Published in final edited form as:
Gene. 2008 February 29; 410(1): 44–52. doi:10.1016/j.gene.2007.11.014.

Functional genetic variation in aminopeptidase A (*ENPEP*): lack of clear association with focal and segmental glomerulosclerosis (FSGS)

Stephen Tonna[a], **Savita V. Dandapani**[a], **Andrea Uscinski**[a], **Gerald B. Appel**[b], **Johannes S. Schlöndorff**[a], **Kang Zhang**[c], **Bradley M. Denker**[a], and **Martin R. Pollak**[a,*]

[a]Renal Division, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts, USA

[b]Renal Division, Columbia-Presbyterian Medical Center, New York, NY, USA

[c]Johan A. Moran Eye Center, University of Utah, Salt Lake City, USA

Abstract

The aminopeptidase A (APA) ectopeptidase is an integral membrane-bound zinc metalloprotease that cleaves aspartic and glutamic acidic residues from the N-terminus of a number of protein substrates that includes angiotensin II. Angiotensin II, the most vasoactive component of the renin-angiotensin-aldosterone (RAAS) pathway, can contribute to renal disease by causing an increase in arterial blood pressure leading to glomerular injury and fibrosis. APA is expressed in many organs, including the kidney where it localizes mainly to the podocyte cell membrane and brush borders of the proximal tubule cells. Antibodies directed to the APA peptide can induce an acute massive albuminuria in wild type BALB/c mice after intravenous injection.

We examined whether variants in the APA encoding gene (*ENPEP*) are more frequent in individuals with the proteinuric disease focal and segmental glomerulosclerosis (FSGS) compared to control individuals. The *ENPEP* coding sequence was resequenced in 188 FSGS patients and 48 controls. Genetic variants were further genotyped in 181 individuals without any known kidney disease. We then examined the effect of the non-synonymous coding variants identified on their cell surface APA activity after transfection in COS-1 cells.

Several of these *ENPEP* variants lead to reproducibly altered APA activity. However, we did not see a clear correlation between the presence of a functional *ENPEP* variant and FSGS. However, the existence of these variants with marked effect on APA activity suggests that both rare and common variation in *ENPEP* may contribute to the development of renal and hypertensive disorders and warrants further study.

Keywords

Private non-conservative allele; Single nucleotide polymorphisms; DNA sequence analysis; Mutation; hypertension

* Corresponding author: Harvard Institutes of Medicine, 4 Blackfan Circle, Boston MA 02115, USA, Email: E-mail: mpollak@rics.bwh.harvard.edu.

1. Introduction

Damage to the glomerular podocytes can occur through a variety of genetic, environmental and physiological mechanisms. Genetic insults include mutations in genes that encode major proteins of the podocyte slit diaphragm and cytoskeleton such as the *NPHS1* (nephrin), *NPHS2* (podocin), *ACTN4* (α-actinin-4) and *TRPC6* (transient receptor potential-canonical 6 ion channel) genes (Boute, et al., 2000; Kaplan, et al., 2000; Kestila, et al., 1998; Reiser, et al., 2005; Winn, et al., 2005). Environmental causes such as the human immunodeficiency virus-1 (HIV) (Barisoni, et al., 1999; Kaufman, et al., 2007; Ross, et al., 2005; Schwartz, et al., 2001; Zuo, et al., 2006), and glucose-induced injury (via type 1 and type 2 diabetes) (Mogensen, 1976; Mogensen, 1984; Nosadini and Tonolo, 2003) have been well described, while physiological triggers include (but are not be limited to) elevated blood pressure (Forbes, et al., 2002; Ichihara, et al., 2006; Kretzler, et al., 1994; Nagase, et al., 2006; Szokol, et al., 1979).

The renin-angiotensin-aldosterone system (RAAS) is a central effector in the control of blood pressure. The RAAS is involved in the regulation of blood volume and systemic vascular resistance, which together regulate blood pressure (Bogatzki, 1964; Conn, et al., 1965; Mulrow and Ganong, 1962; Nahum, 1965a; Nahum, 1965b; Tigerstedt and Bergman, 1898). Drugs targeting the RAAS (angiotensin I-converting enzyme (ACE) inhibitors or angiotensinogen II receptor type 1 antagonists) have been shown to reduce hypertension and decrease proteinuria (Collier, et al., 1973; Gavras, et al., 1974; Pickering and Prinzmetal, 1940) with a concomitant reduction in rate of renal injury in chronic kidney disease. These drugs have been shown to be effective, with a range of effects, in a majority of patients (Corvol and Plouin, 2002; Croog, et al., 1990).

The Aminopeptidase A (APA) ectopeptidase is a integral membrane-bound member of the zinc metalloprotease family (Jongeneel, et al., 1989; Wu, et al., 1990) that cleaves aspartic or glutamic acidic residues from the N-terminus of a large variety of protein substrates, including angiotensin II (Wolf, et al., 1997; Zini, et al., 1996), cholecystokinin-8 (Migaud, et al., 1996), neurokinin B and chromogranin A (Goto, et al., 2006) as part of their respective metabolic pathways. The protein consists of 3 domains; a 17-amino acid cytosolic N-terminal domain, a 22 amino acid transmembrane hydrophobic domain and a 906 amino acid extracellular C-terminal domain (Li, et al., 1993; Nanus, et al., 1993; Wu, et al., 1990). The protein is encoded by *ENPEP*, a 20 exon gene located at chromosome 4q25 (Li, et al., 1997; Wang, et al., 1996).

The APA peptide is expressed in the many organs, but its expression profile in the kidney has been extensively studied, were it is expressed on the podocyte cell membrane and brush borders of the proximal tubule epithelial cells. Faint expression has also been observed in the juxtaglomerular cells, endothelial cells of the peritubular capillaries and in the pars media of the arteries (Assmann, et al., 1992; Dijkman, et al., 2006; Mentzel, et al., 1996b).

The degradation of the angiotensin II peptide to angiotensin III is mediated directly by APA in both the brain and the kidney (Wolf, et al., 1997; Zini, et al., 1996), but other proteins can metabolize angiotensin II in vitro (neutral endopeptidase (Walter, et al., 1980), prorylendopeptidase (Gafford, et al., 1983) and angiotensinogen converting enzyme 2 (Tipnis, et al., 2000)). Angiotensin II is thought to be the most vasoactive component of the RAAS (Wolf, et al., 1997) and can contribute to renal insufficiency by causing an increase in arterial pressure and inducing glomerular injury and fibrosis in a variety of human kidney diseases (Brenner, et al., 2001; Copelovitch, et al., 2007). Interestingly, certain antibodies to the APA protein can induce an acute form of proteinuria in wild-type BALB/c mice that eventually resolves (Assmann, et al., 1992; Mentzel, et al., 1996a). A combination of two antibodies

(ASD-37 and ASD-41) can induce a massive and acute albuminuria in BALB/c mice, that starts 6 hours after administration and peaks to approximately 60mg/ml at 8 hours and subsides at day 7 (Dijkman, et al., 2003; Mentzel, et al., 1999). These antibodies either bind to the C-terminal 39 amino acids of the APA peptide (ASD-37), or very close to its active site (at amino acids 448-585 in the case of ASD-41)(Gerlofs-Nijland, et al., 2003). Further evidence that the anti-APA antibodies cause albuminuria by affecting APA activity has been provided by studies that have administrated them into APA homozygous null mice: these mice do not develop proteinuria (Lin, et al., 1998).

The expression pattern of podocin, CD2AP, actin and nephrin in the podocyte of mice changes after injection of ASD-37 and ASD-41 (Dijkman, et al., 2003). Both podocin and CD2AP have a more granular staining pattern 6 hours after injection that changes to a more normal appearance after 7 days (during which time albumin excretion is low) (Dijkman, et al., 2003). Twenty-four hours post injection, podocyte-specific nephrin staining in the mice is decreased compared to non-treated mice, and actin aggregates into a more granular pattern (Dijkman, et al., 2003).

The albuminuria and renal alterations induced by the injection of the ASD-37/41 anti-APA antibodies do not appear to be mediated directly by angiotensin II. This has been demonstrated using a combination of methods (Gerlofs-Nijland, et al., 2001; Mentzel, et al., 1999). Nevertheless, intrarenal angiotensinogen II levels are greatly elevated in the ASD37/41 induced proteinuria (Gerlofs-Nijland, et al., 2001). Experiments showing either that ASD-37 (but not ASD-41) decreases APA activity *in vitro* (Mentzel, et al., 1999), and mice injected with the ASD-37/41 antibodies respond to triple-therapy using enalapril (ACE inhibitor), losartan (angiotensin II type 1 receptor antagonist) and a β-blocker illustrates that defective APA enzyme activity per se does not contribute to albuminuria, and that the renal damage may be more related to systemic blood pressure (Mentzel, et al., 1999). In addition, angiotensinogen (*AGT*) homozygous-gene knock out mice injected with ASD-37/41 also developed massive albuminuria, thus further demonstrating that albuminuria in the double-injected mice is not dependent on angiotensin II action (Gerlofs-Nijland, et al., 2001). These results thus lead to the proposal that structural alteration of the APA peptide on the podocyte cell membrane may also directly cause renal disease.

We were therefore interested in investigating whether genetic mutations in the APA encoding gene (*ENPEP*) can either contribute to or cause the proteinuria podocyte disorder focal and segmental glomerulosclerosis (FSGS) in humans as it is possible that humans that carry alleles that alter the coding region of APA may have an increased susceptibility to FSGS. From the direct sequencing of the entire *ENPEP* coding sequence in 188 patients and 48 controls, we identified five private non-conservative alleles in the cases, and two of these in control samples. We also identified three known single nucleotide polymorphisms (SNPs) and four novel ones. The frequency of private non-conservative alleles did not differ significantly between cases and controls, but we did identify an increased frequency of both W413X(1239G>A) and Q213R (639A>G) single nucleotide polymorphism (SNP) alleles in cases compared to normal, while the I32V(96A>G) SNP allele had an increased frequency in normals compared to cases. We were interested to know whether the private non-conservative alleles affected the enzyme function of the APA peptide, as these types of variants may be important in conferring susceptibility to cardiovascular and hypertensive disease. Several of these variants produced significant and reproducible effects on APA function.

2. Materials and Methods

2.1 Patients: Index cases and sporadics with FSGS

Subjects were recruited under the aegis of a protocol approved by the Human Research Committee at the Brigham and Women's Hospital. One hundred and eighty-eight unrelated individuals diagnosed with FSGS on the basis of persistent proteinuria (>300mg protein/24 hours) and renal biopsy were studied. Of these 93 (49.5%) had a familial history of this disease. The remaining 95 (50.5%) were sporadic cases that did not have a family history of disease. Individuals with radiologic, clinical, or histopathologic findings consistent with secondary forms of FSGS were excluded from the analysis. Most of these patients were Caucasian (n= 110, 59%) while the remaining 78 where of either African American (n= 24, 13%) Hispanic (n= 25, 13%), Asian (n= 4, 2%), or of unknown ethnicity (n= 25, 13%). In addition, 48 control DNA samples from families with FSGS (spouses of affected individuals) were directly sequenced for *ENPEP*. The ethnic frequencies of the control samples were closely matched to that present in the sporadic subjects (Table 1).

2.2. ENPEP sequencing analysis

Genomic DNA from each of the 188 patients and 48 control samples was extracted from peripheral blood cells using the QIAmp DNA blood kit (QIAGEN Inc., Valencia, California, USA). Total genomic DNA (20-25ng) was amplified using primers designed from the analysis of the available genomic sequence (*Homo sapiens* chromosome 1 BAC clone RP11-545A16, GeneBank accession number **AL160286**). High-throughput capillary sequencing instrumentation and Sanger dideoxy DNA sequencing was used for mutation detection. Amplified DNA was sequenced with both a nested forward and reverse primer (Table S1).

2.3. Mutation validation

Novel variants that changed the amino acid sequence were studied for their frequency in a cohort of 181 control samples (or 362 non-diseased control alleles) and co-segregation with disease in the respective families. In addition, a further 186 control samples (or 372 alleles) from Utah (with type 2 diabetes) were genotyped for the E686K (2058G>A) variant in exon 14 *ENPEP*. The mean duration of diabetes in the Utah patients was 22 years, and 27% (50/186) had end-stage renal disease (ESRD) while the remaining 73% (136/186) did not. In both instances, genotyping was performed using the MALDI-TOF Sequenom based SNP genotyping. The novel sequence variants identified in this study were submitted to the EMBL nucleotide database.

2.4.1. Generation of Green fluorescent protein (GFP) and Discosoma red fluorescent protein (DsRed) C-terminal-tagged cDNA ENPEP clones—The full-length human GATEWAY *ENPEP* cDNA clone (Invitrogen Life Technologies, Carlsbad, CA, USA) was cloned into the *Hind*III and *BamH*I sites of the pEGFP-N1 vector (Clontech, Mountain View, CA, USA). Correct cloning was confirmed by direct sequencing. The full-length cDNA was then subcloned into the *Hind*III and *BamH*I sites of the pDsRed-Monomer-N1 vector (Clontech, Mountain View, CA, USA).

2.4.2. Site-directed mutagenesis—The QuickChange site-directed mutagenesis kit (Stratagene, LaJolla, CA, USA) was used to produce all alleles that were identified from the direct sequencing of the *ENPEP* gene. These were inserted into the full-length *ENPEP* pEGFP-N1 vector using 39-mer oligonucleotide primers containing the desired nucleotide change flanked by unmodified sequence. Mutagenesis was performed according to the manufacturers protocol. Briefly, the mutagenesis was done with an amplification reaction using the *ENPEP* cDNA as a template, the 39-mer primers and *Pfu* DNA polymerase. Single clones were selected

and sequenced to identify and verify the alleles. The plasmids were resequenced to ensure that no additional nucleotides were inadvertently altered.

2.5. Electron microscopy and Western-blot analysis

COS-1 cells grown on either 6-well culture plates (Corning Incorporated, Corning, NY, USA) or 14 mm glass bottom coated microwell culture dishes (MatTek Corporation, Ashland MA, USA) were transfected with 1μg of each full-length wild-type pDsRed-Monomer-N1 and 1μg of variant pEGFP-N1-*ENPEP* plasmid DNA. COS-1 cells were used as a basis of these assays as they have previously been reported not to express any endogenous APA activity (Ofner and Hooper, 2002).

Transfected COS-1 cells grown on 14mm glass bottom coated microwell culture dishes were visualized for their GFP expression using a blue light FITC filter that detected the excitation peak of 488nm and peak emission of 509nm of the GFP molecule.

Cells grown on 6-well culture plates were transiently co-transfected with 1μg of each full-length wild-type pDsRed-Monomer-N1 and 1μg of variant pEGFP-N1-*ENPEP* plasmid DNA, lysed in ice-cold 20mM HEPES, pH 7.0 buffer containing 0.5% Triton X-100 buffer, 2mM EGTA, 4mM EDTA, 30mM sodium fluoride, 40mM b-glycerophosphate, 20mM sodium pyrophosphate, 1mM orthovanadate and a protease inhibitor mixture tablet (Complete, Roche Applied Science). The cell lysate was mixed with an equal volume of 4X electrophoresis sample buffer and boiled for 5 minutes. The proteins were loaded into a 10% (w/v)-SDS acrylamide gel and transferred to a PVDF-membrane using a wet transfer method. The membranes were blocked by incubation in 5% nonfat dry milk in TBST overnight at 4°C. The primary antibody (anti-GFP; Abcam, Cambridge MA, USA, # ab290) was diluted in 5% nonfat dry milk in TBST and incubated with the PVDF-membrane. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with a Chemiluminescent substrate (Pierce, Rockford, IL, USA).

2.6. Aminopeptidase A cell surface activity assay

To assess the functional significance of the variant aminopeptidase A alleles, we used a protocol designed by Ofner and Hooper (Ofner and Hooper, 2002). We transfected wild-type or variant pEGFP-N1-*ENPEP* plasmids into 6-well culture plates (Corning Incorporated, Corning, NY, USA) of COS-1 cells (African green monkey kidney cells, ATCC Inc., VR, USA) using the FuGENE 6 transfection reagent (Roche Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. For this purpose, 1μg of plasmid DNA was used and the total amount of DNA was thus constant in each well. The transfections were performed in triplicate independent reactions, on three different occasions (thus each construct was tested nine times for its APA activity level). The COS-1 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, 100μgml^{-1} streptomycin and maintained in 5% CO_2 and at 37°C.

Forty-eight hours after transfection, cells were removed from 37°C and incubated at 15°C for 4 hours. Growth media was removed and the cells were then washed twice in 500μl of ice-cold 0.1M Tris/HCl (pH 7.4)/1mM $CaCl_2$, prior to adding the same volume of 0.1M Tris/HCl (pH 7.4)/1mM $CaCl_2$ supplemented with 2.5mM α-glutamic acid ρ-nitroanilide (Peptide Institute Inc., Ina Minohshi, Osaka JAPAN). At intervals of 20, 40 and 80 minutes, 100μl substrate containing media was removed and aliquoted to a freshly-sterilized 1.7ml microcentrifuge tube (American Bioanalytical, Natick MA, USA), then centrifuged at 8,000 rpm for 15 minutes at room temperature. The supernatant was then dispensed in a 96-well microtitre plate and the concentration of cleaved ρ-nitroanilide was measured based on its absorbance at 405nm.

A co-expression assay was developed to asses how the private non-conservative *ENPEP* variants demonstrated in cases affected the wild type aminopeptidase A activity. For these experiments, 1μg of full-length wild-type and private non-conservative *ENPEP* plasmid DNA were co-transfected into COS-1 cells grown on the 6-well culture plates. The results of these aminopeptidase A activity assays were compared to a control sample that consisted of 1μg of each full-length wild-type pEGFP-N1-*ENPEP* and polymorphic allele (I32V pEGFP-N1-*ENPEP*) plasmid DNA that was found not to affect aminopeptidase A activity.

3. Results

3.1. Identification of ENPEP alleles

By direct sequencing of the entire coding region of the APA encoding gene (*ENPEP*) in 188 patients with FSGS and 48 control samples, we identified 15 different nucleotide variants that lead to either nonsense (n=1) or missense substitutions (n= 14) (Table 2a and 2b). Four of the nucleotide variants that affected the coding sequence (Q213R (639A>G), V218A (654A>C), W413X(1239G>T) and S861R(2583C>G)) were previously reported in the public SNP database dbSNP (www.ncbi.nlm.nih.gov/sites/entrez). Thus, of the nucleotide variants that either caused premature stop codons or non-synonymous coding sequence changes, eleven are previously non-described.

3.2. Private non-conservative ENPEP alleles

Seven private non-conservative alleles were identified in the heterozygous state (Table 2b). Variants leading to amino acid changes Q435E (1305C>G), D622N (1866G>A), A676T (2028G>A) and E687D (2061(G>T) were identified in four unrelated FSGS patients with sporadic disease, while the E686K (2058G>A) was demonstrated in a patient with familial FSGS. The K923I (2769A>T) and R925G (2775A>G) alleles were present in two unrelated control DNA samples sequenced.

All of the private non-conservative alleles identified in FSGS were also absent from all 362 non-diseased control alleles genotyped for these specific variants. Likewise, the variants K923I and R925G were not identified in any of the other 362 non-diseased control alleles genotyped, and were also not found in any of the 188 FSGS patients sequenced. In addition, despite the finding that E686K was identified in a proband with biopsy-confirmed FSGS, this allele was not present in another affected relative with biopsy-confirmed disease. This allele was also absent in a further 372 control alleles isolated from 186 unrelated individuals from the same ethnicity and geographic location as the family (Utah).

We also identified rare non-synonymous alleles in two of the control individuals sequenced. Both the K923I (2769A>T) and R925G (2775A>G) alleles (Table 2a) were present in two unrelated control samples and were not present in any of the 188 FSGS patients (or 376 alleles) analyzed. The frequency of private non-conservative alleles was not more common in cases compared to controls (frequencies of 2.6% and 4% respectively, p= 0.583, chi-square analysis). However, the small numbers of cases and controls makes the power of this analysis inadequate for identifying small but significant differences.

3.3. ENPEP single nucleotide polymorphisms (SNPs)

Four known SNPs also predicted to cause missense amino-acid substitutions were also observed by resequencing (Table 2b): Q213R (639A>G), V218A (654A>C), W413X (1239G>T) and S861R(2583C>G). We also identified four additional non-synonymous variants present in multiple unrelated individuals that we did not observe in the public SNP databases: I32V (96A>G), R159S (477G>T), E172Q (516G>C) and Y544F (1632A>T).

We examined the frequencies of all of these polymorphisms in cases and controls. Variant I32V (96A>G) was found to have an allele frequency of 5.6% in the controls compared to 2.1% of cases (p= 0.013 Chi-square analysis). Both Q213R (639A>G) and W413X (1239G>T) had a higher allele frequency in cases than controls (Table 3) but only the Q213R (639A>G) reached statistical significance (11% in cases compared to 6% in controls, p= 0.012 Chi-square analysis). This p value is not statistically different after correction for multiple hypothesis testing. There was no significant difference in the frequencies of the other polymorphisms (V218A, S861R, R159S, E172Q and Y544F) between cases and controls (Table 2b).

3.4. Generation of pEGFP-tagged ENPEP alleles and cell surface aminopeptidase-A (APA) activity assay

We next investigated whether the private non-conservative alleles we identified affected APA activity. We cloned the entire wild-type (WT) cDNA of the APA peptide into the pEGFP-N1 vector to create the pEGFP-N1-WT *ENPEP* plasmid and used this to generate each of the 15 alleles that affected the coding region by site directed mutagenesis. In addition, the WT *ENPEP* cDNA was subcloned from the pEGFP-N1-WT *ENPEP* plasmid into the pDsRed-Monomer-N1 vector.

3.5. Imaging and Western Blot analysis

Each plasmid produced GFP epifluorescence located predominantly at the cell membrane, with the exception of the W413X truncation variant that produced extremely low levels of GFP appearing mostly as vesicles in the cell. When co-expressed with an equal amount of pDsRed-Monomer-N1-WT *ENPEP* plasmid DNA, the expression pattern of the pEGFP-N1 *ENPEP* plasmids appeared identical to cells expressing the pEGFP-N1-WT plasmid (Figure S1).

A crude cell lysate was prepared from untransfected cells and from those transfected with 1μg of pDsRed-Monomer-N1-WT *ENPEP* plasmid and either an equal amount of pEGFP-N1-WT *ENPEP*, or each of the 15 pEGFP-tagged *ENPEP* constructs that affected the coding region. Western blot using a commercially available anti-GFP rabbit antibody (Abcam Inc., Cambridge MA, USA) detected the expected polypeptide of approximately 147 kDa in all cell lysates except from the W413X-transfected cells (Figure S2).

3.6. Aminopeptidase A assay

We performed transient tranfections using each of the 15 plasmids containing both private non-conservative as well as the “non-private” alleles into COS-1 cells using untransfected cells as a control. Forty-eight hours after transfection and incubation at 37°C, the cells were incubated at 15°C for 4 hours, to suppress the export of newly synthesized protein from the endoplasmic reticulum (Ofner and Hooper, 2002). The cells were then returned to 37°C and the amount and activity of APA was measured directly using a cell surface activity assay developed by Ofner and Hooper (2002) (Ofner and Hooper, 2002). These experiments were performed in two groups. The first group consisted of assays of private non-conservative alleles, while the second assayed the activity of the non-private variants (Figure 1a and 1b). After 20 minute time intervals (but not exceeding 90 minutes) APA activity was measured for all plasmids in both groups and the APA activity level was compared after 40 minutes. Assays were performed using independent triplicate transfections, and these assays were also repeated in three separate experiments.

Among the private variants, the A676T allele caused a gain of cell surface activity by 27% compared to wild type, whereas E686K, R925G, Q435E, and E687D exhibited a partial loss of activity, with reductions of 71%, 61%, 30%, and 23%, respectively. E686K activity was comparable to that of untransfected cells. Activity of APA expressing the D622N or K923I alleles was not different from wild type (Figure 1a).

Among the polymorphisms tested, R159S plasmid produced increased APA activity compared to wild type (33%), whereas W413X, E172Q and V218A showed decreased activity (69%, 56% and 14% lower than wild type respectively). The I32V, E172Q, Q213R, Y544F and S861R APA variants did not have a measurable difference when compared to wild type (Figure 1b).

We also performed co-transfection experiments with both mutant and wild-type *ENPEP* plasmids to determine if any of the alleles had a dominant-negative effect on APA activity. We transfected 1μg of pEGFP-N1-WT *ENPEP* DNA with an equal amount of pEGFP-N1-A676T, E686K, E687D, W413X, Q435E or D622N. Untransfected cells were used as a negative control and a cell transfected with 1μg of wild-type and I32V plasmid DNA was used as a positive control, as I32V was shown not to affect cell surface APA activity. This experiment did not demonstrate any statistically significant differences compared to the WT/I32V positive control. There was a modest increase in activity of the A676T/WT transfected cells, consistent with our earlier results. None of the variants behaved as a dominant-negative allele (Figure 2).

4. Discussion

The APA peptide has been directly implicated in the degradation of angiotensinogen II to angiotensinogen III in the brain and the kidney (Wolf, et al., 1997; Zini, et al., 1996). Antibodies directed against APA can induce acute proteinuria in BALB/c wild type mice (Assmann, et al., 1992; Mentzel, et al., 1996a). This proteinuria has been shown to be independent on angiotensin II (Gerlofs-Nijland, et al., 2001; Mentzel, et al., 1999). Mice with a targeted deletion of the angiotensinogen (*AGT*) gene also develop heavy albuminuria to comparable levels to the BALB/c wild type mice after injection of ASD37/41 (Gerlofs-Nijland, et al., 2001). This has lead to the notion that structural alterations of the APA peptide can cause the acute albuminuria. We were therefore interested in investigating whether humans with the proteinuria podocyte disorder FSGS can carry *ENPEP* mutations that may increase their susceptibility to glomerular injury.

From the sequencing of 188 patients with FSGS and 48 controls we identified 15 alleles that caused amino acid changes. Five of these (Q435E (1305C>G), D622N (1866G>A), A676T (2028G>A), E686K (2058G>A) and E687D (2061(G>T)) were found in cases and in no controls (5/188, 3%). We also found two private non-conservative alleles in two normals sequenced (2/48, 4.2%). Private non-conservative alleles were not more common in cases compared to normals (p= 0.583, Chi square analysis), although such a comparison is underpowered. In addition, the frequency of non-synonymous alleles did not differ between familial and sporadic cases with FSGS (p= 0.355, Chi-square analysis).

Two SNPs were found to have a higher allele frequency in cases (Q213R) or controls (I32V). When corrected for the multiple variants examined, these differences are not statistically significant. Nevertheless, these differences suggest that larger studies designed to determine if certain alleles of *ENPEP* confer susceptibility to or protection from proteinuric kidney disease may be worthwhile. Also of note, W413X was found at higher allele frequency in FSGS cases compared to normals (1.1% compared to 0.3% respectively) but, again, this difference was not statistically significant. This variant produces a truncated APA peptide that leads to loss of a GFP signal from western blotting and a loss of APA cell surface activity in vivo. Genotyping these I32V, Q213R and W413X *ENPEP* SNPs in large well-controlled groups with and without proteinuric kidney disease seems warranted.

We were interested to explore the enzymatic activity of alleles harboring the non-synonymous variants we identified. Six of the seven “private” variants studied lead to altered enzyme activity of APA (86%), compared to four of the eight SNPs (50%). These included both variants leading

to increased as well as decreased APA activity. Two of the *ENPEP* variants, W413X and E686K lead to nearly complete loss of APA activity.

It is possible that private non-conservative variation in *ENPEP* may cause kidney dysfunction in combination with alleles in other genes that are known to cause inherited forms of FSGS, or other alleles that disrupt related pathways. None of the families we screened had disease attributed to mutations in either of the *NPHS2*, *ACTN4*, *CD2AP*, or *TRPC6* genes from prior analysis. The identified *ENPEP* non-synonymous alleles demonstrated did not segregate with disease in the respective families (Table 2a and 2b). As would be expected, the private non-conservative *ENPEP* alleles we identified affected APA activity more frequently than more common polymorphisms (or SNPs). These variants can lead to either increased or decreased activity.

Several lines of evidence indicate that the APA protein is involved in experimental acute proteinuria via antibodies directed to particular regions of the peptide (Assmann, et al., 1992; Dijkman, et al., 2003; Gerlofs-Nijland, et al., 2001; Gerlofs-Nijland, et al., 2003; Mentzel, et al., 1996a; Mentzel, et al., 1999), and perhaps even the acceleration of slowly progressive FSGS in mice that ectopically express the Thy1.1 antigen on podocytes (Assmann, et al., 2002). Yet other studies have shown that the APA peptide may be involved in salt-induced renal damage in male Dahl salt-sensitive rats (which have a higher renal mRNA expression for the protein compared to male Dahl salt-resistant controls mice that have elevated APA mRNA and this is thought to protect them against the hypertension and glomerulosclerosis that develops in the salt-sensitive rats) (Nomura, et al., 2005).

Along these same lines, homozygous mice that have targeted deletion of *ENPEP* (Lin, et al., 1998) have elevated baseline blood pressure, which can be increased by continual infusion of angiotensinogen II to a greater extent compared to wild type mice (Mitsui, et al., 2003). In addition, high blood pressure caused by hyperactive brain RAAS in the DOCA-salt sensitive rat can be reduced for up to 24 hours by a selective inhibitor of APA that is able to transgress the blood-brain barrier (Fournie-Zaluski, et al., 2004). Thus, the APA protein has been implicated in proteinuria and salt-induced hypertension. There appears to be a non-trivial incidence of human *ENPEP* nucleotide changes that lead to private non-conservative alleles that have functional effects. Although we were unable to show a clear relationship between the presence of a functional ENPEP variant and FSGS, such functional variants remain strong candidate for having phenotypic effects in humans. It is still unclear whether private non-conservative alleles are more common in patients with end-stage renal disease or diseases that cause cardiovascular or hypertension events, but we believe that further resequencing of *ENPEP* in a cohort of cases with well defined renal and cardiovascular diseases may be justified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health (to DK54931 to M.R.P) and the Juvenile Diabetes Research Foundation (to S.T).

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Abbreviations

ACE

angiotensin I-converting enzyme

APA
aminopeptidase A

$CaCl_2$
calcium chloride

DNA
deoxyribonucleic acid

EDTA
ethylenediamine tetraacetic acid

EGTA
ethylene glycol tetraacetic acid

EMBL
European molecular biology laboratory

FSGS
focal and segmental glomerulosclerosis

HEPES
(4-2-hydroxyethyl)-1-piperazineethanesulfonic acid

HCI
hydrochloric acid

ng
nanogram

nm
nanometer

Pfu
pyrococcus furiosus

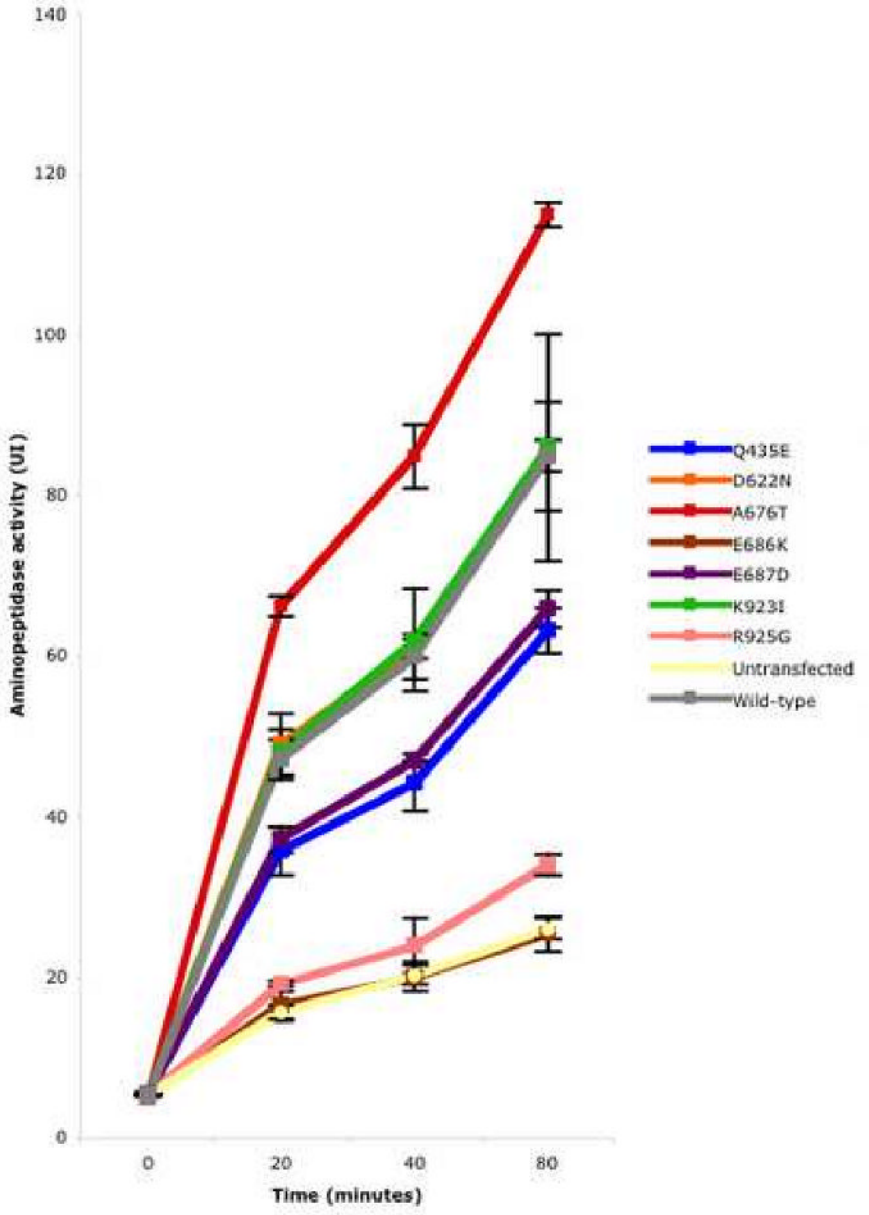
RAAS
renin-angiotensinogen-adosterone pathway

SDS
sodium dodecyl sulfate

TBST
tris-buffered saline tween 80

µg
micro gram

Figure 1a



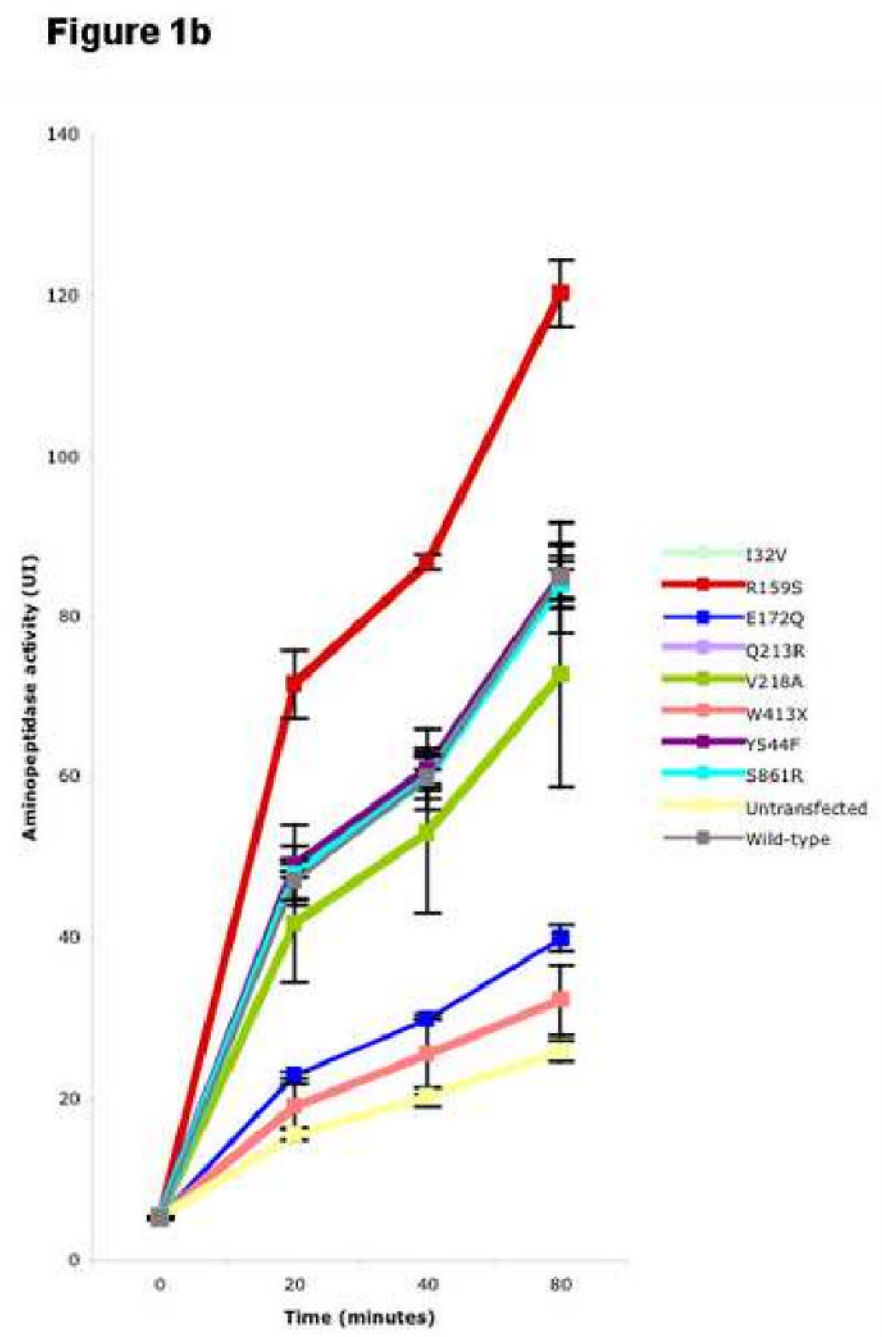


Figure 1.

a. COS-1 cells transfected with either 1μg total DNA of wild-type *ENPEP* or *ENPEP* constructs with private coding sequence variants were grown in six-well plates. Following transfection, cells were incubated at room temperature for 4 hours before returning them to 37°C. After the indicated time intervals, the amount of cell surface activity of aminopeptidase A (APA) was assayed using α-glutamic acid ρ-nitroanilide as substrate. These assays showed that A676T had a gain of cell surface activity by 27% compared to wild-type, whereas E686K, R925G Q435E and E687D had decreased activity, with reductions of 71%, 61%, 30% and 23% respectively. E686K activity was comparable to that of untransfected cells. The D622N and K923I alleles were not distinguishable from wild-type. The results are the means +/-standard

error of the mean (S.E.M) of triplicate measurements after independent transfections on the same day. IU: mmol hydrolysis of α-glutamic acid ρ-nitroanilide /min.

b. COS-1 cells transfected with either 1μg total DNA of wild-type or single nucleotide polymorphism (SNP) *ENPEP* constructs were grown in six-well plates. Following transfection, the cells were incubated at room temperature for 4 hours before returning them to 37°C. After the indicated time intervals, the amount of cell surface activity of aminopeptidase A (APA) was assayed using α-glutamic acid ρ-nitroanilide as substrate. The R159S plasmid had increased activity compared to wild-type (33%), whereas, W413X, E172Q and V218A had decreased cell surface activity (69%, 56% and 14% lower compared to wild-type respectively). Activity of the I32V, E172Q, Q213R, Y544F and S861R APA variants were not distinguishable from wild-type. The results are the means +/-standard error of the mean (S.E.M) of triplicate measurements after independent transfections on the same day. IU: mmol hydrolysis of α-glutamic acid ρ-nitroanilide /min.

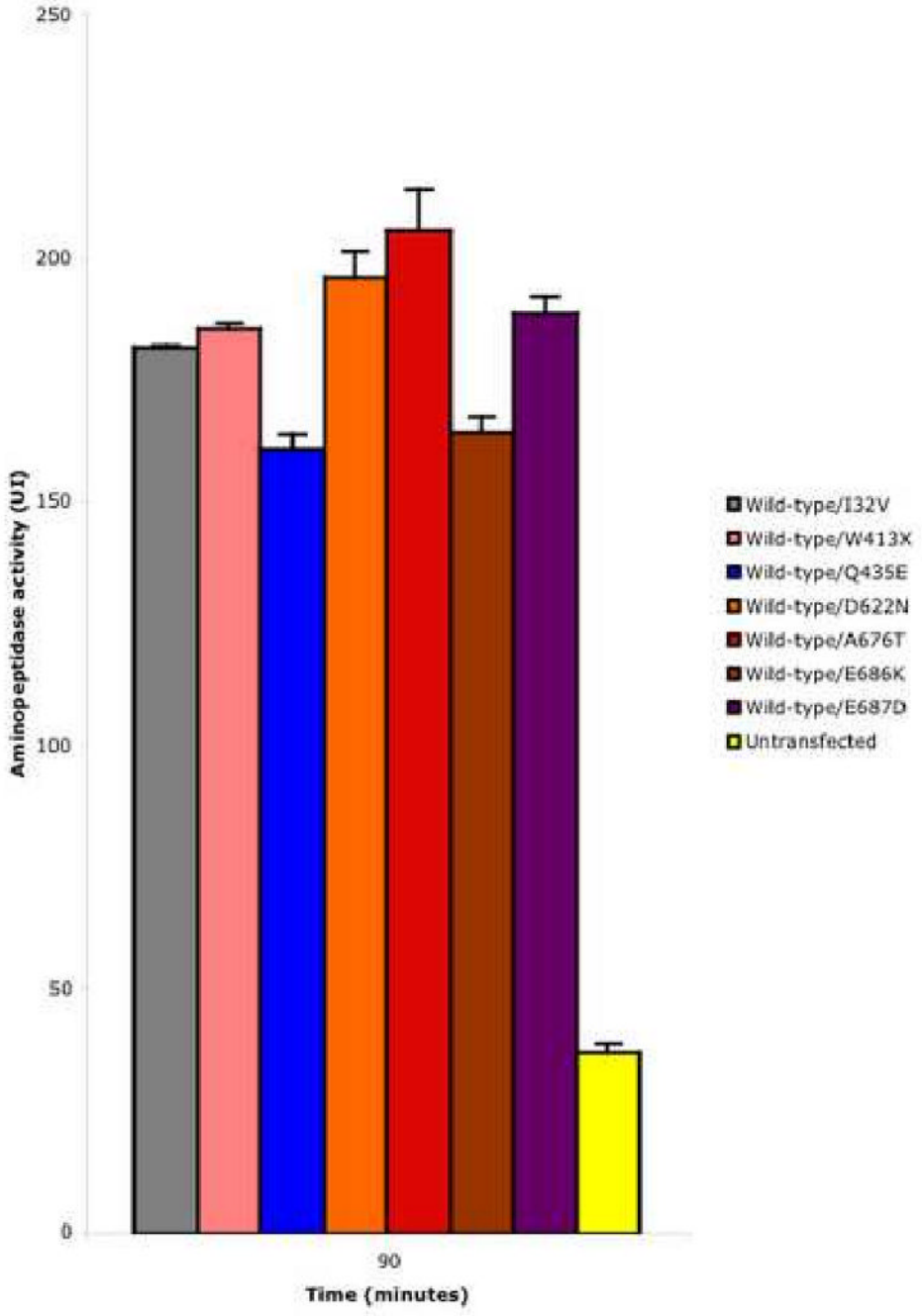


Figure 2.

pEGFP-N1 *ENPEP* plasmids containing I32V, W413X, Q435E, D622N, A676T, E686K and E687D were expressed in COS-1 cells along with an equal DNA quantity (1μg) wild-type *ENPEP* plasmid DNA. These results showed that after 90 minutes, the wild-type cell surface activity of the Aminopeptidase A (APA) peptide was higher when co-expressed with A676T, but lower with E686K, and Q435E and unchanged with W413X and E687D. Untransfected cells had a small endogenous cell surface APA activity. The results are the means +/-standard error of the mean (S.E.M) of triplicate measurements after independent transfections on the same day. IU: mmol hydrolysis of α-glutamic acid ρ-nitroanilide /min.

Table 1

Clinical and biopsy characteristics of the 188 patients screened for *ENPEP* mutations

	Male	Female	Caucasian	Black	Hispanic	Asian
Familial FSGS (n= 93)	40	53	47	2	15	4
'Sporadic' FSGS (n= 95)	48	47	63 (66%)	22 (23%)	10 (11%)	0
Total FSGS	88	100	110	24	25	4
Controls	-	-	32 (67%)	13 (27%)	3 (6%)	-

FSGS: focal and segmental glomerulosclerosis

Table 2a

Private non-conservative alleles detected in *ENPEP*

Nucleotide change	Effect on coding sequence	Exon	Individual	Allele frequency in cases	Allele frequency in controls
1305C>G	Q435E	6	CPMC-93	1/376 (0.2%)	0/354
1866G>A	D622N	12	CPMC-24	1/376 (0.2%)	0/348
2028G>A	A676T	14	CPMC-35	1/376 (0.2%)	0/354
2058G>A	E686K	14	FG-BO-111	1/376 (0.2%)	0/690
2061G>T	E687D	14	CPMC-46	1/376 (0.2%)	0/354
2769A>T	K923I	20	FG-ER-1180	0/376	1/96 (1%)
2775A>G	R925G	20	FG-CM-1180	0/376	1/96 (1%)

All variants were demonstrated in the heterozygous state only

Table 2b

Polymorphisms[1] detected in *ENPEP*

Type of mutation	Nucleotide change	Effect on coding sequence	Exon	Variant Status[2]	Allele frequency in FSGS	Allele frequency in controls
nonsense	1239G>T	W413X	6	h	4/376 (1.1%)	1/354 (0.3%)
missense	96A>G	I32V	1	h	8/376 (2.1%)	20/354 (5.6%)
missense	477G>T	R159S	1	h	1/376 (0.3%)	1/344 (0.3%)
missense	516G>C	E172Q	1	h	2/376 (0.5%)	6/354 (1.7%)
missense	639A>G	Q213R	1	h	42/376 (11%)	21/354 (6%)
missense	654A>C	V218A	2	h H	100/376 (26.6%) 38/376 (10.1%)	109/354 (30.8%) 29/354 (8.1%)
missense	1632A>T	Y544F	10	h H	2/376 (0.5%) 1/376 (0.3%)	7/364 (2%) 0/364
missense	2583C>G	S861R	18	h	6/376 (1.6%	7/354 (2%)

[1] h: heterozygous; H: homozygous

[2] Polymorphism defined here as a variant detected in two or more unrelated individuals