determined by enzyme immunoassay[6,18,22]. In type III citrullinemia almost no ASS activity was detected in the liver, kidney or cultured skin fibroblasts even when much higher concentrations of the substrates were used for the assay[5,6,18]. A very small amount of the CRIM (cross-reactive immune materials) having the same size as normal ASS protein, however, could be detected in the liver of type III patients by means of a sensitive enzyme immunoassay[14,18,22], suggesting that ASS mRNA of normal size is produced by the normal splicing process in the tissues of patients with abnormal splicing mutation[17,18].

(3) Diagnosis

In general, CTLN1 is characterized by laboratory findings such as a marked elevation of serum/plasma citrulline (2,500 ± 1,040 nmol/ml, control range: 20-40), a decrease of plasma arginine (58 ± 31 nmol/ml, control: 80-130) and hyperammonemia. CTLN1 is diagnosed by the findings of 40 to 200 times normal plasma citrulline, a decrease of plasma arginine, and a decreased ASS activity in all tissues and cells. At present, we are able to clinically detect 32 mutations in ASS gene of CTLN1 patients using genomic DNA by PCR/Southern (a combination of PCR and restriction enzyme digestion) and are performing prenatal diagnosis and carrier detection.

3.3. Adult-onset type II citrullinemia (CTLN2)

(1) Clinical features

The most characteristic feature of CTLN2 is the late onset of serious and recurring symptoms, varying from 11 to 72 years old (34.4 ± 12.8, n=102)[41]. We represent the age of onset as the age when a significant clinical abnormality first appeared[2,4,7,8,10-13,20-25,41-50], although it is somewhat arbitrary since several CTLN2 patients have some sort of difficulty during infancy or early childhood. Most CTLN2 individuals suffered from a sudden disturbance of consciousness associated with flapping tremor, disorientation, restlessness, drowsiness and coma, and the majority died mainly of cerebral edema within a few years of onset.

Many patients have been found at clinics for psychiatry and neurology because they show mental and neurological symptoms such as nocturnal delirium, behavioral aberrations, convulsive seizures, delusions, and/or loss of memory. In infancy, most of them show no symptoms. Delayed mental or physical development has occurred in some. The progress of the psychoneurotic symptoms can generally be divided into three periods[21]. In the first period, there are recurring disturbances of consciousness lasting from one night to several days. In the second period, the episodic disturbances become more frequent and bizarre behavior begins to appear, including manic episodes,

echolalia and frank psychosis. At this stage motor weakness or paralysis of the limbs is often seen, as well as dysarthria. Patients become difficult to arouse. Muscle spasms, myoclonus and hyperreflexia as well as pathologic reflexes occur. In acute cases, systemic convulsive seizures already appear in this period. In the third period, a highly wasted condition occurs and the brain shows neurological changes of the pseudoulegyric or ischemic type.

(2) Other findings

Many CTLN2 patients show an unbalanced diet. In half of the patients, a peculiar fondness for beans, peanuts, egg, fish and/or meat has been noted from early childhood, usually associated with a dislike for rice, vegetables, fruits and sweets. None have been alcoholics; in fact, several have shown intolerance for alcohol. Many patients are thin (low body weight), several have emaciation without other physical abnormalities, and some show endocrine abnormality in the form of a slight pituitary dwarfism. The ratio of male to female was 77 to 26 in 103 patients examined in our laboratory[41]. It has also been found that a few CTLN2 patients are associated with pancreatitis (10% of the patients examined in our laboratory), hepatoma (8%) or hyperlipidemia (5%).

CTLN2 patients show almost no or, if any, mild liver disfunctions. Portal-systemic shunts have been excluded by angiography in all of the CTLN2 patients. Pathological findings in the liver include fatty infiltration and mild fibrosis. Patients present abnormal behaviour with neurological symptoms that closely resemble those of hepatic encephalopathy, but brain CT is normal. Electroencephalograms (EEG) show diffuse slow waves with the occasional appearance of triphasic waves. Neuropathologically, characteristic findings at autopsy are brain edema with cerebellar tonsilar herniation, laminoid necrosis with spongy formation in the cerebral cortex, and Alzheimer type II glia.

(3) Biochemical investigations

The most prominent characteristics of CTLN2 are that a quantitative decrease of ASS protein (15.4 ± 12.6 % of control, n=96)[41] is observed specifically in the liver but not in the kidney or cultured skin fibroblasts[4,10-13], resulting in hyperammonemia, an increase of serum citrulline (521 ± 290 nmol/ml, n = 96), a slight increase of serum arginine (232 ± 167 nmol/ml, n = 91), and a significant elevation of urinary argininosuccinate excretion (106 ± 40 nmol/mg of creatinine, n=9; control: 21.3 ± 6.4, n=11)[41-43].

It has been reported that hyperammonemia is not present consistently in citrullinemia and, if present at all, occurs only during the postprandial period. In CTLN2 patients, blood ammonia frequently shows diurnal rhythms, probably due to

dietary intake: it is low in the morning and increases in the evening, and the diurnal rhythms disappear during fasting[44]. Serum citrulline levels of CTLN2 patients (5-30 times those of the controls) are not as high as those of CTLN1 patients (usually more than 40 times those of controls) and often fluctuate during their clinical courses[42].

One big difference between CTLN2 and CTLN1 is that the serum arginine levels of CTLN2 patients are higher than in controls, whereas CTLN1 patients are arginine-deficient [3,41,42]. This phenomenon can be explained by the difference in ASS activity in the kidney between CTLN2 and CTLN1, and by a widely accepted theory about the origin of the circulating arginine[27,28]. According to the theory, circulating arginine is derived from arginine synthesized by the action of ASS and ASL on citrulline in the kidney, which is synthesized from glutamate via ornithine in the intestine under normal conditions. Under citrullinemic conditions, citrulline is released in large quantities from the liver owing to the urea cycle defect. Citrulline accumulated in the circulation cannot be converted to arginine in the case of CTLN1 patients because they have a defect of ASS in the kidney, but citrulline is converted efficiently to circulating arginine in the kidney of CTLN2 patients because they have normal renal ASS activity. This is supported by the finding that the concentration of argininosuccinate, the product of the defective enzyme (ASS), was found to be elevated in the urine of CTLN2 patients[43].

It is also a prominent characteristic of CTLN2 that the serum levels of alanine, serine, glycine and branched-chain amino acids are significantly lower than in the controls[42], in spite of the fact that serum alanine levels are usually higher in hyperammonemia. As a result, the ratio of threonine to serine is characteristically higher (2.43 ± 1.12, $n=83$)[41,42], and the ratio of branched-chain amino acids to aromatic amino acids lower than the controls[42]. The mechanisms underlying such changes in serum amino acids except citrulline and arginine remain unclear.

(4) Abnormal localization of ASS in the lobulus

In the controls, ASS was located only in hepatocytes, not in bile duct cells or endothelial cells, and was distributed almost homogeneously in the lobulus with slightly denser staining in the periportal region than in the pericentral region where glutamine synthetase is located[3,45,46]. This distinct distribution of the urea cycle enzymes and glutamine synthetase may play a role in the nitrogen metabolism of the liver[51].

CTLN2 is characterized by a liver-specific deficiency of ASS protein. One question was whether the decreased ASS content in the liver of CTLN2 patients represented an even decrease in all hepatocytes or an uneven decrease. Immunohistochemical analysis with the antisera to ASS revealed that the reduction in ASS protein does not invariably occur homogeneously in the liver of CTLN2 patients.

There were two types of ASS protein distribution[3,45,46]; one homogeneous and the other clustered. The clustered type of ASS distribution was seen in the liver of CTLN2, but not in CTLN1, hepatitis, liver cirrhosis or hepatoma. Furthermore, the clustered enzyme distribution was observed only with ASS, not with arginase or aldolase B in CTLN2 livers.

The ASS-specific heterogeneous or clustered distribution was observed in 14 out of 25 CTLN2 patients examined[46]. There were no differences between the homogeneous and clustered types in age, sex, hepatic ASS activity or serum citrulline level. Nevertheless, the clinical prognosis was quite different: the fatality of the CTLN2 patients who belong to the clustered heterogeneous type was much higher than that of the homogeneous type[46]. The cause of the uneven distribution of ASS in the liver of CTLN2 patients and its relation to the pathogenesis of CTLN2 remain unresolved.

(5) Increased expression of hepatic PSTI

Differential mRNA display analysis, which was introduced to understand the detailed pathophysiology and pathogenesis, revealed that the pancreatic secretory trypsin inhibitor (PSTI) gene was highly expressed in the liver of CTLN2 patients[47]. We also found that the concentration of PSTI protein was higher in the liver of CTLN2 patients (0.368 ± 0.954 μg/g liver, n=52) than controls (0.002 ± 0.001, n=28) and there was a significant increase in serum PSTI level (73.2 ± 66.2 ng/ml, n=29, control: 11.5 ± 2.5, n=28) with no change in the other serum markers of pancreatitis, cancer or acute-phase response, such as elastase 1, trypsin, phospholipase A2, α-fetoprotein, CA19-9 or C-reactive protein[41]. In addition to the serum amino acid pattern, increase of serum PSTI level is useful as a diagnostic marker for CTLN2. However, the mechanisms underlying the increase in hepatic PSTI mRNA remain unresolved.

(6) Liver transplantation

Most CTLN2 patients present mental and neurological symptoms such as the sudden appearance of behavioral aberrations, tremor, disorientation, restlessness and coma. The biochemical findings, which include hyperammonemia, hypercitrullinemia, a mild elevation in serum arginine and an increase in urinary argininosuccinate excretion, result from a specific decrease in hepatic ASS level. However, there have been no effective pharmacological treatments, and the disease management is difficult because the clinical course of the patients is unpredictable and many patients undergo sudden deterioration with progressive cerebral edema. Liver transplantation remains the only therapeutic option for the majority of CTLN2 patients, who would otherwise face possible death from brain edema[48-50]. Since the first liver transplantation was performed in 1988 at the University of Pittsburgh[48], 15 CTLN2 patients have been

treated with liver transplantation. All the metabolic abnormalities (ammonia, citrulline, arginine and PSTI levels in the blood) were corrected and the neurological symptoms disappeared[7,41,48-50], supporting the hypothesis that the CTLN2 is a liver-specific disorder. The first patient treated with the liver transplantation is still alive, in employment, and has no symptoms (Kumashiro R, personal communication).

3.4. Molecular genetics of CTLN2

(1) Identification of the disease gene

As summarized in Fig. 1, CTLN2 occurs in association with decreased ASS activity and protein in the liver, however, there are no apparent abnormalities in the hepatic ASS mRNA of CTLN2 patients[7,23,24]. RFLP analysis of 16 affected CTLN2 patients from consanguineous marriages suggested that the abnormality is not within the ASS gene locus[7]. We have found that 20% of patients are apparently from consanguineous parents, and siblings have been affected, indicating that CTLN2 is probably an autosomal recessive disorder. Since 1977, we have analyzed over 130 CTLN2 patients; the availability of DNA samples in CTLN2 patients from 18 consanguineous families greatly facilitated our genetic analyses, allowing us to use homozygosity mapping to delimit the critical region for the disease[8]. The SLC25A13 gene causing CTLN2 was identified in chromosome 7q21.3 by positional cloning and found to encode a putative calcium-binding mitochondrial carrier protein[8]. The human SLC25A13 gene spanned 160 kb of genomic DNA organized into 18 exons (Fig. 2)[8,26]. SLC25A13 is predicted to encode a 74 kDa protein comprising 675 amino acid residues, which we have named citrin[8]. In an autosomal recessive disease, homozygosity mapping is a powerful method to identify the disease gene, since only a small number of patients from consanguineous marriages are required for the analysis.

(2) Mutations in SLC25A13 gene of CTLN2

As shown in Fig. 3, we identified five distinct mutations in SLC25A13 gene of CTLN2 patients and confirmed their causative role in the disease[8]. The mutations include; [I] 851del4: a 4 bp deletion in exon 9, predicting a frameshift and introduction of a stop codon at position 286, leading to premature truncation of the protein. [II] IVS11+1G>A: a substitution at the 5'-end of intron 11 resulting in abnormal splicing and deletion of exon 11 in mRNA. This causes a loss of 53 amino acids (codons 340-392) within the first hydrophilic loop between the TM1 and TM2 domains of citrin. [III] 1638ins23: a 23 bp insertion in exon 16 that results in a frameshift at codon 554 and the addition of 16 new amino acids. A stop codon is introduced at position 570, leading to premature truncation of the C-terminus of citrin. [IV] S225X: a substitution

at position 674 in exon 7 that changes serine to a stop codon at position 225 and predicts premature truncation of the protein. [V] IVS13+1G>A: a substitution at the 5'-end of intron 13 resulting in abnormal splicing and the deletion of 27 amino acids (codons 411-437) between TM2 and TM3.

These mutant citrin could not locate into the mitochondria membrane after losing their C-terminal half and extension or after having their mitochondrial transmembrane spanning structures destroyed (Fig. 3). In a preliminary experiment by Western blot analysis with anti-human citrin antibody, we detected no cross-reactive immune material band in the liver of CTLN2 patients. This, together with the biochemical data showing decreased ASS protein in CTLN2 patients, suggests that citrin not only functions as a carrier protein but also plays a role in stabilizing ASS protein (Fig. 4).

(3) DNA diagnosis of CTLN2

Before identifying the SLC25A13 gene, we had diagnosed patients suffering from type II citrullinemia under criteria described previously[3-6,10-13,41-43] (including their symptoms, hyperammonemia, increased serum citrulline, arginine, ratio of threonine to serine and PSTI levels, and decreased hepatic ASS protein levels). Now, we have found 5 mutations in the SLC25A13 gene of CTLN2 patients and have established the diagnosis for each mutation using genomic DNA[8].

Using diagnostic methods for the 5 mutations of SLC25A13 gene, we tested about 100 CTLN2 patients who had been diagnosed with biochemical and enzymatic experiments. We found that 90% of the patients had one or more of the 5 mutations and 85% were diagnosed as compound heterozygotes or homozygotes (Yasuda et al., manuscript in preparation). The results show without a doubt that the SLC25A13 gene is the cause of adult-onset type II citrullinemia, and this is clearly in agreement with the classification of CTLN2 as diagnosed under our previous criteria. Through these findings, we are now able to say conclusively that CTLN2 is a genetic disease.

Surprisingly, 5 out of 21 patients from consanguineous union were found to be compound heterozygotes[8]. This suggests a very high incidence of the mutant genes among the Japanese population. On the basis of the proportion of consanguinity (about 20%), the incidence of CTLN2 has been calculated to be approximately 1 in 100,000[7]. In recent preliminary searching of general Japanese population, we found that the frequency of heterozygotes is 1-2 in 100 (Yamaguchi et al., manuscript in preparation). From the rates of carrier detected, the frequency of homozygotes with abnormal SLC25A13 genes is calculated to be 1-2 in 20,000.

4. Citrin, a New Calcium Binding Protein

4.1. Comparison of citrin with aralar

(1) Protein structure

The predicted SLC25A13 protein, designated citrin, consists of a polypeptide of 675 amino acids (74 kDa) and a bipartite structure: a C-terminal half with the characteristic features of the MCF members and an N-terminal extension harboring four EF-hand domains (Figs. 2 and 3)[8], and is closely related to a calcium-binding mitochondrial solute carrier protein, aralar[52]. The amino acid sequence of citrin is most similar to the human aralar (678 aa, 74 kDa) with 77.8% identity and to the C. elegans protein (Q21153) with 53.7% identity[8]. The alignment of citrin with aralar revealed a high degree of sequence conservation between both proteins with the exception of the N-terminal half and C-terminal end: in the N-terminal half, the residue 1-325 of citrin is 69.9% identical to 1-323 of aralar; in the C-terminal half, 326-625 of citrin is 86.7% identical to 324-623 of aralar; in the C-terminal end, 626-675 of citrin is 61.8% identical to 624-678 of aralar.

(2) Tissue distribution

The primary structure of citrin (SLC25A13, chromosome 7q21.3)[8,26] is most similar to that of aralar (SLC25A12, chromosome 2q24)[53], however, the expression pattern of SLC25A13 mRNA encoding citrin largely differed from that of SLC25A12 mRNA encoding aralar in tissue distribution and also in mRNA size (Fig. 2)[8]. The 981 bp fragment derived from the 5'-region of the citrin cDNA was used as a specific probe for Northern blot analysis, of which nucleotide sequence was 67% identical to the 975 bp sequence of aralar cDNA. Northern blot analysis using a blot of poly(A)$^+$RNA from human tissues revealed that SLC25A13 mRNA as a transcript of approximately 3.4 kb was expressed predominantly in the liver, moderately in the kidney, the heart, the pancreas and the placenta, slightly in the brain and skeletal muscle, but not in the lung. The same result was obtained with the 934 bp fragment from the 3'-noncoding region of SLC25A13 cDNA as probe, and the nucleotide sequence was 49.8% identical to the 900 bp of the aralar noncoding region. In contrast, SLC25A12 mRNA expression was not observed in the liver but was more prevalent in the heart, brain and skeletal muscle having two major bands of 2.8 and 3.3 kb in size. A less intense but apparently specific hybridization band 6.6 kb in size was also observed in Northern blots for aralar.

4.2. Mitochondrial localization

The C-terminal half of citrin as well as aralar has also a substantial similarity with MCF proteins (20-30% identity). All MCF members appear to consist of a tripartite

structure with each of the three repeated segments being about 100 residues in length. Each repeat contains two TM spanners. The typical mitochondrial carrier signature, GX3GX8PX(D/E)X(I/L/V)(K/R)X(R/K)XQX20-30GX4(Y/W)(R/K)GX9P, was included in the C-terminal half of citrin as well as in aralar[8,52,54]. Since the hydrophobic profile of the mitochondrial carriers shows six potential membrane-spanning helices, with both N- and C-termini of the protein and the loops between TM regions 2-3 and 4-5 possibly facing the cytosol, we were able to predict the structure of citrin localized in the mitochondria (Figs. 3 and 4) on the basis of the sequence homology and the hydrophobic profile.

It is well known that most MCF members are located in the inner membrane of the mitochondria[37]. We detected citrin in the mitochondria by using the expression of GFP-fusion protein in cultured cells and in the mitochondrial inner membrane obtained from subfractionation of mouse and rat liver by Western blot analysis using anti-citrin antibody (Iijima et al., manuscript in preparation). Our results agree with the demonstration by del Arco et al. using expression of Flag-tagged aralar2/citrin in HEK-293T cells[54]. The mitochondrial localization of aralar was also clarified by a transfection experiment using COS cells and detected in the brain by anti-aralar anibody[52].

4.3. Calcium binding property

There is a striking difference in the N- and C-terminal extension (Figs. 2 and 3), especially in the extreme long extension in N-terminal half, between citrin and aralar proteins and other MCF proteins (28-38 kDa). Aralar was originally cloned as a member of a subfamily of mitochondrial carrier that binds calcium[52]. Members of the calcium-binding mitochondrial solute carrier subfamily have a bipartite structure: the N-terminal half harbours four EF-hand domains whereas the C-terminal half has the characterictic features of MCF members. The presence of calcium binding domains in citrin, aralar and other calcium-binding mitochondrial carrier members[55] indicates that these proteins may be involved in calcium-regulated metabolite transporters.

On the other hand, searching the protein databases with the amino acid sequence of citrin revealed relationships with different Ca^{2+}-binding proteins[56], such as troponin C (P21798 and P02590), calcineurin B subunit (P42322), neuron specific calcium-binding protein (P41211) and vitamin D-dependent calcium-binding protein (P29377). The sequence of residues 68-78 in citrin was found in EF-hand Ca^{2+}-binding domain protein (BL00018) and the sequences 58-90 and 92-124 were in S-100/ICaBP type Ca^{2+}-binding protein (BL00303B). The N-terminal half of citrin contains four

possible EF-hand domains (residues 28-39, 66-77, 100-111 and 171-182), which are comparable to those in aralar and conserved in the other calcium-binding proteins.

To examine the possibility that the N-terminal half of citrin could bind calcium, we expressed recombinant His-tagged proteins using the pET system and tested their ability to bind $^{45}Ca^{2+}$ in an overlay assay. As shown in Fig. 5, one major protein band of around 35-38 kDa corresponding to the predicted size of N-half citrin (35 kDa, 1-284) and aralar (38 kDa, 1-312), appears in lysate of E. coli and purified samples, but not in C-half citrin (44 kDa, 285-675). Similar results have been demonstrated by del Arco et al. using His-tagged N-terminal regions of aralar2/citrin (9-278)[54]. Furthermore, del Arco et al. reported that by eliminating the first EF-hand domain of aralar2/citrin, the protein (37-278) loses its calcium binding capacity[54]. These results indicate that the N-terminal half of citrin is able to bind calcium and that the first EF-hand domain is required. This supports the notion that neither the third nor fourth EF-hand binds calcium and that calcium binding by EF-hands 1 and 2 is prevented by eliminating one of these EF-hands, as observed in other calcium binding proteins[57]. These results suggest that citrin shares with aralar the function of a calcium-binding mitochondrial solute carrier.

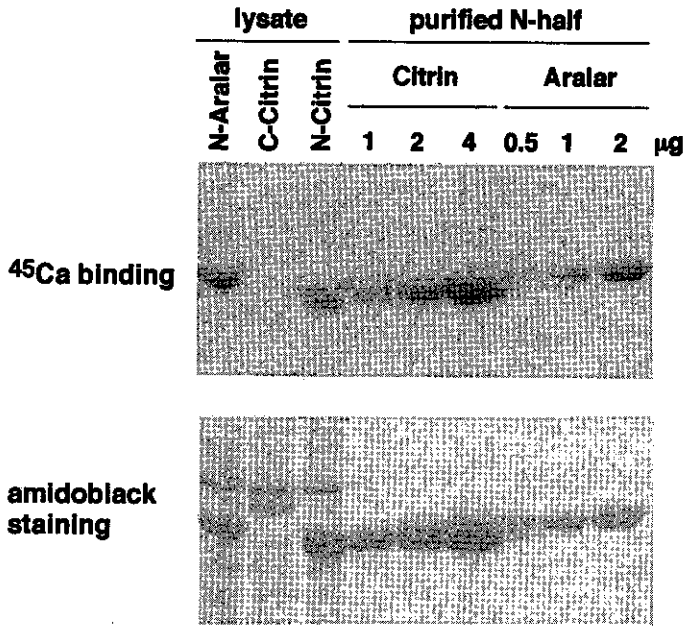

Fig. 5. Calcium binding activity by ^{45}Ca overlay assay. The His-tagged proteins; N-half citrin (1-284, 35 kDa), N-half aralar (1-312, 38 kDa) and C-half of citrin (285-675, 44 kDa) were expressed in E. coli, and purified. Aliquots of lysates or purified samples were separated by SDS-polyacrylamide gel electrophoresis, transfered to nitrocellulose membranes, and assayed for ^{45}Ca-binding. After exposure of the membrane, the transfered proteins were detected by amidoblack staining.

5. Perspectives

5.1. Are CTLN2 patients found only among Japanese?

CTLN2 appears to be almost exclusively found in Japan with a frequency of more than 1 in 100,000[7], but possible cases from Europe[58,59] and China[60] have also been reported. After publication of our study on SLC25A13 gene[8], we were able to analyze two Chinese patients with citrullinemia and found that the patients were diagnosed as CTLN2 with the same mutation as identified in Japanese CTLN2 patients (Hwu et al., manuscript in preparation). So it seems that CTLN2 is not restricted to Japan but exists at least in Asia. This suggests the possibility of a common ancestor among these CTLN2 patients. It is now possible to detect CTLN2 patients in other countries without difficulty, because we have sequenced the SLC25A13 gene[8,26].

5.2. Possible function of citrin

An interesting aspect of urea cycle function is the fact that enzymatic components in both mitochondrial matrix and cytosol appear to cluster in association with the mitochondrial membrane[61]. This has been proposed as an explanation for the apparent preferential use of endogenously generated intermediates by the enzymes of the urea cycle, a phenomenon known as "metabolic channeling". Citrin may have a central role in these interactions (Fig. 4). ASS and the two subsequent activities of the urea cycle may be arranged around the mitochondria, contributing to the channeling of urea cycle intermediates. On the other hand, Demarquoy et al. reported that ASS is associated with mitochondrial membrane, and the activity was mainly in mitochondrial fraction of fetal and newborn rat liver and in cytoplasmic fraction of adult rat liver[62]. It is possible that citrin forms a complex in some way with the three "soluble" cytoplasmic enzymes (ASS, ASL and ARG) of the urea cycle, especially ASS within the hepatocytes. If so, the loss of the organization in the channeling by the abnormal citrin would lead to a reduction of ASS protein possibly through its destabilization and/or degradation.

5.3. Role of Ca^{2+} in regulating citrin transporter

The supposition that SLC25A13 is indeed a calcium-binding solute carrier is based primarily on its sequence characteristics[8]. Citrin contains a high degree of amino acid identity with membrane-bound proteins of the mitochondrial solute carrier family and the same four EF-hand domains closest conserved in other calcium-binding proteins. Neither of these proteins have an obvious N-terminal mitochondrial import sequence. As observed with aralar[52] and efinal[55], citrin may also act as a calcium-

binding/transducing protein whereby its N-terminal half confers calcium sensitivity to the activity of its C-terminal half or to other proteins with which it may interact. Citrin and aralar, although structurally similar, have markedly different expression patterns. SLC25A13 is mainly expressed in the liver, kidney and pancreas, while SLC25A12 mRNA appears to be primarily expressed in the heart, brain and skeletal muscle[8]. Even if citrin and aralar serve to transport the same mitochondrial solutes, the specificity of CTLN2 pathology to the liver would still be explainable by the different tissue expression profiles of the genes.

The enzymes involved in citrulline-arginine-nitric oxide metabolism, ASS, ASL and nitric oxide synthase are expressed in rat pancreas islets and neurons[38-40]. It may be interesting that in the presence of stimulatory glucose, citrulline and argininosuccinate at physiological concentrations increase cytosolic Ca^{2+} concentration in rat β-cells[40]. On the other hand, mitochondrial Ca^{2+} transport is mediated by a complex system comprising at least three separate mechanisms[63]. Ca^{2+} influx is mediated primarily via a very fast Ca^{2+} uniporter which is energetically downhill, and efflux is mediated via both Na^{+}-independent and Na^{+}-dependent efflux mechanisms which are energetically uphill. Both Na^{+}-independent and Na^{+}-dependent transport takes place in mitochondria from a wide variety of tissues. However, Na^{+}-independent transport dominates in the liver and kidneys, whereas Na^{+}-dependent transport dominates in the heart, skeletal muscle and brain[63]. It is interesting that two independent Ca^{2+} efflux mechanisms exist in the mitochondrial inner membrane and the different tissue distribution between these two transport systems is similar to the difference of expression between citrin and alarar.

5.4. Unresolved phenomena

Although SLC25A13 gene has now been identified as the disease gene of CTLN2[8], many questions remain unresolved: (1) What is the function of citrin? (2) How do mutant citrin result in CTLN2? (3) Is there any effect of environmental factors, dietary and/or hormonal, on the formation of pathophysiology in CTLN2? What are those factors and mechanisms? (4) Why does non-liver-specific citrin cause liver-specific ASS deficiency? (5) What is the mechanism which increases the expression of PSTI gene? Furthermore, the cause of the clustered distribution of ASS protein in the liver of CTLN2 patients and the reason why CTLN2 is late-onset disease remain unresolved.

The identification of the disease gene for adult-onset type II citrullinemia provides us with a powerful molecular tool to clarify the mechanisms of various phenomena found in CTLN2 patients. The identification of the mutations in the SLC25A13 gene

of CTLN2 patients now allows us to establish simple and cost-effective carrier detection and diagnostic screening tests.

Acknowledgements

We thank M Gore for editorial assistance and S Nagata and A Sumiyoshi for secretarial assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, the Kodama Foundation for Research in Medical Science, and Health Sciences Research Grants in Research on Human Genome and Gene Therapy from the Ministry of Health and Welfare in Japan.

References

1. McKusick VA. Citrullinemia. in Mendelian Inheritance in Man (ed McKusick VA) vol. 3, pp.2093-2095 (Johns Hopkins University Press, Baltimore, 1998)
2. Walser M. Urea cycle disorders and other hereditary hyperammonemic syndrome. in The Metabolic Basis of Inherited Disease (eds Stanbury JB, Wyngaarden JB, Frederikson DS, Goldstein JL, Brown MS) pp.402-438 (McGraw-Hill, New York, 1983)
3. Saheki T, Kobayashi K, Inoue I. Hereditary disorders of the urea cycle in man: biochemical and molecular approaches. Rev Physiol Biochem Pharmacol 108: 21-68 (1987)
4. Saheki T, Ueda A, Hosoya M, Kusumi K, Takada S, Tsuda M, Katsunuma T. Qualitative and quantitative abnormalities of argininosuccinate synthetase in citrullinemia. Clin Chim Acta 109: 325-335 (1981)
5. Saheki T, Nakano K, Kobayashi K, Imamura Y, Itakura Y, Sase M, Hagihara S, Matuo S. Analysis of the enzyme abnormality in eight cases of neonatal and infantile citrullinemia in Japan. J Inherit Metab Dis 8: 155-156 (1985)
6. Saheki T, Kobayashi K, Ichiki H, Matuo S, Tatsuno M, Imamura Y, Inoue I, Noda T, Hagihara S. Molecular basis of enzyme abnormalities in urea cycle disorders: with special reference to citrullinemia and argininosuccinic aciduria. Enzyme 38: 227-232 (1987)
7. Kobayashi K, Shaheen N, Kumashiro R, Tanikawa K, O'Brien WE, Beaudet AL, Saheki T. A search for the primary abnormality in adult-onset type II citrullinemia. Am J Hum Genet 53: 1024-1030 (1993)

8. Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H, Crackower MA, Kondo I, Tsui L-C, Scherer SW, Saheki T. The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. Nature Genet 22: 159-163 (1999)
9. McMurray WC, Mohyuddin F, Rossiter RJ, Rathbun JC, Valentine GH, Koegler SJ, Zarfas DE. Citrullinuria: a new aminoaciduria associated with mental retardation. Lancet i: 138 (1962)
10. Saheki T, Tsuda M, Takada S, Kusumi K, Katsunuma T. Role of argininosuccinate synthetase in the regulation of urea synthesis in the rat and argininosuccinate synthetase-associated metabolic disorder in man. Adv Enzyme Regulation 18: 221-238 (1980)
11. Saheki T, Ueda A, Iizima K, Yamada N, Kobayashi K, Takahashi K, Katsunuma T. Argininosuccinate synthetase activity in cultured skin fibroblasts of citrullinemic patients. Clin Chim Acta 118: 93-97 (1982)
12. Saheki T, Ueda A, Hosoya M, Sase M, Nakano K, Katsunuma T. Enzymatic analysis of citrullinemia (12 cases) in Japan. Adv Exp Med Biol 153: 63-76 (1983)
13. Saheki T, Sase M, Nakano K, Yagi Y. Arginine metabolism in citrullinemic patients. in Guanidines (eds Mori A, Cohen BD, Lowenthal A) pp.149-158 (Plenum, New York, 1985)
14. Kobayashi K, Ichiki H, Saheki T, Tatsuno M, Uchiyama C, Nukada O, Yoda T. Structure of an abnormal messenger RNA for argininosuccinate synthetase in citrullinemia. Hum Genet 76: 27-32 (1987)
15. Kobayashi K, Jackson MJ, Tick DB, O'Brien WE, Beaudet AL. Heterogeneity of mutations in argininosuccinate synthetase causing human citrullinemia. J Biol Chem 265: 11361-11367 (1990)
16. Kobayashi K, Rosenbloom C, Beaudet AL, O'Brien WE. Additional mutations in argininosuccinate synthetase causing citrullinemia. Mol Biol Med 8: 95-100 (1991)
17. Kobayashi K, Shaheen N, Terazono H, Saheki T. Mutations in argininosuccinate synthetase mRNA of Japanese patients, causing classical citrullinemia. Am J Hum Genet 55: 1103-1112 (1994)
18. Kobayashi K, Kakinoki H, Fukushige T, Shaheen N, Terazono H, Saheki T. Nature and frequency of mutations in the argininosuccinate synthetase gene that cause classical citrullinemia. Hum Genet 96: 454-463 (1995)
19. Kakinoki H, Kobayashi K, Terazono H, Nagata Y, Saheki T. Mutations and DNA diagnoses of classical citrullinemia. Hum Mutat 9: 250-259 (1997)
20. Miyakoshi T, Takahashi T, Kato M, Watanabe M, Ito C. Abnormal citrulline

metabolism of Inose-type hepatocerebral disease. Shinkeikagaku (Japanese) 7: 88-91 (1968)
21. Inose T. Hepatocerebral degeneration, a special type. J Neuropath Exp Neurol 11: 401-408 (1952)
22. Imamura Y, Kobayashi K, Yamashita T, Saheki T, Ichiki H, Hashida S, Ishikawa E. Clinical application of enzyme immunoassay in the analysis of citrullinemia. Clin Chim Acta 164: 201-208 (1987)
23. Sase M, Kobayashi K, Imamura Y, Saheki T, Nakano K, Miura S, Mori M. Level of translatable messenger RNA coding for argininosuccinate synthetase in the liver of the patients with quantitative-type citrullinemia. Hum Genet 69: 130-134 (1985)
24. Kobayashi K, Saheki T, Imamura Y, Noda T, Inoue I, Matuo S, Hagihara S, Nomiyama H, Jinno Y, Shimada K. Messenger RNA coding for argininosuccinate synthetase in citrullinemia. Am J Hum Genet 38: 667-680 (1986)
25. Tsujii T, Morita T, Matsuyama Y, Matsui T, Tamura M, Matsuoka Y. Sibling cases of chronic recurrent hepatocerebral disease with hypercitrullinemia. Gastroenterologia Japonica 11: 328-340 (1976)
26. Sinasac DS, Crackower MA, Lee JR, Kobayashi K, Saheki T, Scherer SW, Tsui L-C. Genomic structure of the adult-onset type II citrullinemia gene, *SLC25A13*, and cloning and expression of its mouse homologue. Genomics, 62: 289-292 (1999)
27. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. Am J Physiol 241: E473-E480 (1981)
28. Funahashi M, Kato H, Shiosaka S, Nakagawa H. Formation of arginine and guanidinoacetic acid in the kidney in vivo: their relations with the liver and their regulation. J Biochem 89: 1347-1356 (1981)
29. de Jonge WJ, Dingemanse MA, de Boer PAJ, Lamers WH, Moorman AFM. Arginine-metabolizing enzymes in the developing rat small intestine. Pediatr Res 43: 442-451 (1998)
30. Li MX, Nakajima T, Fukushige T, Kobayashi K, Seiler N, Saheki T. Aberrations of ammonia metabolism in ornithine carbamoyltransferase-deficienct spf-ash mice and their prevention by treatment with urea cycle intermediate amino acids and an ornithine aminotransferase inactivator. Biochim Biophys Acta 1455: 1-11 (1999)
31. Gamble JG, Lehninger AL. Transport of ornithine and citrulline across the mitochondrial membrane. J Biol Chem 248: 610-618 (1973)
32. Bradford NM, McGivan JD. Evidence for the existence of an ornithine/citrulline antiporter in rat liver mitochondria. FEBS Lett 113: 294-298 (1980)
33. Palmieri L, de Marco V, Iacobazzi V, Palmieri F, Runswick MJ, Walker JE.

Identification of the yeast ARG-11 gene as a mitochondrial ornithine carrier involved in arginine biosynthesis. FEBS Lett 410: 447-451 (1997)
34. Indiveri C, Tonazzi A, Stipani I, Palmieri F. The purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria: electrical nature and coupling of the exchange reaction with H^+ translocation. Biochem J 327: 349-356 (1997)
35. Inoue I, Saheki T, Kayanuma K, Uono M, Nakajima M, Takeshita K, Koike R, Yuasa T, Miyatake T, Sakoda K. Biochemical analysis of decreased ornithine transport activity in the liver mitochondria from patients with hyperornithinemia, hyperammonemia and homocitrullinuria. Biochim Biophys Acta 964: 90-95 (1988)
36. Camacho JA, Obie C, Biery B, Goodman BK, Hu C-A, Almashanu S, Steel G, Casey R, Lambert M, Mitchell GA, Valle D. Hyperornithinaemia-hyperammonaemia-homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter. Nature Genet 22: 151-158 (1999)
37. Walker JE. The mitochondrial transporter family. Curr Opinion Structure Biol 2: 519-526 (1992)
38. Nakagawa S, Mizuma M, Ichiki H, Saheki T. Immunocytochemical demonstration of argininosuccinate synthetase in the neuronal structures of the intestinal tract and pancreas of the Japanese monkey. Acta Histochem Cytochem 24: 209-213 (1991)
39. Isayama H, Nakamura H, Kanemaru H, Kobayashi K, Emson PC, Kawabuchi M, Tashiro N. Distribution and co-localization of nitric oxide synthase and argininosuccinate synthetase in the cat hypothalamus. Arch Histol Cytol 60: 477-492 (1997)
40. Nakata M, Yada T, Nakagawa S, Kobayashi K, Maruyama I. Citrulline-argininosuccinate-arginine cycle coupled to Ca^{2+}-signaling in rat pancreatic β-cells. Biochem Biophys Res Commun 235: 619-624 (1997)
41. Kobayashi K, Horiuchi M, Saheki T. Pancreatic secretory trypsin inhibitor as a diagnostic marker for adult-onset type II citrullinemia. Hepatology 25: 1160-1165 (1997)
42. Saheki T, Kobayashi K, Miura T, Hashimoto S, Ueno Y, Yamasaki T, Araki H, Nara H, Shiozaki Y, Sameshima Y, Suzuki M, Yamauchi Y, Sakazume Y, Akiyama K, Yamamura Y. Serum amino acid pattern of type II citrullinemic patients and effect of oral administration of citrulline. J Clin Biochem Nutr 1: 129-142 (1986)
43. Saheki T, Kobayashi K, Inoue I, Matuo S, Hagihara S, Noda T. Increased urinary excretion of argininosuccinate in type II citrullinemia. Clin Chim Acta 170: 297-304 (1987)
44. Yajima Y, Hirasawa T, Saheki T. Diurnal fluctuation of blood ammonia levels in

adult-type citrullinemia. Tohoku J Exp Med 137: 213-220 (1982)
45. Saheki T, Yagi Y, Sase M, Nakano K, Sato E. Immunohistochemical localization of argininosuccinate synthetase in the liver of control and citrullinemic patients. Biomed Res 4: 235-238 (1983)
46. Yagi Y, Saheki T, Imamura Y, Kobayashi K, Sase M, Nakano K, Matuo S, Inoue I, Hagihara S, Noda T. The heterogeneous distribution of argininosuccinate synthetase in the liver of type II citrullinemic patients: its specificity and possible clinical implications. Am J Clin Pathol 89: 735-741 (1988)
47. Kobayashi K, Nakata M, Terazono H, Shinsato T, Saheki T. Pancreatic secretory trypsin inhibitor gene is highly expressed in the liver of adult-onset type II citrullinemia. FEBS Lett 372: 69-73 (1995)
48. Todo S, Starzl TE, Tzakis A, Benkov KJ, Kalousek F, Saheki T, Tanikawa K, Fenton WA. Orthotopic liver transplantation for urea cycle enzyme deficiency. Hepatology 15: 419-422 (1992)
49. Yazaki M, Ikeda S, Takei Y, Yanagisawa N, Matsunami H, Hashikura Y, Kawasaki S, Makuuchi M, Kobayashi K, Saheki T. Complete neurological recovery of an adult patient with type II citrullinaemia after living related partial liver transplantation. Transplantation 62: 1679-1681 (1996)
50. Kawamoto S, Strong RW, Kerlin P, Lynch SV, Steadman C, Kobayashi K, Nakagawa S, Matsunami H, Akatsu T, Saheki T. Orthotopic liver transplantation for adult-onset type II citrullinemia. Clin Transplantation 11: 453-458 (1997)
51. Häusinger D. Nitrogen metabolism in liver: structural and functional organization and physiological relevance. Biochem J 267: 281-290 (1990)
52. del Arco A, Satrústegui J. Molecular cloning of aralar, a new member of the mitochondrial carrier superfamily that binds calcium and is present in human muscle and brain. J Biol Chem 273: 23327-23334 (1998)
53. Crackower MA, Sinasac DS, Lee JR, Herbrick J-A, Tsui L-C, Scherer SW. Assignment of the SLC25A12 gene coding for the human calcium-binding mitochondrial solute carrier protein aralar to human chromosome 2q24. Cytogenet Cell Genet 87: 197-198 (1999)
54. del Arco A, Agudo M, Satrústegui J. Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitable tissues. Biochem J 345: 725-732 (2000)
55. Weber FE, Minestrini G, Dyer JH, Werder M, Boffelli D, Compassi S, Wehrli E, Thomas RM, Schulthess G, Hauser H. Molecular cloning of a peroxisomal Ca^{2+}-dependent member of the mitochondrial carrier superfamily. Proc Natl Acad Sci

USA 94: 8509-8514 (1997)
56. Kawasaki H, Kretsinger RH. Calcium-binding proteins. Protein Profile 1: 343-517 (1994)
57. Vito P, Lacaná E, D'Adamio L. Interfering with apoptosis: Ca^{2+}-binding protein ALG-2 and Alzheimer's disease gene *ALG-3*. Science 271: 521-525 (1996)
58. Vidailhet M, Levin B, Dautrevaux M, Paysant P, Gelot S, Badonnel Y, Pierson M, Neimann N. Citrullinemie. Arch Franc Ped 28: 521-532 (1971)
59. Roerdink FH, Gouw WLM, Okken A, Van del Blij JF, Haan GL, Hommes FA, Huisjes HJ. Citrullinemia, report of a case, with studies on antenatal diagnosis. Pediatr Res 7: 863-869 (1973)
60. Chow WC, Ng HS, Tan IK, Thum TY. Case report: recurrent hyperammonaemic encephalopathy due to citrullinaemia in a 52 year old man. J Gastroenterol Hepatol 11: 621-625 (1996)
61. Cheung C-W, Cohen NS, Raijman L. Channeling of urea cycle intermediates *in situ* in permeabilized hepatocytes. J Biol Chem 264: 4038-4044 (1989)
62. Demarquoy J, Fairand A, Gautier C, Vaillant R. Demonstration of argininosuccinate synthetase activity associated with mitochondrial membrane: characterization and hormonal regulation. Mol Cell Biochem 136: 145-155 (1994)
63. Gunter TE, Gunter KK, Sheu S-S, Gavin CE. Mitochondrial calcium transport: physiological and pathological relevance. Am J Physiol 267: C313-C339 (1994)