Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 237-242

MESSAGE AMPLIFICATION PHENOTYPING OF AN INHERITED δ-AMINOLEVULINATE DEHYDRATASE DEFICIENCY IN A FAMILY WITH ACUTE HEPATIC PORPHYRIA

Nobuhiro Ishida*, Hiroyoshi Fujita*, Teruhisa Noguchi§, Manfred Doss#, Attallah Kappas* and Shigeru Sassa*

*The Rockefeller University Hospital, New York, N.Y. 10021

§The Suntory Institute for Biomedical Research, Osaka, Japan

#Philipp University, Marburg, Federal Republic of Germany

Received August 6, 1990

The molecular basis of the enzymatic defect responsible for acute hepatic porphyria due to δ-aminolevulinate dehydratase (ALAD) deficiency was investigated in a family including a proband with the acute disease. In order to delineate the mutation in the proband, cDNA for deficient ALAD was synthesized from the proband's cells. The ALAD phenotype was studied by message amplification phenotyping with total RNA extracted from lymphoblastoid cells of the proband and his family members. Two independent mutant alleles of ALAD were identified in the proband's cells. One mutant allele was shown to result in an amino acid substitution at residue 274 ($Ala^{274} \rightarrow Thr$). Message amplification phenotyping studies have also permitted us to define the ALAD phenotype of each subject in the family. This is the first mutation to be recognized in the human ALAD gene.

δ-Aminolevulinate dehydratase (E.C.4.2.1.24; ALAD) is a cytosolic enzyme in the heme biosynthetic pathway that catalyzes the condensation of two molecules of δ-aminolevulinate to form a monopyrrole, porphobilinogen. The enzyme activity is present in excess in normal cells and a partial deficiency of this enzyme activity is not associated with any clinical consequences (1). However, a marked enzyme deficiency in acute hepatic porphyria due to ALAD deficiency (ADP), as well as marked inhibition of the enzyme activity by succinylacetone, result in the development of a clinically severe hepatic porphyria (1). Four cases of ADP have been described to date (2,3), but no studies on the molecular defect of ALAD in this disorder have been reported. In this study, we investigated the molecular basis of the enzymatic defect of ALAD in a proband with ADP, and have carried out a study on the ALAD phenotype in the members of the proband's family. The results of this study have permitted us to define the first mutation to be recognized in the human ALAD gene.

0006-291X/90 $1.50

MATERIALS AND METHODS

Cell cultures: Isolation, transformation with EB-virus and cultivation of lymphoblastoid cells were carried out as described previously (4).

Synthesis of ALAD cDNA: Poly(A)$^+$ RNA was prepared from EB-virus-transformed lymphoblastoid cells, as described previously (5,6). First strand of cDNA was primed with oligo(dT)$_{12\text{-}18}$ (Pharmacia, Uppsala, Sweden) and synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Pharmacia). Second strand of cDNA was synthesized as described by Gubler et al. (7).

Polymerase chain reactions (PCR): Two oligomers for amplification, ALAD1: 5'-CCGGAA TTCCAACCAACTGATGCCC, and ALAD2: 5'-GTTCTAGAGCCTGGCACTGTCCTCC, were chosen according to the published sequence of the ALAD cDNA (8), and designed with EcoRI site and XbaI site at the 5' terminus respectively. These oligomers correspond to the 5' untranslated and the 3' untranslated regions of ALAD cDNA, respectively. Amplifications were performed twice independently, and further steps were processed separately. Using a DNA Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT, USA), 5 cycles of amplification were carried out employing a thermal cycle program (94°C, 1 min; 60°C, 2 min; 72°C, 3 min), followed by 35 cycles of amplification using another program (94°C, 1 min; 57°C, 2 min; 72°C, 3 min, with an extension of 4 sec for each cycle).

Cloning of PCR products: PCR products were digested with EcoRI and XbaI, and cloned into pGEM4z vector (Promega, Madison, WI, USA) to transform JM109 cells. Existence of ALAD cDNA was confirmed by colony hybridization using rat ALAD cDNA as a probe (gift of Dr. T.R. Bishop) (9).

Sequencing of cDNAs: Appropriate restriction fragments were subcloned into M13mp18 or M13mp19. DNA sequencing was carried out by the dideoxy chain-termination method using the genetically engineered T_7 DNA polymerase (Pharmacia, Sequenase version 2.0; United States Biochemical, Cleveland, OH, USA) and [α-^{35}S]-dATP (10,11). Sequence data were analyzed using DNASIS software (Hitachi, San Bruno, CA, USA).

Message Amplification Phenotyping: This study was performed according to the method described previously (12,13). Total RNA was purified according to Cathala et al. (14). The cDNA was synthesized in a 20 µl reaction mixture containing 1 µg of total RNA in the reverse transcriptase reaction buffer (Bethesda Research Laboratories [BRL], Gaithersberg, MD, USA), 5 mM dithiothreitol, 5 pM p(dN)$_6$ (Pharmacia), 1 mM each of dNTP (dATP, dCTP, dGTP, dTTP), 20 units of rRNasin (Promega), 200 units of MMLV reverse transcriptase (Superscript; BRL). Samples were incubated at 45°C for 1 h, and the incubation was terminated by heating at 95°C for 5 min followed by quick-chilling on ice. Target sequences were amplified in a 100 µl reaction mixture diluting the reaction in the Taq buffer (Perkin-Elmer-Cetus), 1 µM of each primer (ALAD1 and ALAD2), 1 unit of PerfectMatch Polymerase enhancer (Stratagene, La Jolla, CA, USA), 2.5 units of Taq polymerase (AmpliTaq; Perkin-Elmer-Cetus). Forty-five cycles of amplification were carried out using a thermal cycle program (94°C, 1 min; 57°C, 2 min; 72°C, 3 min, with an extension of 4 sec for each cycle).

Oligonucleotide hybridization: Ten µl, i.e., one-tenth volume of the amplified samples, were subjected to electrophoresis on 1.2%[w/v] agarose gel to evaluate the efficiency of the reaction, and processed further for hybridization with an oligomer. Oligomers were labelled with ^{32}P at the 5' termini by phosphorylation using T_4 polynucleotide kinase (Pharmacia). The hybridization was carried out as described previously (15).

RESULTS AND DISCUSSION

Cloning of the mutant ALAD cDNA: In order to examine the mutation of the ALAD gene, cDNA was synthesized by MMLV reverse transcriptase from poly(A)$^+$ RNA purified from EB-virus transformed lymphoblastoid cells of the proband. ALAD cDNA sequence was amplified by PCR, and cloned into pGEM4z vector to construct libraries. The amplification and the construction of libraries were performed twice independently in order to affirm the

results. One clone each was isolated from each library, and termed pADPg2.1, and pADPg2.2. Determination of nucleotide sequences of these clones indicated that there were two different mutations in the ALAD gene of the proband's cells, as judged by comparison with the published normal sequence for ALAD (8). One was a substitution of C for T at nucleotide residue 168, while the other was a substitution of A for G in the normal ALAD gene at nucleotide residue 820. The former substitution had no effect on amino acid residues, while the latter resulted in an amino acid change from Ala to Thr at amino acid residue 274. Thus we conclude that the mutation in the ALAD gene responsible for enzyme deficiency, termed 'G2', was the one which led to amino acid substitution at residue 274. pADPg2.1 had 2 additional base substitutions, one was T for C at nucleotide residue 414, and the other was C for T at nucleotide residue 463. Neither base substitution, however, resulted in amino acid changes. pADPg2.2 also had an additional substitution of A for G at nucleotide residue 164. These three base substitutions are likely to be the result of an artefact known to occur with PCR (16), since the other clone had a normal residue in the corresponding nucleotide sequence.

Characterization of the ALAD phenotype in the proband: In order to determine the phenotype of the proband, clones isolated from each library were hybridized with an oligomer specific for the G2 mutation. The synthetic oligonucleotide of 17 bases containing the substitution $G^{820} \rightarrow A$ (Fig. 1) hybridized with 2 out of 5 clones, and 2 out of 4 clones isolated from each library, respectively (data not shown). The fact that approximately 50% of these clones hybridized with the probe confirms that the nucleotide substitution at residue 820 represents one of the mutant alleles that characterize the abnormal ALAD gene in the proband. In addition, the fact that approximately one-half of the clones did not hybridize with the probe strongly suggests that there must be yet another mutant ALAD allele in the proband's cells, which could be termed 'non-G2', since his cells had hardly any enzyme activity.

This study thus represents the first demonstration of a molecular defect of the ALAD gene in a patient with ADP. Previous studies on ADP suggested that probands for this condition might be homozygous for the ALAD defect (2,3). Our findings in this study, however, clearly show that the proband in this family is doubly heterozygous for two independent mutant alleles for ALAD deficiency (Fig. 3).

ALAD phenotype in the family: Using the specific oligomer (Fig. 1) as the probe, we carried out studies on the ALAD phenotype in other members of the proband's family.

```
5'-TCCCTCTCGCCGTGTAC-3' :Normal
                *
5'-TCCCTCTCACCGTGTAC-3' :G2 Mutation
```

Figure 1. The specific oligonucleotides used for hybridization with amplified RNA by PCR. The asterisk indicates the position of the base change.

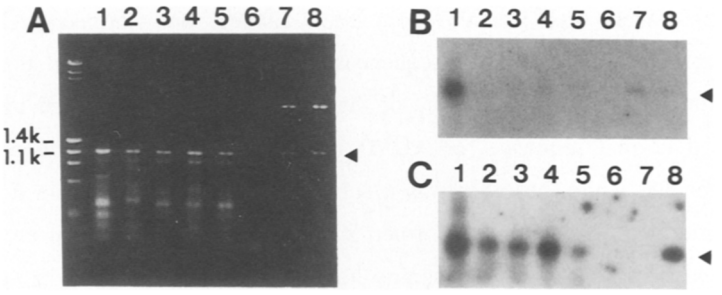

Figure 2. Message amplification phenotyping studies in members of the proband's family. Complementary DNA was synthesized in the presence or absence of total RNA purified from EB-virus-transformed lymphoblastoid cells. PCR was carried out using the synthetic oligonucleotides, ALAD1 and ALAD2. The amplified DNA was fractionated by agarose gel electrophoresis and hybridized with the specific oligomers. The gel also contained a G2-mutation positive clone, pADPg2.1, and a negative clone, pADPg1.1, isolated from the proband's library. Arrowheads indicate the position of ALAD cDNA. **A**: PCR products fractionated on 1.2% agarose gel, **B**: An autoradiogram of PCR products hybridized with the oligomer specific for the G2 mutation, **C**: An autoradiogram of PCR products hybridized with the oligomer specific for the normal sequence. **1**: proband, **2**: mother, **3**: sister, **4**: brother, **5**: normal subject, **6**: without RNA, **7**: pADPg2.1, **8**: pADPg1.1

Both the proband's RNA and pADPg2.1 hybridized, as expected, with the oligomer specific to the G2 mutation (Fig. 2B, lanes 1 and 7). However, other members of the family, i.e., mother, sister and brother who were heterozygous for the ALAD deficiency (2,4), as well as a normal subject, did not hybridize with the probe (Fig. 2B, lanes 2-5). All members examined, including the proband, had a normal residue at this site in the sequence (Fig. 2C, lanes 1-4). These results suggest that these subjects share the non-G2 allele for ALAD

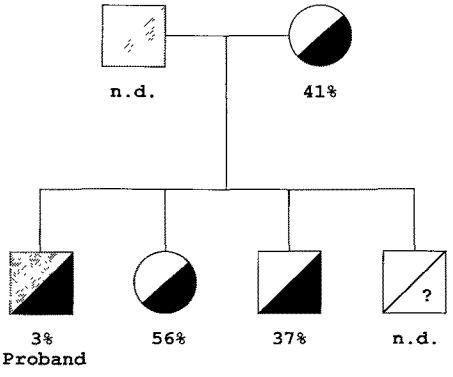

Figure 3. The pedigree of the ALAD phenotype in the proband's family. The ALAD phenotype of family members was determined by oligomer-specific hybridization. ALAD activity in lymphoblastoid cells for each subject (4) is also shown, as the percentage of the normal mean. Dotted, solid, and open areas represent the mutant allele with the G2 mutation, the mutant allele with the presumed non-G2 mutation, and the normal allele, respectively. The phenotype of the youngest brother and that of the father could not be determined, for reasons noted in the text. Their heterozygosity, however, is unequivocally documented by abnormally decreased ALAD activity in their erythrocytes (≈ 50% of the normal mean) (2). The father's phenotype was deduced from his heterozygosity for ALAD deficiency, as well as from the type of mutation found in his wife and in the proband.

deficiency (Fig. 3). A G2-negative clone, pADPg1.1, also isolated from the proband's library, hybridized with the oligomer specific for the normal sequence (Fig. 2C, lane 8). Thus it is highly likely that pADPg1.1 represents the non-G2 mutant allele of the proband's ALAD gene. Studies on the characterization of pADPg1.1 are in progress in our laboratories. It should also be noted that the G2 mutation in the proband must have been inherited from his father (Fig. 3), since his mother did not carry this allele (Fig. 2B, lane 2). For reasons beyond our control, it was not possible to conduct further studies of the father or the youngest brother in this family.

The G2 mutation resulted in a substitution from Ala to Thr at amino acid residue 274. This site is located down-stream of the lysine residue in the presumed catalytic site of the enzyme (8). The substitution from Ala to Thr is expected to cause a relatively minor change in the conformation of the enzyme, since these amino acids are very similar. This point mutation, however, must have a significant effect on enzyme activity, since ALAD activity in erythrocytes (2) and lymphocytes of the proband (4) was close to nil. Availability of pADPg2.1, which has the only one amino acid substitution at this residue, should enable us to examine the role of this amino acid in regulating the enzyme activity by expressing the abnormal gene in appropriate systems; such studies are underway.

ACKNOWLEDGMENTS

We are grateful to Drs. Hubert de Verneuil and Hiroshi Nakazato for their helpful advice, and to Dr. Terry R. Bishop for providing us with rat ALAD cDNA. This work was supported in part by grants from U.S.P.H.S. DK-32890, ES-01055, The Suntory Fund for Biomedical Research, and Deutsche Forschungsgemeinschaft (DO 134). The excellent technical assistance of Ms. Luba Garbaczewski is gratefully acknowledged.

REFERENCES

1. Kappas, A., Sassa, S., Galbraith, R.A. & Nordmann, Y. (1989) In The Metabolic Basis of Inherited Disease, 6th ed. (Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D., eds.), McGraw-Hill Book Co., New York, pp.1305-1365.
2. Doss, M., Benkmann, H.-G. & Goedde, H.-W. (1986) Clin. Genet. 30, 191-198.
3. Hassoun, A., Verstraeten, L., Mercelis, R. & Martin, J.-J. (1989) J. Clin. Chem. Clin. Biochem. 27, 781-786.
4. Sassa, S., Fujita, H., Doss, M., Hassoun, A., Verstraeten, L., Mercelis, R. & Kappas, A. (1990) Eur. J. Clin. Invest. in press.
5. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
6. Aviv, H. Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412.
7. Gubler, U. & Hoffman, B.J. (1983) Gene 25, 263-321.
8. Wetmur, J.G., Bishop, D.F., Cantelmo, C., Desnick, R.J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 7703-7707.
9. Bishop, T.R., Cohen, P.J., Boyer, S.H., Noyes, A.N., Frelin, L.P. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5586-5572.
10. Sanger, F., Nicklen, S. & Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467.
11. Messing, J. Crea, R., Seeburg, P.H. (1981) Nucl. Acids Res. 9, 309-321.

12. Brenner, C.A, Tam, A.W., Nelson, P.A., Engelman, E.G., Suzuki, N., Fry, K.E. & Larrick, J.W. (1989) Biotechniques 7, 1096-1103.
13. Rappolee, D.A., Wang, A., Mark, D. & Werb, Z. (1989) J. Cell. Biochem. 39, 1-11.
14. Cathala, G., Savouret, J.F., Mendez, B., West, B.L., Karlin, M., Martial, J.A. & Baxter, J.D. (1983) DNA 2, 329-335.
15. Wood, W.I., Gitshier, J., Lasky, L.A. & Lawn, R.M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1585-1588.
16. Tindall, K.R. & Kunkel, T.A. (1988) Biochemistry 27, 6008-6013.