Human Molecular Genetics, 2005, Vol. 14, No. 8 ***1019–1027***
doi:10.1093/hmg/ddi094
Advance Access published on March 3, 2005

A unique exonic splice enhancer mutation in a family with X-linked mental retardation and epilepsy points to a novel role of the renin receptor

Juliane Ramser[1,7], Fatima E. Abidi[2], Celine A. Burckle[3], Claus Lenski[1], Helga Toriello[4], Gaiping Wen[5], Herbert A. Lubs[2,6], Stefanie Engert[1], Roger E. Stevenson[2], Alfons Meindl[1,7,*], Charles E. Schwartz[2] and Genevieve Nguyen[3]

[1]Institute of Human Genetics, Ludwig-Maximilians-University, Munich 80336, Germany, [2]J.C. Self Research Institute, Greenwood Genetic Center, Greenwood, SC 29646, USA, [3]Institut National de la Santé et de la Recherche Médicale INSERM U36, Collège de France, Paris 75231, France, [4]Spectrum Health, Grand Rapids, MI 49503, USA, [5]Institute for Molecular Biotechnology, Jena 07745, Germany, [6]Mailman Center Child Development, University of Miami School of Medicine, Miami, FL 33101, USA and [7]Department of Obstetrics and Gynaecology, Technical University, Munich 81675, Germany

Received December 20, 2004; Revised February 16, 2005; Accepted February 23, 2005 DDBJ/EMBL/GenBank accession nos[†]

The renin–angiotensin system (RAS) is essential for blood pressure control and water–electrolyte balance. Until the discovery of the renin receptor, renin was believed to be mainly a circulating enzyme with a unique function, the cleavage of angiotensinogen. We report a unique mutation in the renin receptor gene (*ATP6AP2*) present in patients with X-linked mental retardation and epilepsy (OMIM no. 300423), but absent in 1200 control X-chromosomes. A silent mutation (c.321C > T, p.D107D) residing in a putative exonic splicing enhancer site resulted in inefficient inclusion of exon 4 in 50% of renin receptor mRNA, as demonstrated by quantitative RT–PCR. Analysis of membrane associated-receptor molecular forms showed the presence of full-length and truncated proteins in the patient. Functional analysis demonstrated that the mutated receptor could bind renin and increase renin catalytic activity, similar to the wild-type receptor, but resulted in a modest and reproducible impairment of ERK1/2 activation. Thus, our findings confirm the importance of the RAS in cognitive processes and indicate a novel specific role for the renin receptor in cognitive functions and brain development.

INTRODUCTION

The renin–angiotensin system (RAS) is classically described as a circulating system with a single final endpoint, the generation of angiotensin II (Ang II), which is considered to be the major, biologically active peptide. This assumption has been based on initial observations that components of the RAS located upstream or downstream of Ang II do not have functional receptors, whereas Ang II controls blood pressure and fluid and salt balance through binding to its receptors AT1 and AT2. However, recent studies indicate that the situation is more complex than originally thought and that, in addition to the endocrine RAS, a tissue RAS appears to have a critical role in physiological processes such as development, learning and memory, tissue growth and in disease processes such as inflammation, macro- and microvascular hypertrophy and vascular remodelling, fibrosis and obesity (1). Moreover, the recent discovery of a functional (pro)

*To whom correspondence should be addressed at: Institute of Human Genetics, Ludwig-Maximilians-University, Goethestr. 29, Munich 80336, Germany. Tel: +49 8941402429; Fax: +49 8941404831; Email: alfons@pedgen.med.uni-muenchen.de
[†]NM_005765, BI599385 and AC092473

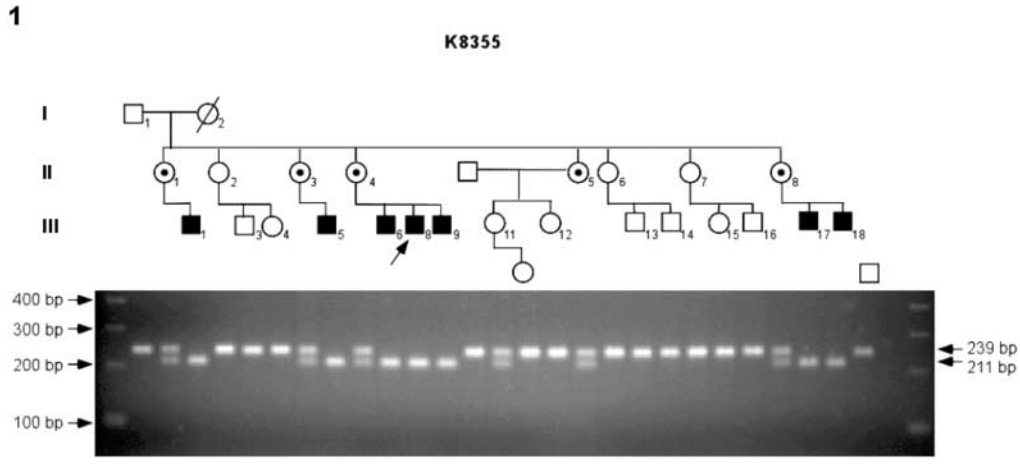

Figure 1. Pedigree of the XMRE family (K8355) and segregation of the c.321C > T transition identified in exon 4 of the *ATP6AP2* gene demonstrated by *Bfa* I restriction digest analysis. Usage of a modified forward primer introduces a C at position c.320, next to the c.321C > T alteration, thereby creating a *Bfa* I site in the sequence carrying the mutation. The designed primer in combination with a specific reverse primer amplifies a product of 239 bp that, after digestion with *Bfa* I, results in two fragments (211 and 28 bp) visualized on a 2.5% agarose gel. Affected males are indicated by black boxes and obligate carrier females with dotted circles. The patient from whom a cell line was utilized is marked with an arrow.

renin receptor (2,3) has forced the emergence of a new concept that casts renin as potentially playing a direct role in the tissue RAS. It is now established that all components of the RAS are also synthesized in the brain, with renin being synthesized by some neuronal populations but mainly by astrocytes (4,5). In the central nervous system, the role of the RAS includes the control of cell growth and death, neuro-endocrine regulation and cognitive properties (4,5). Here, we report the first mutation in the renin receptor that results in X-linked mental retardation (XLMR) and epilepsy (XMRE) and possibly affects the activation of the MAP kinases ERK1/2.

RESULTS

Family K8355 was diagnosed with XLMR and XMRE syndrome (OMIM no. 300432). Clinical findings are described in Materials and Methods and the pedigree is shown in Figure 1. Linkage analysis using 28 markers spaced along the X-chromosome localized XMRE in family K8355 to Xp11.23–Xp21.1 between markers *DXS1003* and *DXS1237*, an interval that is ~29 cM. During the initial phase of these studies, another group independently published linkage data for the same family (6). Their linkage interval was from Xp11.3 to Xp11.4 between markers *DX8054* and *DXS1049*, thereby reducing it at both the proximal and the distal site. This published 18 cM interval was confirmed in our lab by further typing of the two recombinants II-5 and III-1 (Fig. 1).

In order to identify candidate genes for XMRE, we compiled a gene catalogue between markers *DXS8054* and *DXS1049* and found 26 genes in the disease interval. One of the genes in the interval, *ATP6AP2*, encodes the renin receptor (Fig. 2, left). Recently, mutations in the angiotensin II receptor type 2 gene, *AGTR2*, were found to be associated with XLMR (7). Interestingly, seizures were also reported in many affected males. In order to further investigate the role of the RAS in neuronal processes in the central nervous system, we decided to evaluate the renin receptor gene, *ATP6AP2*, as a possible candidate for the XMRE phenotype. The *ATP6AP2* gene is composed of nine exons and encodes a 350 amino acid protein with one single transmembrane domain (2). The gene has been found to be widely expressed, with high expression in brain, heart and placenta, to a lower extent in liver, pancreas and kidney and weak expression in lung and skeletal muscle (2).

A silent C>T transition (c.321C > T) in the *ATP6AP2* gene

Direct genomic sequencing of all nine exons of the *ATP6AP2* gene in an affected male (III-8, Fig. 1) from family K8355 revealed a translationally silent C to T (c.321C > T, p.D107D) transition in exon 4 at nucleotide position 321 (Fig. 3A, left). In order to determine the consequence of this silent substitution at the level of expression of the *ATP6AP2* gene, we performed semiquantitative RT–PCR experiments (Fig. 3B). In patient III-8, the amplification of the cDNA encompassing exons 2–5 revealed the expected 554 bp wild-type fragment (E3–4) as well as a second fragment of 458 bp (E3–5) with similar intensity (Fig. 3B). Sequences for the primer pair used (1F/1R) are given in Materials and Methods ('Reverse transcription experiments'), their positions in the gene are shown in Figure 2. The 458 bp fragment detected in patient III-8 was very faint or not visible in 20 healthy controls (Fig. 3B). In addition, a third fragment with a length of 422 bp was observed in all controls and the patient (Fig. 3B). Analysis of an additional three cDNA samples prepared separately from a lymphoblastoid cell line of patient III-8 revealed the same two abundant fragments (E3–4 and E3–5). Subsequent sequencing of these two fragments demonstrated a C to T mutation in the 554 bp fragment (E3–4) and the loss of exon 4 in the 458 bp fragment (E3–5), hereafter referred to as Δ4-renin receptor (Fig. 3A, right). Furthermore, sequence analysis of the variably expressed 422 bp fragment revealed the lack of exon 2. This minor

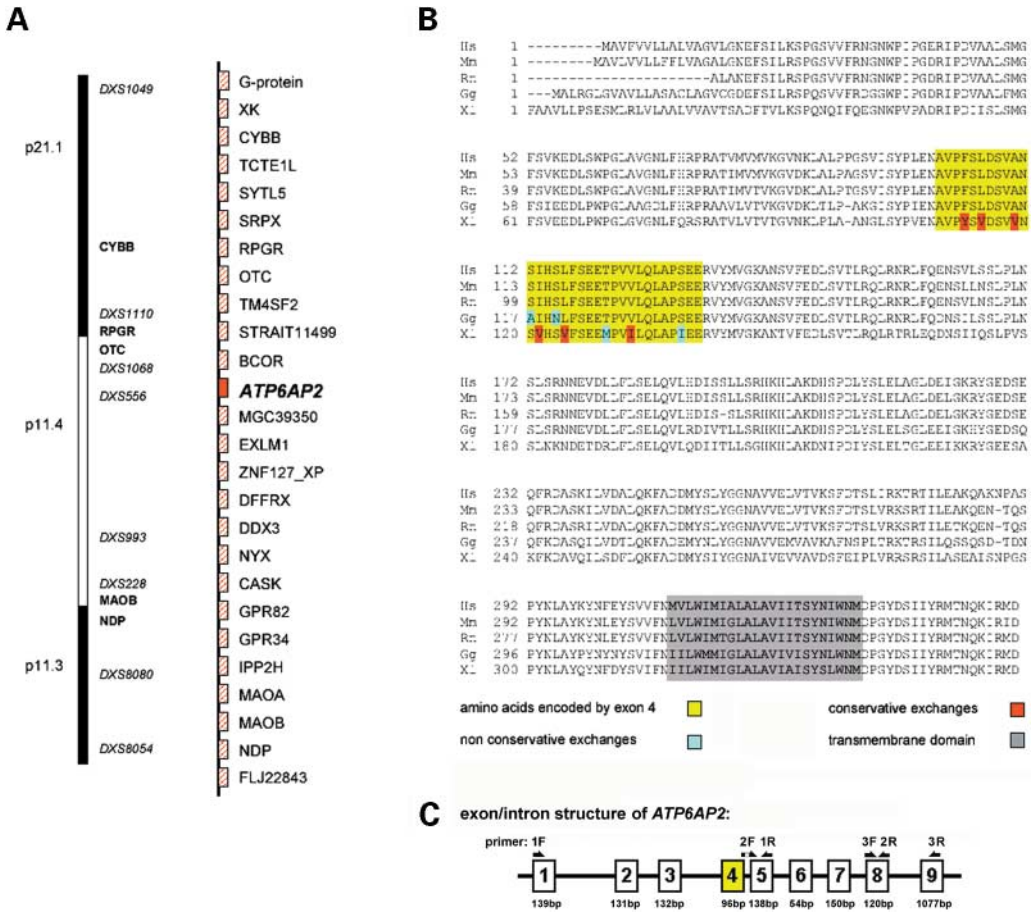

Figure 2. Transcription map of the candidate interval for XMRE and BLAST analysis of the renin receptor gene (*ATP6AP2*). (**A**) In total, 26 genes located in the candidate interval were screened for mutations including *ATP6AP2*. Screening was performed on genomic DNA and cDNA, except for one-exon-genes (*STRAIT11499*, *ZNF127_XP*, *GPR82* and *GPR34*). (**B**) BLAST analysis of the renin receptor gene *ATP6AP2*. Analysis of the renin receptor reveals high conservation of the 32 amino acids encoded by exon 4. Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*. (**C**) Schematic diagram of the exon–intron structure of the *ATP6AP2* gene. The sizes of the exons are given in base pairs. Arrows indicate the position of primers used for reverse transcription experiments. Primers 1F and 1R were used for amplification of the 554 bp cDNA fragment encompassing exons 2–5.

splice variant was found to be present in Genbank (accession no. BI599385).

The involvement of the observed c.321C>T transition in the exon-skipping event was investigated using different approaches. First, further comparison of the genomic DNA sequence from the patient and a healthy control failed to identify any additional substitution in either the 5883 bp intron 3 or the 182 bp intron 4, which flank exon 4. No aberration in the 2.3 kb 5′-UTR region that might affect the expression level of the gene was identified (data not shown). Screening of 600 healthy control males and 300 healthy females (1200 X-chromosomes in total) failed to detect the c.321C>T variant, whereas segregation of the variant within the family K8355 was clearly demonstrated (Fig. 1). The identified c.321C>T transition was found to be absent in 372 chromosomes from normal males tested by restriction digestion and 828 normal X-chromosomes (228 males and 300 females) examined by Denaturing High-Performance Liquid Chromatography (DHPLC). In order to exclude the other genes located in the candidate interval as causative for the disease, we sequenced all the 25 genes we compiled as the gene catalogue for the disease region, either at the genomic or at the RNA level and did not find additional mutations in the family. These included three genes (*RPGR*, *NYX* and *NDP*) in the screening that are presently exclusively associated with different eye diseases.

Mutation screening in an additional nine syndromic and five non-syndromic XLMR families linked to this interval failed to detect other mutations in the *ATP6AP2* gene. Only one of these families contained affected males who exhibit seizures.

The domain encoded by exon 4 is evolutionary conserved

The predicted protein sequences of the renin receptor from different species, derived from public cDNA- and EST-databases, show nearly complete conservation of the amino acid residues encoded by exon 4 (Fig. 2B). The complete identity between human and rodent renin receptor is striking. With the exception of two non-conservative amino acid changes, the sequence over a long stretch of 32 amino acids is remarkably conserved between human and frog (Fig. 2B).

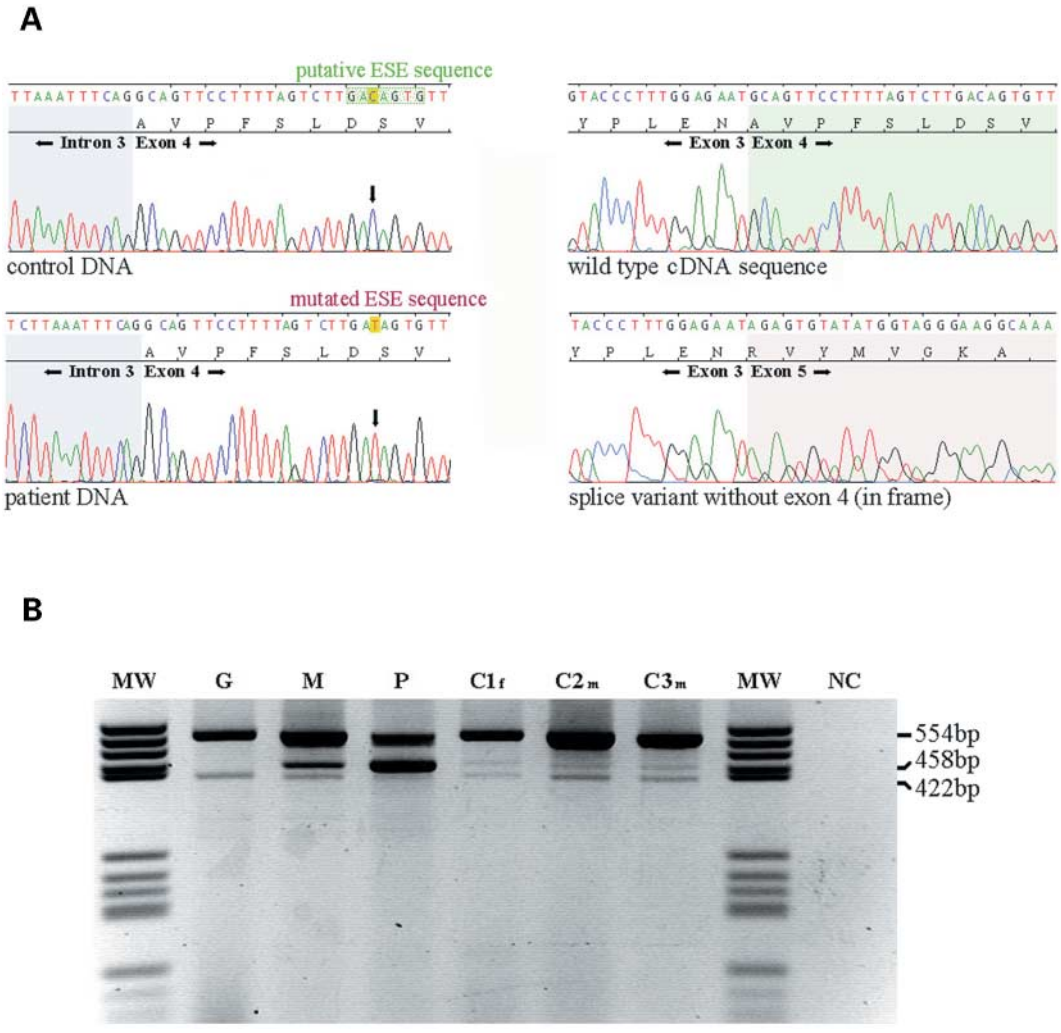

Figure 3. Mutation analysis of cDNA and genomic DNA from the XMRE family. (**A**) Sequence analysis of exon 4 of the *ATP6AP2* gene. (Left) Sequence of the exon/intron boundary of exon 4 in control and patient's genomic DNA. The C > T transition in the 7 bp putative ESE consensus sequence (yellow) is indicated with arrows. (Right) Wild-type cDNA with exon 3 followed by exon 4 and the splice variant lacking exon 4. (**B**) Semiquantitative RT–PCR experiments. Amplification of the cDNA encompassing exons 2–5 of the gene reveals, in addition to the expected 554 bp wild-type fragment, a fragment of similar intensity of 458 bp in the patient (P) lane. M, mother; G, grandfather; P, patient; C, controls; MW, molecular weight marker; NC, negative control.

Δ4-renin receptor expression

To elucidate the altered expression of the renin receptor gene in patient III-8 in more detail, we performed quantitative PCR with the Light Cycler system (Roche Diagnostics). Primer pairs were designed to specifically amplify wild-type and Δ4-fragments (see Materials and Methods and Supplementary Material, Fig. S1). Standard curves for both PCR systems were performed and showed equal efficiencies (Supplementary Material, Fig. S2). The experiments were carried out in triplicate and were repeated four times. The results demonstrated the significant upregulation of the Δ4-variant (E3–5) to >50% of the total expression of the two isoforms in the patient compared with 2.5% on average in control individuals. The levels of total mRNA in the patient and in the controls were normalized by performing dual-colour PCR using the human hypoxanthine-phosphoribosyl-transferase gene (*h-HPRT*) as an internal standard (Supplementary Material, Fig. S3). Interestingly, the levels of the wild-type fragment (E3–4) were very similar in both the patient and the control. This would mean that the amount of total mRNA of the gene was almost twice as high in the patient when compared with the control.

Δ4-receptor synthesis by the patient's cells

We compared the protein forms of the renin receptor expressed by the patient's lymphocytes (affected male III-8) with immortalized lymphocytes from a control subject. Metabolic labelling of the cells with ^{35}S-methionine followed by immunoprecipitation of the receptor showed the presence of two bands of 42 and 39 kDa, corresponding to full-length and Δ4-renin receptors, respectively (Fig. 4A).

Although the real time PCR showed that full-length and Δ4-mRNAs were expressed at similar levels, the results of

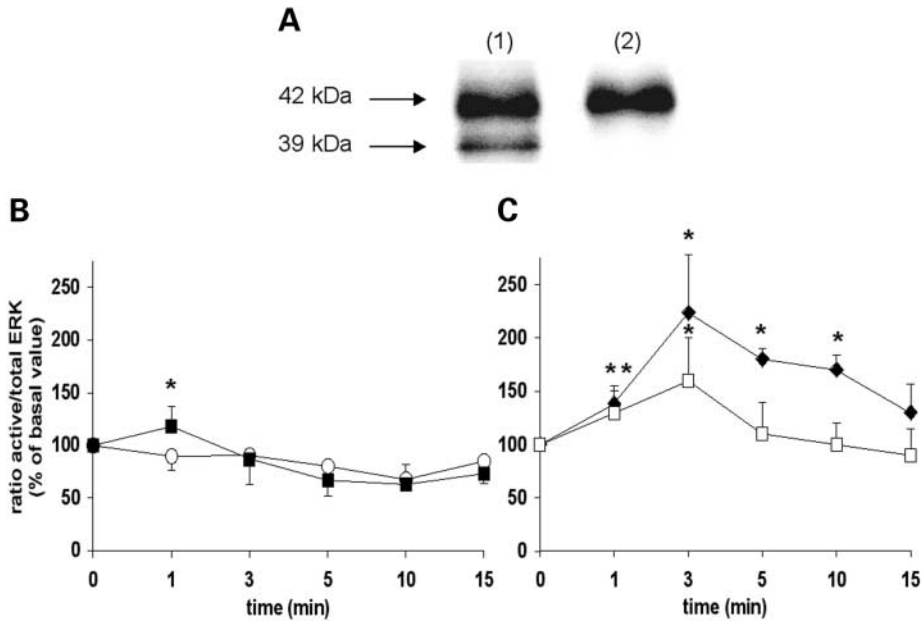

Figure 4. (**A**) Analysis of the protein forms of the renin receptor by metabolic labelling with ^{35}S-methionine and immunoprecipitation. Lane 1: full-length (42 kDa) and Δ4-renin receptors (39 kDa) in patient's cells; lane 2: control lymphoblastoid cells. (**B**) Time course activation of ERK1/2 in the patient's lymphocytes (open circle) and in the control lymphocytes (filled square) stimulated by renin (10 nM) in the presence of 1 μM losartan. The phosphorylation of ERK1/2 was studied by western blot and the intensity of the bands were scanned and analyzed using the NIH IMAGE software (Bethesda, MD, USA) (2). The ratio active/total ERK1/2 was normalized and plotted. (**C**) Time course activation of ERK1/2 in the patient's lymphocytes stimulated by thrombin 10 nM (filled lozenge) and in the human primary mesangial cell (open square) stimulated by renin 10 nM. The values represent mean ± SD of four independent experiments, except for mesangial cells (three experiments). $^{*}P < 0.05$ compared with time 0 (*t*-test).

the immunoprecipitation showed a lower intensity of the 39 kDa band, suggesting that either the translation of the Δ4-renin receptor was less effective or its turnover rate was different from that of the full-length receptor.

Binding of renin and activity of Δ4-receptor

Binding of renin to the Δ4-receptor was studied using ^{125}I-labelled renin. The results showed that the cells of the patient were able to specifically bind renin in a manner comparable to that of EBV-transformed lymphoblastoid cells of a control subject (Fig. 5). The renin receptor has been shown to act as a co-factor for increasing the catalytic efficiency of angiotensinogen cleavage by receptor-bound renin (2). To study the co-factor activity of the Δ4-receptor, the patient's and the control subject's cells were allowed to bind renin, and receptor-bound renin was tested for Ang I generation. The results were compared to Ang I generation by an identical concentration of renin in solution, measured by acid elution of membrane-bound renin from an aliquot of each type of cells (2). Unexpectedly, the mutated receptor retained its co-factor activity and renin bound to the lymphoblastoid cells of the patient displayed comparable activity to that of control cells and was more active than renin in solution (Fig. 5).

Δ4-receptor and ERK1/2 activation

Finally, we compared the characteristics of ERK1/2 activation induced by renin in four independent experiments. In the control cells, ERK phosphorylation was modest, rapid and very transient (Fig. 4B, filled square). The kinetics of ERK1/2 activation induced by renin were comparable to that observed in smooth muscle and mesangial cells (2), with a maximum at 3 min and return to basal value at 5 min (Fig. 4C, open square). Although a weak increase of ERK1/2 phosphorylation was seen in the control lymphocytes 1 min after renin stimulation, no activation could be observed in the patient's lymphocytes after that time (Fig. 4B, open circle). In contrast, a robust and sustained ERK1/2 activation could be measured in the presence of thrombin in the cells of the patient (Fig. 4C, filled lozenge). Overall, the replicated experiments suggest a slightly increased ERK1/2 activation by renin in control (117 ± 19%) compared with patient's cells (90 ± 14%).

DISCUSSION

Besides its classical involvement in cardiovascular and renal physiology, the RAS has been shown to be associated with a wide variety of other events ranging from tissue remodelling, inflammation and atherosclerosis to learning, memory and cognitive functions (4). The existence of an intrinsic RAS was demonstrated in the central nervous system and the eye (5,8). The importance of the RAS in the central nervous system was further demonstrated when some families with XLMR were found to have mutations in a gene of the angiotensin pathway, *AGTR2* (7). However, the role of some of the missense mutations and the frequency of mutations in *AGTR2* in non-specific XLMR are presently a matter of debate (9).

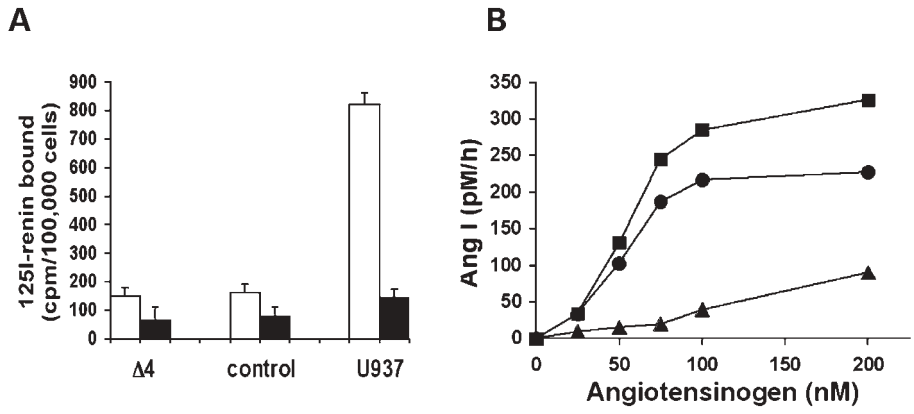

Figure 5. (**A**) ^{125}I-renin binding to patient (Δ4), to control lymphoblastoid cells and to U937 monocytic-macrophages cells. Total binding (empty bars) and non-specific binding (filled bars) were performed in the presence of 100 nM cold renin. The results represent mean ± SD of two independent experiments performed in triplicate. (**B**) Angiotensin I generation by equivalent concentrations of renin (250 pM) bound to patient's cells (square), to control cells (circle) (2×10^6 cells/point) or to renin in solution (triangle) in the presence of increasing concentrations of angiotensinogen.

We show that a silent mutation (c.321C>T, p.D107D) in the *ATP6AP2* gene results in XMRE. Analysis of the genomic sequence affected by the c.321C>T transition revealed that it resides in a putative exonic splice enhancer (ESE) site, which when mutated can cause exon skipping (10–12). Several sequences for putative ESE elements for different specific serine/arginine-rich (SR) proteins have been published. The 7 bp consensus sequence for the SF2/ASF protein has been reported to be (C≫G>A) (A>G>U>C) (C or G) (A>C≫U) (C or G) (G≫U) (A or U>G) (12). The wild-type sequence in our putative ESE motif is GACAGUG, which is converted into GATAGUG in K8355 (Fig. 3A, left). As described in a recent review article (11), silent mutations in putative ESE elements can predispose for exon-skipping events as in the case of proximal spinal muscular atrophy (13). However, a number of missense or silent mutations in different genes causing splice variants resulting in disease phenotypes have been described without revealing a common pattern or strictly conserved ESE motifs (11).

Mutations in such *cis*-elements that are responsible for the correct splicing of genes have also been associated with other disease entities (11). Likewise, in case of the *MAPT* gene, the alteration of a combined ESE/ESS (exonic splicing silencer) element in exon 10 leads to disruption of a proper ratio of two isoforms of the microtubule associated protein tau (in frame inclusion or exclusion of exon 10) that can give rise to fronto-temporal dementia and parkinsonism associated with chromosome 17 (FTDP17) (14).

In support of our result, Amsterdam *et al.* (15) used insertional mutagenesis to show that the *ATP6AP2* gene is one of a group of genes essential for early development in zebrafish. More importantly, the insertional mutation in the *ATP6AP2* gene led to an embryonic phenotype with no pigmentation, underdeveloped liver/gut, a smaller head, central nervous system necrosis by day 5 postfertilization and was lethal. Therefore, a mutation causing complete inactivation of the *ATP6AP2* gene obviously has a more dramatic consequence than the mutation seen in the XMRE family. Furthermore, the organ specific phenotype in the patients might be explained by a rather specific impairment of the renin receptor.

Our experiments indicate that wild-type mRNA of the *ATP6AP2* gene is not diminished in the patient. However, the fact that the Δ4-variant is increased in the patient to a similar level as the wild-type mRNA, makes it likely that functionally impaired renin receptor molecules are generated via dimerization (2). The observation that this altered renin receptor still binds renin and facilitates Ang I generation on the cell surface, indicating that the downstream angiotensin pathway is not involved in the disease, in contrast to the families with AGTR2 deficiency (7). Thus, our hypothesis is that the Δ4-variant of the renin receptor may prevent the recruitment of a yet unknown intracellular substrate that mediates ERK1/2 activation independently of the angiotensin pathway.

Noteworthy, the renin receptor organization is reminiscent to that of cytokine receptors (16–19). It is known that ligand binding induces the dimerization of such cytokine receptors that is accompanied by reorientation of domains within the receptor dimer (16,17). The importance of receptor flexibility and the need for conformational change of the dimer to recruit and activate its target protein have been demonstrated (18,19), and we, therefore, suggest that the Δ4-variant of the renin receptor prevents the flexibility of the receptor and precludes the necessary conformational change of the entire molecule necessary to recruit the postulated intracellular substrate.

The importance of the MAP kinases ERK1 and ERK2 in memory consolidation and long-term potentiation has been shown (20,21). Therefore, it might be reasonable to link the mental retardation and epilepsy observed in the affected males with XMRE to a slightly changed activation of these molecules by the impaired renin receptor. Indeed, these MAP kinases can integrate the signalling triggered by several ligands and receptors to activate downstream effectors such as CREB/CBP and RSK2 (22). Haploinsufficiency of CBP is associated with Rubinstein–Taybi syndrome that includes cognitive deficits presumably resulting from impairments of long-term-potentiation (23). Mutations in the RSK2 protein

are associated with Coffin–Lowry syndrome, another XLMR disorder (24). Finally, it is important to note that renin receptor interaction with renin was recently reported to be essential for human glial cell proliferation and survival (25).

In conclusion, our study provides the first evidence for the importance of the renin receptor in cognitive function. Furthermore, a defect in the *ATP6AP2* gene also gives rise to seizures. Apparently, the consequences of the mutation do not affect the downstream angiotensin system. Additional experiments, such as the analysis of other regulators, involved in the renin pathway and the extension of such experiments to neuronal cells are required to substantiate the proposed direct connection between the renin receptor and MAP kinases ERK1/2.

MATERIALS AND METHODS

Patients

Family K8355 was ascertained when two brothers with seizures and ataxia (III-17, III-18, Fig. 1) were referred for syndrome identification. The family history was significant in that their mother was one of eight females, five of whom had boys with mild to moderate mental retardation. In all, there were eight affected males in one generation. All had seizures associated with variable combinations of speech delay, ataxia, hyperactivity and impulsive, aggressive behaviour. Independently, a group from the Department of Neurology at the University of Michigan ascertained and also clinically characterized this same family and no abnormalities in cardiovascular and renal physiology were described for the affected males (6). On the basis of the constellation of clinical features, these authors designated the syndrome as XLMR and XMRE syndrome (OMIM no. 300423), distinguishable from West syndrome (OMIM no. 308350) based on the degree of retardation and the pattern of seizures (6). Informed consent was obtained prior to participation and these studies were approved by the Institutional Review Committee of Self Regional Healthcare (Greenwood, SC, USA). DNA and RNA were isolated from blood samples and from immortalized cell lines, established from peripheral lymphocytes using standard protocols.

Linkage analysis

Twenty-eight microsatellite markers (available on request), spread along the entire X-chromosome as well as *DXS8054* and *DXS1049*, were used for linkage analysis as described previously (26). The forward primers were synthesized with a fluorescein-tag (Fluore Prime, Amersham) at the 5′ end. Microsatellite polymorphisms were analyzed on the Automated Laser Fluorescent sequencer (ALF, Amersham) using the Fragment manager and the Automated Linkage Preprocessor (ALP) software. Two-point LOD scores were calculated using the Fastlink 3.0 programme (27). The gene frequency for XMRE was set at 0.0001 and the penetrance in affected males was set at 1.0.

DNA sequencing

We obtained the *ATP6AP2* sequences by sequencing BAC RP11-126D17. A 2.3 kb sequence located upstream of the initiation codon and the entire genomic region from exon 3 to exon 5 were obtained by amplifying overlapping fragments from patient III-8 (Fig. 1). Exons 1–2 and exons 6–9 were amplified with the adjacent intronic sequences (available on request). Direct double-strand sequencing was done by using Big Dye 2 kits (Perkin Elmer, Heidelberg) with separation of the fragments on an ABI 377 sequencer.

Mutation screening

The sequences of the primer pairs used for PCR- and sequencing reactions for the additional 25 screened genes (Fig. 2) are available on request.

DHPLC analysis and restriction digest

Exon 4 of controls (228 healthy males and 300 healthy females from Southern Germany) and patients was amplified using the primer sequences 5′-GATGGGAGATACAG ACTTGG-3′ and 5′-ATAAGGAGTGAATGGAATTTG-3′. A double peak for the C → T variant was seen using the WAVE-System (Transgenomics, Omaha, NE, USA) after addition of wild-type DNA under the following conditions: gradient of B-buffer: 51–61%, running temperature: 57°C. Exon 4 from 372 healthy males from South Carolina (USA) and members of the XMRE family was analyzed by a different method. As the c.321C > T change did not create or destroy any restriction enzyme site, a modified primer (ATP Ex 4 des; 5′-AATTTCAGGCAGTT-CCTTTTAGTCTTGC-3′) was designed to introduce a C at position c.320, next to the c.321C > T alteration (28). This primer creates a *Bfa* I site in the sequence carrying the mutation. The designed primer in combination with primer ATP Ex 4: (5′-GTTCCAATACCC AGAGGTA-3′) amplified a product of 239 bp which when digested with *Bfa* I results in two fragments (211 and 28 bp) visualized on a 2.5% agarose gel (Fig. 1). The validity of this approach was shown by analyzing obligate carriers as well as affected and non-affected males from the family also by the DHPLC method.

Reverse transcription experiments

The entire cDNA of the *ATP6AP2* gene was amplified from patient III-8 (Fig. 1) and two healthy controls by generating three overlapping fragments. These fragments were obtained with the following primer pairs: 1F: 5′-ACCTCCTCACG CTGCGGCTG-3′, 1R: 5′-AGGCGATTACGGAGCTGGC G-3′; 2F: 5′-GCTCCCAGTGAGGAAAGAGTG-3′, 2R: 5′-G TCCTTGTCTTCCTAATGAGGG-3′; 3F: 5′-GGTGGGAAT GCAGTGGTAGAG-3′, 3R: 5′-CTATACCACAGTGGGATT CACG-3′. Positions of these primer pairs in the gene are indicated in Figure 2C.

Protein sequence alignment

The predicted protein sequences of different species were aligned using ClustalW Multiple Sequence Alignment software.

Real time PCR

RNA was reverse transcribed using a first Strand cDNA Synthesis Kit (Roche Diagnostics). PCR amplification and detection on the Light Cycler was performed using the DNA hybridization probe format. Reactions were performed in a 20 μl volume using 1 μl cDNA, 2 μl LightCycler-FastStart DNA Master Hybridization Probes Mix (10×, Roche) and 5 μl Detection Mix (including primer and hybridization probes). Primers and probes were designed by TIBMOLBIOL, Berlin, Germany and had the following sequences: forward primer: 5′-CCAGGACTCGCAGTGGGTAA-3′, reverse primer 1: 5′-TGTCAAGACTAAAAGGAACTGCAT-3′, reverse primer 2: 5′-TCCCTACCATATACACTCTATTCTCCAAA-3′, FL-probe: 5′-GGTAGAGCCAGTTTGTTCACTCCCT-3′, LC-probe Red640: 5′-CACCATCACCATGACGGTAGCCC -3′. PCR efficiencies were estimated by performing standard curves using PCR products of the gene in defined quantities (1×10^6 copies, 1×10^5 copies, 1×10^4 copies, 1×10^3 copies, 1×10^2 copies, 2.5×10^1 copies) (Roboscreen, Leipzig, Germany). For dual-colour PCR, the human hypoxanthine-phosphoribosyl-transferase (*h-HPRT*) gene was co-amplified and detected together with the *ATP6AP2* gene in the same capillary. The hybridization probes for the *ATP6AP2* gene were labelled with Red 640, and the h-HPRT PCR with Red 705. Each dual-colour reaction was performed in a 20 μl volume using 4 μl cDNA, following the Roche standard protocols. The LightCycler Color Compensation Set was used to avoid crosstalk between the two detection channels.

Northern hybridization

cDNA of the renin receptor was radiolabelled using the Random Primed DNA Labeling Kit (Roche Diagnostics) and hybridized to membranes containing human multiple tissue cDNA panels (MTC panels from Clontech Laboratories, Palo Alto, CA, USA). Hybridization and washing of the membranes was performed using ExpressHyb hybridization solution (Clontech) according to the recommendation of the manufacturers.

Metabolic labelling and immunoprecipitation

The cells were cultured in DMEM without methionine and supplemented with ^{35}S-methionine (30 μCi/ml) for 36 h. Cell pellets were lysed and the lysate was incubated with a polyclonal antibody to the renin receptor, the complexes were precipitated with protein G sepharose and analyzed by SDS–PAGE and fluorography (2). The antibody used was raised against two peptides corresponding to amino acids 221–235 and 327–350 (2).

Study of the renin receptor functions

The kinetics of Ang I generation by receptor-bound renin and renin in solution and the analysis of ERK1/2 activation were studied as described previously (2).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We would like to express our gratitude to members of family K8355 for their participation. Tonya Moss established and maintained the lymphoblastoid cell line and prepared mRNA. We thank J. Gecz for providing DNA from four XLMR families. We also thank O. Brandau, E. Clauser, P. Corvol, A. Hardcastle and J.-D. Sraer for stimulating discussions. This paper is dedicated to the memory of Ethan Francis Schwartz (1996–1998). This work was supported by the German Ministry for Research and Education (BMBF, 01KW9974) and the European community (QLG2-CT-1999-00791) (A.M.), by a grant from NICHD (HD26202) (C.E.S.) and in part by the South Carolina Department of Disabilities and Special Needs. C.B. was supported by a grant from the French Academy of Medicine.

REFERENCES

1. Dzau, V.J., Bernstein, K., Celermajer, D., Cohen, J., Dahlof, B., Deanfield, J., Diez, J., Drexler, H., Ferrari, R., van Gilst, W. *et al.* (2001) The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data. *Am. J. Cardiol.*, **88**, 1L–20L.
2. Nguyen, G., Delarue, F., Burckle, C., Bouzhir, L., Giller, T. and Sraer, J.D. (2002) Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J. Clin. Invest.*, **109**, 1417–1427.
3. Nguyen, G., Delarue, F., Berrou, J., Rondeau, E. and Sraer, J.D. (1996) Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int.*, **50**, 1897–1903.
4. McKinley, M.J., Albiston, A.L., Allen, A.M., Mathai, M.L., May, C.N., McAllen, R.M., Oldfield, B.J., Mendelsohn, F.A. and Chai, S.Y. (2003) The brain renin–angiotensin system: location and physiological roles. *Int. J. Biochem. Cell Biol.*, **35**, 901–918.
5. Bader, M. and Ganten, D. (2002) It's renin in the brain: transgenic animals elucidate the brain renin–angiotensin system. *Circ. Res.*, **90**, 8–10.
6. Hedera, P., Alvarado, D., Beydoun, A. and Fink, J.K. (2002) Novel mental retardation–epilepsy syndrome linked to Xp21.1–p11.4. *Ann. Neurol.*, **51**, 45–50.
7. Vervoort, V.S., Beachem, M.A., Edwards, P.S., Ladd, S., Miller, K.E., de Mollerat, X., Clarkson, K., DuPont, B., Schwartz, C.E., Stevenson, R.E. *et al.* (2002) AGTR2 mutations in X-linked mental retardation. *Science*, **296**, 2401–2403.
8. Deinum, J., Derkx, F.H., Danser, A.H. and Schalekamp, M.A. (1990) Identification and quantification of renin and prorenin in the bovine eye. *Endocrinology*, **126**, 1673–1682.
9. Bienvenu, T., Poirier, K., van Esch, H., Hamel, B., Moraine, C., Fryns, J.P., Ropers, H.H., Beldjord, C., Yntema, H.G. and Chelly, J. (2003) Rare polymorphic variants of the *AGTR2* gene in boys with non-specific mental retardation. *J. Med. Genet.*, **40**, 357–359.
10. Caceres, J.F. and Kornblihtt, A.R. (2002) Alternative splicing: multiple control mechanisms and involvement in human disease. *Trends Genet.*, **18**, 186–193.

11. Cartegni, L., Chew, S.L. and Krainer, A.R. (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat. Rev. Genet.*, **3**, 285–298.
12. Fairbrother, W.G., Yeh, R.F., Sharp, P.A. and Burge, C.B. (2002) Predictive identification of exonic splicing enhancers in human genes. *Science*, **297**, 1007–1013.
13. Lorson, C.L., Hahnen, E., Androphy, E.J. and Wirth, B. (1999) A single nucleotide in the *SMN* gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl Acad. Sci. USA*, **96**, 6307–6311.
14. Varani, L., Hasegawa, M., Spillantini, M.G., Smith, M.J., Murrell, J.R., Ghetti, B., Klug, A., Goedert, M. and Varani, G. (1999) Structure of tau exon 10 splicing regulatory element RNA and destabilization by mutations of frontotemporal dementia and parkinsonism linked to chromosome 17. *Proc. Natl Acad. Sci. USA*, **96**, 8229–8234.
15. Amsterdam A., Nissen, R.M., Sun, Z, Swindell, E.C., Farrington, S. and Hopkins, N. (2004) Identification of 315 genes essential for early zebrafish development. *Proc. Natl Acad. Sci. USA*, **101**, 12792–12797.
16. Livnah, O., Stura, E.A., Middleton, S.A., Johnson, D.L., Jolliffe, L.K. and Wilson, I.A. (1999) Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science*, **283**, 987–990.
17. Jiang, G. and Hunter, T. (1999) Receptor signaling: when dimerization is not enough. *Curr. Biol.*, **9**, R568–R571.
18. Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell*, **103**, 211–225.
19. Moriki, T., Maruyama, H. and Maruyama, I.N. (2001) Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. *J. Mol. Biol.*, **311**, 1011–1026.
20. Adams, J.P. and Sweatt, J.D. (2002) Molecular psychology: roles for the ERK MAP kinase cascade in memory. *Annu. Rev. Pharmacol. Toxicol.*, **42**, 135–163.
21. Bozon, B., Kelly, A., Josselyn, S.A., Silva, A.J., Davis, S. and Laroche, S. (2003) MAPK, CREB and zif268 are all required for the consolidation of recognition memory. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **358**, 805–814.
22. Impey, S., Obrietan, K. and Storm, D.R. (1999) Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron.*, **23**, 11–14.
23. Alarcon, J.M., Malleret, G., Touzani, K., Vronskaya, S., Ishii, S., Kandel, E.R. and Barco, A. (2004) Chromatin acetylation, memory, and LTP are impaired in CBP+/− mice: a model for the cognitive deficit in Rubinstein–Taybi syndrome and its amelioration. *Neuron.*, **42**, 947–959.
24. Merienne, K., Jacquot, S., Pannetier, S., Zeniou, M., Bankier, A., Gecz, J., Mandel, J.L., Mulley, J., Sassone-Corsi, P. and Hanauer, A. (1999) A missense mutation in RPS6KA3 (RSK2) responsible for non-specific mental retardation. *Nat. Genet.*, **22**, 13–14.
25. Juillerat-Jeanneret, L., Celerier, J., Chapuis, B.C., Nguyen, G., Wostl, W., Maerki, H.P., Janzer, R.C., Corvol, P. and Gasc, J.M. (2004) Renin and angiotensinogen expression and functions in growth and apoptosis of human glioblastoma. *Br. J. Cancer*, **90**, 1059–1068.
26. Abidi, F., Hall, B.D., Cadle, R.G., Feldman, G.L., Lubs, H.A., Ouzts, L.V., Arena, J.F., Stevenson, R.E. and Schwartz, C.E. (1999) X-linked mental retardation with variable stature, head circumference, and testicular volume linked to Xq12–q21. *Am. J. Med. Genet.*, **85**, 223–229.
27. Cottingham, R.W., Idury, R.M. and Schaffer, A.A. (1993) Faster sequential genetic linkage computations. *Am. J. Hum. Genet.*, **53**, 252–263.
28. Haliassos, A., Chomel, J.C., Tesson, L., Baudis, M., Kruh, J., Kaplan, J.C. and Kitzis, A. (1989) Modification of enzymatically amplified DNA for the detection of point mutations. *Nucleic Acids Res.*, **17**, 3606.