MITOCHONDRIAL MYOPATHY AND SIDEROBLASTIC ANEMIA (MLASA): MISSENSE MUTATION IN THE PSEUDOURIDINE SYNTHASE 1 (PUS1) GENE IS ASSOCIATED WITH THE LOSS OF tRNA PSEUDOURIDYLATION

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Running title: Loss of tRNA pseudouridylation in MLASA

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A missense mutation in the PUS1 gene affecting a highly conserved amino acid has been associated with Mitochondrial Myopathy and Sideroblastic Anemia (MLASA)1, a rare autosomal recessive oxidative phosphorylation disorder. The PUS1 gene encodes the enzyme Pseudouridine Synthase 1 (Pus1p) that is known to pseudouridylate tRNAs in other species. Total RNA was isolated from lymphoblastoid cell lines established from patients, parents, unaffected siblings, and unrelated controls and the tRNAs were assayed for the presence of pseudouridine (Ψ) at the expected positions. Mitochondrial and cytoplasmic tRNAs from MLASA patients are lacking modification at sites normally modified by Pus1p, whereas tRNAs from controls, unaffected siblings, or parents all have Ψ at these positions. In addition, there was no Pus1p activity in an extract made from a cell line derived from a patient with MLASA. Immunohistochemical staining of Pus1p in cell lines showed nuclear, cytoplasmic, and mitochondrial distribution of the protein, and there is no difference in staining between patients and unaffected family members. MLASA is thus associated with absent or greatly reduced tRNA pseudouridylation at specific sites, implicating this pathway in its molecular pathogenesis.

Following transcription, all stable RNAs are processed and nucleotides are modified. The most abundant modification is pseudouridine (Ψ), formed by the action of pseudouridine synthases. A number of these modification enzymes have been characterized from several species and one that has been particularly well studied is Pseudouridine synthase 1 (Pus1p). Pus1p has been characterized from yeast (1), mouse (2), and C. elegans (3) and homologues have been identified in many eukaryotes, including humans (2). Pus1p has been shown to modify positions 27, 28, 34, and 36 in tRNAs in vitro and in vivo (1,2). Additional sites in tRNAs have been identified for yeast Pus1p in vivo (1). Knockouts of genes for Pus1p in yeast (4) or C. elegans (3) have a wild-type phenotype, in spite of the fact that the presence of Ψ stabilizes base-pairing and base stacking (5,6) and the sites of pseudouridylation are highly conserved in tRNAs (7). In yeast if the deletion of the PUS1 gene is combined with deletions of the gene for Pseudouridine synthase 4 (Pus4p, tRNA Ψ55 synthase) or with the gene for Los1p (part of the nuclear pore complex), either combination is lethal (4,8). In addition, when Pus1p and Pus4p are deleted in yeast there is a defect in the transport of certain tRNAs out of the nucleus (8).

Genetic analysis has implicated a missense mutation in the PUS1 gene as the cause of Mitochondrial Myopathy and Sideroblastic Anemia (MLASA, [MIM 600462])2, a rare autosomal recessive disorder of oxidative phosphorylation and iron metabolism (9,10). This mutation, a replacement of an arginine with a tryptophan at position 116,
affects an amino acid in Pus1p that is highly conserved throughout evolution. This amino acid is part of the active site of the enzyme and is near a critical aspartate (position 118) that has been shown to be necessary for catalysis in several families of pseudouridine synthases (11-13).

We speculated that Pus1p in patients with MLASA was likely to be enzymatically inactive and therefore cellular tRNAs would lack \( \Psi \)s that are formed by the enzyme. We found that tRNAs from patients do lack \( \Psi \)s that are known to be formed by Pus1p and that there is no Pus1p activity in an extract made from a cell line derived from a patient. In addition, immunohistochemical staining of Pus1p in cells showed nuclear, cytoplasmic, and mitochondrial distribution of the protein, and there was no difference in the pattern of staining between patients and unaffected family members.

Materials and Methods

**Assays for pseudouridine.** Total RNA (10 µg) from lymphoblastoid cell lines (10) was treated with either 0 (CMCT-) or 0.167 M 1-cyclohexyl-3(2-morpholinoethyl) carbodi-imide metho-p-toluenesulphonate (CMCT+) for 15 minutes at 37°C, isolated, and treated with sodium bicarbonate as described (14,15). A portion of these treated RNAs (1 µg) were used in primer extension reactions using AMV RT and the \(^{32}\)P-end-labeled primers 5’ATTCGAACCTGCGCGGGAAAC3’ for cytoplasmic tRNA\(^{5\Psi}\)(UGA) and 5’GAGTTGTTCTCTTAAATC3’ for mitochondrial tRNA\(^{5\Psi}\)(UUU) as described (15). The products were separated on denaturing (8.3 M urea) 10% polyacrylamide gels (19:1: acrylamide:bis-acrylamide). Sequencing reactions using the labeled primer and a plasmid containing the human tRNA gene were electrophoresed along with the primer extension reactions (3,15,16). The dried gels were exposed to Kodak XAR film.

The plasmids used in the sequencing reactions were constructed by generating a fragment using the polymerase chain reaction (PCR) with primers from the 5’ and 3’ ends of human tRNA genes and human DNA from one of the control cell lines as the template (17,18).

The primers used to generate the fragments for: cytoplasmic tRNA\(^{5\Psi}\)(UGA) are 5’GTAGTCGTGGCAGGTGAC3’ (forward) and 5’CGTACTGCAGGATTCG3’ (reverse); mitochondrial tRNA\(^{5\Psi}\)(UUU) are 5’CAGTGAAAGCTAATCAGAC3’ (forward) and 5’CAGTGAAAGCTAATCAGAC3’ (reverse); mitochondrial tRNA\(^{5\Psi}\)(UGA) are 5’GAAAGGTACCTGAGGCAC3’ (forward) and 5’AAAGGAAGGAAATCGAC3’ (reverse); and cytoplasmic tRNA\(^{5\Psi}\)(UUU) are 5’GCCCGGATAGCTAGTCG3’ (forward) and 5’CGCCCGAAACAGGGACTTG3’ (reverse). These fragments were inserted into pGEMT (Promega, Madison, WI) and sequences of the resultant recombinants were confirmed by sequencing (16).

Nuclear extracts were made as described (19) from cell lines derived from a MLASA patient (−/−), a heterozygote (+/−), and an unaffected sibling (+/+). Labeled tRNA\(^{5\Psi}\)(UGA) was transcribed in vitro by T7 RNA polymerase in the presence of \(^{32}\)P-GTP, using the plasmid pHtS (20) digested with BstN1 as a template (21). This labeled substrate (43,000 cpm, 0.41 ng) was incubated with each of the extracts (30 µg total protein in 20 mM Heps, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), recombinant mPus1p (0.7 µg), or Lac protein (0.7 µg) for 2 hours at 37°C as described (2,22). The RNA was isolated from the reactions and gel purified on a 10% polyacrylamide/8.3 M urea gel (23). The recovery of counts was variable for the five samples and was considerably worse for the samples where the tRNA\(^{5\Psi}\) was incubated with extracts (see legend for Figure 4 for relative values). Each RNA sample was then digested with RNaseT, and chromatographed on thin-layer chromatography (TLC) plates using isobutyric acid:ammonium hydroxide (25%):water (50:1.1:28.9:v/v/v) in the first dimension and isopropanol:conc. HCl:water (70:15:15:v/v/v) in the second dimension (2,24). The TLCs were then exposed to a phosphoimaging screen for 48 hours and scanned using a BioRad Phosphoimager FX.

** Immunohistochemistry.** To experimentally determine the intracellular localization of the human Pus1p protein, polyclonal antibodies against human Pus1p were raised by
immunization of rabbits with the synthetic peptide CEKYNQRFGNDGLHE (GenBank accession number NP_079491, internal amino acids 313-326) using PolyQuick™ custom antibody services of ZYMED Laboratories Inc. (San Francisco, CA). EBV-transformed lymphoblastoid cell lines from patient with MLASA, a parent, and an unaffected sibling, as well as the K-562 cell line (American Type Culture Collection, ATCC, Manassas, VA) and Human Skeletal Muscle cells (Cell Applications Inc, San Diego, CA) were cultured and incubated with polyclonal rabbit anti-Pus1p antibody and mouse anti-human mitochondria monoclonal antibody (Chemicon, Temecula, CA). The signals were visualized using secondary goat anti-rabbit Alexa fluor® 568 and goat anti-mouse Alexa fluor® 488 antibodies (Molecular Probes, Invitrogen). An Olympus AX70 fluorescence microscope equipped with an Apogee KX-2E digital camera and Image Pro and Focus Control computer programs, were used to visualize and capture the cells mounted in Vectashield mounting DAPI containing medium (Vector Laboratories, Burlingame, USA). The confocal images were taken with an Olympus EV300 confocal unit attached to the microscope. Excitation was made with an ArKr ion laser at 488nm and 567nm. Cw laser power was maintained in the range of 3-7 mW.

RESULTS

To determine the pseudouridylation state of the tRNAs in affected and unaffected individuals, total RNA was isolated from the 12 lymphoblastoid cell lines established from patients with MLASA, their unaffected parents and siblings, as well as unrelated controls (10). The RNA samples were used in a primer extension assay for Ψ that involves chemically treating the total RNA with CMCT (1-cyclohexyl-3(2-morpholinoethyl) carbodi-imide metho-p-toluenesulphonate) which modifies N-3 of uridine and Ψ and N-1 of guanosine. This chemical modification is then reversed with mild base on U and G residues but not Ψ residues. This modification of Ψ will cause reverse transcriptase (RT) to stop during the specific primer extension phase of the assay. The products are electrophoresed on a 10% polyacrylamide/8.3 M urea gel along with sequencing lanes using the same end-labeled primer (14,15).

Two tRNAs that were assayed in the samples were human cytoplasmic tRNASer(UGA) and human mitochondrial tRNAlys(UUU) (see Figure 1). With tRNASer(UGA) there are three Ψs at positions 28, 39, and 55 (25). The Ψ at 28 is formed by Pus1p (1,2) and the other two modifications are formed by Pus3p and Pus4p respectively (22,26,27). Human mitochondrial tRNAlys(UUU) has two Ψs at 27 and 28 of the tRNA (28), both residues are potentially the product of Pus1p modification.

The results of the primer extension assay using a primer specific for tRNASer(UGA) are shown in Fig. 2. Stops to RT that are found in the CMCT+ lanes but not in the CMCT– lanes are indicative of a Ψ at positions 28 and 39 (see arrows on side of panel). RT stops just before the covalently modified nucleotide and additional stops to RT found in both lanes are due to the secondary structure of the tRNA. The stop to RT at position 28 is missing in RNA samples 3, 4, 7, and 9, indicating that the tRNASer(UGA) from those samples is lacking Ψ at position 28. These RNA samples are from patients with MLASA (−/−). All of the other RNA samples (1, 2, 5, 6, 8, 11, 12, 13) have Ψ at position 28 of this tRNA, and these samples are from unaffected parents, siblings, and controls (either +/- or +/+). There is a stray spot in CMCT+ lane for sample #9 in the approximate position where the strong stop for Y28 should be, but when this sample was assayed again there was no band at this position (data not shown).

All of the samples have a Ψ at position 39, which was expected since this uridine is modified by Pus3p (22,26). The primer used hybridizes to the region of the tRNA that contains position 55, formed by Pus4p (27), and so the modification state of that position was not determined.

Next the primer extension assay was used to determine whether the modification of mitochondrial tRNAlys(UUU) was affected by the loss of Pus1p activity. Figure 3 shows the results with the 12 RNA samples and, as with
tRNA<sup>Ser</sup>(UGA), the tRNAs from the patients (samples 3, 4, 7, and 9) do not have strong stops to RT just before position 27 and 28. The other samples do have very strong stops prior to position 28 and slightly weaker but still prominent stops prior to position 27. In the patient samples there is a very slight increase in band intensity in the CMCT+ lanes before position 27 but that may be due to the possibility that the unmodified tRNA adopts a slightly different secondary structure from the modified tRNA<sup>Ψ<sub>Ψ<sub>Ψ</sub></sub>(UUU). Never the less, it is obvious that the intensity of the band increases in samples 1, 2, 5, 6, 8, 11, 12, and 13. In addition, there is no hint of a stop just before position 28 in the patient samples, so the loss of modification at position 28 is unequivocal.

Two other tRNAs were assayed, mitochondrial tRNA<sup>Ser</sup>(UGA) and cytoplasmic tRNA<sup>Ψ<sub>Ψ<sub>Ψ</sub></sub>(UUU) and again the modifications formed by Pus1p are missing in RNA samples from the MLASA patients and present in the samples from the parents, unaffected siblings, and controls (data not shown).

The Pus1p activity of nuclear extracts (19) made from three cell lines was tested by incubating <sup>32</sup>P-GTP labeled tRNA<sup>Ser</sup>(UGA) with either the extracts, recombinant mPus1p, or a control recombinant Lac protein isolated under the same conditions as mPus1p (2). The isolated RNA from the reactions was digested with RNaseT2 and chromatographed on thin-layer chromatography (TLC) plates in two dimensions (2,24). A phosphoimager scan of the five TLCs is shown in Figure 4. Even though tRNA<sup>Ser</sup>(UGA) has three <sup>Ψ</sup>s (Fig. 1), only the <sup>Ψ</sup> at position 28 has a G residue 3’ to the modified nucleotide. This is important because RNAseT2 leaves 3’ phosphates and therefore the <sup>Ψ</sup> at position 28 will be the only one labeled in this experiment. If enzymes such as Pus3p or Pus4p in the extracts modify the other uridines at 39 and 55 (22,26,27), these will not appear on the TLC since they will not be labeled. A spot for <sup>Ψ</sup> can be seen in the TLC where tRNA<sup>Ser</sup>(UGA) was incubated with mPus1p as well as in the TLCs where the extracts made from cell lines from +/- individual or when the Lac control protein was used in the reaction.

To determine the cellular localization of human Pus1p, dual-label immunohistochemistry was employed. Previously published experiments using Saccharomyces cerevisiae, Schizosaccharomyces pombe, and mouse homologs of human Pus1p have shown their predominantly nuclear localization (4,29,30). Publicly available prediction programs classified Pus1p as a likely mitochondrial enzyme (10) and Pus1p activity was found in a mitochondrial extract that was shown to be free of cytosolic contaminants (31). In Figures 5A and 5B, the distribution of Pus1p is shown with red fluorescence, whereas the location of the mitochondria is indicated with green fluorescence. Pus1p is found in all the cells including cells from patients with MLASA (see Fig. 5A, Row 2). Human Pus1p protein is clearly present in the nucleus, throughout the cytoplasm, and appears to co-localize with the mitochondrial marker (Panels C and D) in all cell types, as shown on Figure 5A. The presence of Pus1p in mitochondria was confirmed using confocal microscopy of EBV-transformed lymphocytes from an unaffected family member (see Figure 5B). Clearly, Pus1p shown in red (panel A) and mitochondrial marker shown in green (panel B) co-localize when merged (panel C) as demonstrated by the yellow color of the mitochondria organelles. These results support Pus1p involvement in cytoplasmic and mitochondrial tRNA processing, which occur in the nucleus and mitochondria respectively.

DISCUSSION

Why might the absence of <sup>Ψ</sup> in tRNAs result in MLASA? The tRNA database<sup>5</sup> (25) shows that 29 cytoplasmic tRNA genes (out of 36 listed) and 18 mitochondrial tRNA genes (out of 27 listed, including variants) will have uridines at positions 27 and/or 28 of the tRNA and are potential substrates for Pus1p. Of the sequenced cytoplasmic tRNAs listed (17 total) there are eight with <sup>Ψ</sup>s identified at positions 27 or 28, and three more with uridines in at least one of those positions. Pseudouridine is known to affect the structure of tRNAs, affecting base-stacking (5) in the anticodon loop and when it
appears in stems or the anticodon, it strengthens base-pairing (6,32-34). When modeled with RNA oligomers, the presence of Ψ in U2 small nuclear RNA causes a change in the structure of the lariat branch point of pre-messenger RNA (35,36). A functional requirement for Ψ in U2 snRNA has also been shown in reconstitution of splicing systems (37-39). The loss of Ψ in tRNAs may alter the structure of the tRNA and have an impact on its role in the translation process. This impact may affect the interaction of the tRNA with its cognate amino-acyl tRNA synthetase and the fit of the unmodified tRNA in the P or A site of the ribosome. It may also affect the transport of the tRNA out of the nucleus to the cytoplasm, since it has been shown that tRNA transport is affected after the combined loss of Pus1p and Pus4p activities in yeast (8).

In mitochondria the role of Pus1p may even be more important. Several tRNA genes in human mitochondria (Gly, one Leu, Lys, Phe, one Ser, Thr, and Tyr) have no uridine at position 55. By contrast, every gene for cytoplasmic tRNAs predicts that there will be a uridine at this position and all sequenced cytoplasmic tRNAs have a Ψ at position 55 (25). Therefore these mitochondrial tRNAs will not have a Ψ at position 55 and four of these mitochondrial tRNAs (Gly, one Leu, Lys, and Phe) are potential substrates for Pus1p. If the modification at positions 27 or 28 is also missing, as in the patients with MLASA, then the structure and function of the tRNA may be compromised.

This is not the first human disorder that can be attributed to a defect in pseudouridylation. One form of Dyskeratosis congenita (X-linked) has been shown to be due to the loss or mutation of the Dyskerin protein (40), which is a homologue of the yeast protein Cbf5p, the pseudouridine synthase responsible for ribosomal RNA modification (41). Dyskerin is part of the telomerase complex and its loss results in a destabilization of the complex (42). However, a mouse model of the disease was created when mutations affecting the pseudouridylation activity of Dyskerin but not telomerase binding activity were engineered (43), suggesting that the loss of rRNA pseudouridylation contributes to the disorder.

Recently it was shown that mouse Pus1p (mPus1p) acts as a regulator of nuclear receptor activity through the modification of Steroid Receptor RNA Activator (SRA) (30). The pseudouridine synthase activity of mPus1p is required for this co-activation. The synthase modifies SRA and it is part of the nuclear receptor complex that interacts with the response element on DNA (30). It cannot be excluded that some aspects of MLASA may be due to the loss of this activity of Pus1p.

Future studies will focus on whether the loss of modification affects the function, location, or quantity of certain tRNAs in the nucleus, cytoplasm, or mitochondria. Using the cell lines from MLASA patients and controls, the efficiency of the cytoplasmic as well as mitochondrial translational machinery will be investigated.

REFERENCES
FOOTNOTES

*We thank the individuals with MLASA and their families for their cooperation. We would also like to thank H. Gross for the pHtS plasmid, D. Acuna for help with immunohistochemistry, and Dr. S. Wachsmann-Hogiu for help with confocal microscopy. This work is funded by a grant to J.R.P. from a Research and Productive Scholarship Grant from the University of South Carolina and a grant to N. F.-G. from the National Institutes of Health/National Institute on Deafness and other Communication Disorders (R01DC01402).

1The abbreviations used are: CMCT, 1-cyclohexyl-3(2-morpholinoethyl) carbodi-imide metho-p-toluenesulphonate; MLASA, Mitochondrial Myopathy and Sideroblastic Anemia; Ψ, pseudouridine; Pus1p, Pseudouridine synthase 1; Pus3p, Pseudouridines synthase 3; Pus4p, Pseudouridine synthase 4; PUS1, Pus1p gene; RT, reverse transcriptase; AMV, Avian myoblastosis virus; PCR, polymerase chain reaction; TLC, thin-layer chromatography; SRA, steroid receptor RNA activator; PMSF, phenyl methyl sulfonylfloride; DTT, dithiothreitol.

2Electronic Database Information
The tRNA database, http://www.uni-bayreuth.de/departments/biochemie/trna/

FIGURE LEGENDS

Fig. 1. Structure of tRNAs used in Ψ mapping assays. The structures of (A) human cytoplasmic tRNA^{Ser}(UGA) (25), and (B) human mitochondrial tRNA^{Lys}(UUU) (28) are shown with known Ψ residues boxed and indicated by position. Additional modifications are: 1-methyladenosine (m1A), N6-threoninocarbonyladenosine (t^6A), 3-methylcytosine (m3C), 4-acetylcytosine (ac4C), 2-methylguanosine (m2G), 2'-O-methylguanosine (Gm), 2'-O-methyluridine (Um), 5-methyluridine (T), dihydrouridine (D), 5-taurinomethyl-2-thiouridine (tm5s2U).

Fig. 2. The modification of the uridine at position 28 in cytoplasmic tRNA^{Ser}(UGA) is absent in RNA samples from MLASA patients. The assay is described in the Materials and Methods section. Only a portion of the autoradiograph is shown in each panel. The RNA sample number is at the bottom of each panel and arrows to the left of the panels indicate the location of the strong stops to RT that are referred to in the text. The sequence on the right reflects that of the tRNA. The – or + in the CMCT row above the autoradiograph denotes either no treatment or treatment with 0.167 M CMCT, respectively. Samples that show Pus1p activity have a (+) and those that do not have a (–) in hPus1p phenotype above the autoradiograph. The genotype, with respect to the wild type allele (C at position 656 in human Pus1p mRNA) of the individual that the cell line was derived from, is given at the very top of the figure.

Fig. 3. The modification of the uridines at positions 27 and 28 in mitochondrial tRNA^{Lys} is absent in samples from MLASA patients. The assay is described in detail in the Materials and Methods section. Only a portion of the autoradiograph is shown. The RNA sample number is at the bottom of the panel and arrows to the right of the panel indicate the location of the strong stops to RT that are referred to in the text. The sequence just left of sample #8 reflects that of the tRNA. The – or + in the CMCT row above the autoradiograph denotes either no treatment or treatment with 0.167 M CMCT, respectively. Samples that show Pus1p activity have a (+) and those that do not have a (–) in hPus1p phenotype above the autoradiograph. The genotype, with respect to the wild type allele (C at position 656 in human Pus1p mRNA) of the individual that the cell line was derived from, is given at the very top of the figure.

Fig. 4. Pus1p activity is absent in extracts made from a cell line derived from a patient with MLASA. The assay is described in Material and Methods. RNaseT2 digestion leaves 3’ phosphates and the
positions of the major nucleotides (Ap, Cp, Gp, and Up) are indicated on the mPus1p TLC and the Ψp spot is indicated on the TLCs where it appears. The numbers of counts loaded on each of the TLCs are not equal and based on data generated from the phosphoimager scan the relative amount of radioactivity of each sample are mPus1p 1.00, Lac 0.95, +/- 0.65, and +/-, 0.32.

Fig. 5. Intracellular localization of Pus1p by immunohistochemistry. Double labeling with mitochondria specific marker was used. (A) The Pus1p protein localizes mostly to the nucleus, distributes throughout the cytoplasm and co-localizes with the mitochondrial marker (Panels Cand D) in all cell types. Panels: A. Primary rabbit anti-Pus1p antibody is labeled in red with secondary goat anti-rabbit Alexa fluor® 568 antibodies. B. Primary mouse anti-human mitochondria monoclonal antibody is labeled in green with secondary goat anti-mouse Alexa fluor® 488 antibodies. C. Merge A and B. D. Merge A, B, the nuclei are labeled with DAPI (blue). Row 1: EBV-transformed lymphocyte cell line from unaffected sibling, row 2: EBV-transformed lymphocyte cell line from patient with MLASA, row 3: K-562 cell line, row 4: Human muscle cell line. Scale bars = 20µm. (B) Confocal microscopy of EBV-transformed lymphocyte cell line from unaffected family member confirms co-localization of Pus1p protein with the mitochondrial marker. Panels: A. Primary mouse anti-human mitochondria monoclonal antibody is labeled in green with secondary goat anti-mouse Alexa fluor® 488 antibodies. B. Primary rabbit anti-Pus1p antibody is labeled in red with secondary goat anti-rabbit Alexa fluor® 568 antibodies. C. Merge A and B.
Figure 1A

Human cytoplasmic tRNA$^{\text{Ser}}$(UGA)
Figure 1B

Human mitochondrial tRNA_{Lys}
Figure 2

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**Figure 3**

[Image of gel electrophoresis with RNA samples and genotype/phenotype data]
Mitochondrial myopathy and sideroblastic anemia (MLASA): Missense mutation in the pseudouridine synthase 1 (PUS1) gene is associated with the loss of tRNA pseudouridylation

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J. Biol. Chem. published online March 16, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500216200

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