAS2 protein from mitochondria. The crystal structure of ALAS from $ALAS_{RC}$ provided critical information about the mechanisms for catalytic reaction of ALAS [45,46]. However, determination of the crystal structure of mammalian ALAS2 should await additional investigation on the function of the C-terminal region of ALAS2 protein.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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