Pantetheinase activity of membrane-bound Vanin-1: lack of free cysteamine in tissues of Vanin-1 deficient mice

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Abstract Pantetheinase (EC 3.5.1.-) is an ubiquitous enzyme which in vitro has been shown to recycle pantothenic acid (vitamin B5) and to produce cysteamine, a potent anti-oxidant. We show that the Vanin-1 gene encodes pantetheinase widely expressed in mouse tissues: (1) a pantetheinase activity is specifically expressed by Vanin-1 transfectants and is immunodepleted by specific antibodies; (2) Vanin-1 is a GPI-anchored pantetheinase, and consequently an ectoenzyme; (3) Vanin-1 null mice are deficient in membrane-bound pantetheinase activity in kidney and liver; (4) in these organs, a major metabolic consequence is the absence of detectable free cysteamine; this demonstrates that membrane-bound pantetheinase is the main source of cysteamine in tissues under physiological conditions. Since the Vanin-1 molecule was previously shown to be involved in the control of thymus reconstitution following sublethal irradiation in vivo, this raises the possibility that Vanin/pantetheinase might be involved in the regulation of some immune functions maybe in the context of the response to oxidative stress.

Key words: Vanin; Pantetheinase; Ectoenzyme; Immunoregulation; Oxidative stress

1. Introduction

Pantetheinase (EC 3.5.1.-) catalyzes the hydrolysis of D-pantetheine in the dissimilative pathway of coenzyme A (CoA) and acyl carrier protein [1]. The hydrolytic activity is highly specific, only one of the amide bonds of pantetheine being hydrolyzed to give cysteamine and pantothenic acid in vitro, whereas some related compounds are not substrates [2–4]. Pantetheinase activity was found in many tissues from mammals and birds [5,6] but its biological role has never been explored in vivo. The pig kidney enzyme was purified [3,7] and the biochemical analysis revealed its kinetic and catalytic features [1,2], its inhibition by disulfides [8], its thermal resistance [9] and its stability versus chemical denaturation [10].

The mouse Vanin-1 has initially been characterized as a membrane molecule expressed by a subset of thymic stromal cells and involved in the homing of bone marrow precursor cells into the thymus [12]. The anti-Vanin-1 407 monoclonal antibody (mAb) injected during the reconstitution of the thymus inhibits repopulation without affecting cell differentiation per se. Vanin-1 is the prototypic member of a larger family containing at least three genes in man (i.e. VNN1, VNN2, VNN3), two in mouse (i.e. Vanin-1, Vanin-3) and one *Drosophila* homologue [13,14].

Recently, partial sequences of the pig enzyme were reported [11] and on the basis of sequence similarities, the identity between pantetheinase and mouse Vanin-1 has been postulated.

We report here the in vitro and in vivo demonstration that the Vanin-1 molecule is a membrane pantetheinase, which is ubiquitously expressed in mouse tissues. Vanin-1 and pig pantetheinase molecules show significant sequence similarities and Vanin-1 transfectants gain a pantetheinase activity, which has the same physicochemical properties as those described for pig pantetheinase. Vanin-1 and pantetheinase are highly expressed in the kidney where their expression is lost in Vanin-1 deficient mice. Furthermore, in vivo, the biological function of pantetheinase deals with the recycling of pantothenic acid (vitamin B5) and is thought to be a source of cysteamine in tissues. Using the Vanin-1 null mice, we report here the first experimental demonstration that directly correlates the pantetheinase activity with the tissue level of free cysteamine, a powerful anti-oxidant.

2. Materials and methods

2.1. Northern blot analysis and reverse transcription (RT)-polymerase chain reaction (PCR) analysis

Total RNA was isolated from mouse tissues using the TriZol reagent (Gibco BRL, Life Technologies). 20 μg of each RNA sample was run on formaldehyde denaturing agarose gels. After transfer onto nylon membranes (Amersham, France), hybridizations were performed with probes encompassing the whole Vanin-1 cDNA sequence as a ^{32}P-labelled DNA probe.

1 μg of total RNA was isolated using the TriZol reagent (Gibco BRL, Life Technologies) from mouse tissues or sorted cell populations and reverse-transcribed with random hexamers using the Superscript II reverse transcriptase (Gibco BRL, Life Technologies). Semi-

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quantitative PCR experiments were performed as described using actin as an internal standard [15]. Primers used in these PCR experiments are: Vanin-1 forward primer: 5′-CGGTGCAGGAGAGACTCAGC-3′ and reverse primer: 5′-GCCAAATGAGGAAGGACGTC-3′.

2.2. Vanin-1 deficient mice

A λ phage hybridizing with a Vanin-1 probe was isolated from a mouse 129/Ola library (kindly provided by M. Djabali). It contains a 7 kb *Eco*RI fragment corresponding to sequences located 2 kb upstream of the ATG codon and used as the 5′ homology region in the final construct, a 4.5 kb *Eco*RI fragment encompassing proximal 5′ non-coding sequences and the first exon which was replaced by a neomycin cassette after homologous recombination, and a 1.7 kb *Eco*RI–*Not*I fragment containing the second exon of the Vanin-1 gene and used as the 3′ homology region. The pIC19R/MC1-TK vector [16] was modified by insertion into the *Sal*I site of a *Xho*I fragment containing the pGK-neo cassette without loxP sites: this vector is called pIC-TK-neo. The 5′ and 3′ homology DNA segments were introduced into the blunted *Hin*dIII and *Bam*HI sites of pIC-TK-neo, respectively. E14 (129/Ola strain) ES cells were electroporated with 20 μg of targeting vector DNA linearized at a *Sal*I site located at the 3′ end of the construct. 24 h later, cells were selected with 300 μg/ml G418 and 2 μM gancyclovir. Homologous recombinants were tested by hybridization of *Hin*dIII-digested genomic DNA with an external PCR probe corresponding to the third Vanin-1 exon. The presence of an unique integration event was checked with a neomycin probe. Recombinant ES cells were injected in BALB/c blastocysts, and heterozygotes were genotyped by Southern blot and PCR using the 5′ primers for neomycin 5′-CGAATTCGCCAATGACAAGACG-3′ and endogenous sequences 5′-GTTCTCCAATCCCAAAGTGC-3′ combined with the 3′ primer 5′-ATTGATTCACCACTCAATCCCTG-3′. The Northern blot analysis was performed with total kidney RNA, using a Vanin-1 cDNA probe following conventional procedures.

2.3. Protein analysis

Immunohistochemistry and in situ hybridization on kidney sections were performed as described [15].

For biochemical analysis, transfected cells were plated at 30% confluence in 25 cm^2 dishes and grown for 48 h. Supernatants (10–11 ml) were collected and ultracentrifuged. Cell supernatant was either mixed with Laemmli buffer or immunoprecipitated. Adherent cells were lysed for 30 min at 4°C in buffer containing 20 mM Tris, 140 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 0.5% DOC and a cocktail of protease inhibitors (complete Mini, Boehringer Mannheim–Roche). Cell lysates were harvested and the insoluble fraction removed by centrifugation. Immunoprecipitations from cell lysates, supernatants or solubilized mouse kidney membrane extracts were performed using protein G-Sepharose coupled to the rat IgG1,κ anti-mouse Vanin-1 407 mAb (clone H202-407-7-4, [12]), or control IgG1 mAb. All samples were submitted to SDS–PAGE, transferred onto nylon membranes, and submitted to Western blot analysis with either a rabbit anti-Vanin-1 antiserum (AS90) prepared against an internal peptide Vanin-1-specific sequence (YAPDSPRVFHYDRKTQEGK) and revealed with protein A-HRP (Pierce) and ECL (Amersham).

2.4. Bioinformatics

All Vanin sequences are accessible through EMBL database under the following numbers: Vanin-1: AJ132098, Vanin-3: AJ132103, VNN1: AJ132099, VNN2: AJ132100, VNN3: AJ238982, *Drosophila* homologous sequence: AJ276261. Biotinidase sequence is accessible under the following GenBank number: U03274. These sequences have been shortened to match the known corresponding peptidic sequences of pig pantetheinase [11]. A distance-base phylogenetic tree was generated using ClustalX (PHYLIP format) and edited using TreeView 1.5.

2.5. Enzymatic assays

The analysis of Vanin-1 enzymatic activity was performed on tissue or cell extracts. Most enzymatic assays were performed with the mouse thymic epithelial Vanin-1-transfected (MTE1D/VH) or parental (MTE1D) cell lines [12,17]. When indicated, cytosol and membrane fractions were prepared from 80×10^6 cells. Membrane pellets were solubilized in phosphate-buffered saline 1% DOC.

Pantetheinase from mouse kidney and liver was extracted as previously reported for the pig enzyme [3]. Pantetheinase hydrolyzing activity was spectrophotometrically followed (296 nm) as previously reported [18].

One unit of enzymatic activity is defined as the amount of enzyme necessary to produce 1 μmol of aminoethyl-cysteine ketimine in 1 min at 30°C.

On cell extracts, inhibition experiments were performed by treating samples for 10 min with 4,4′-dithiodipiridine (8.0 μM), pantetheine (2.0 μM), iodoacetate and iodoacetamide (10.0 μM) at pH 8.0 and activity was recorded before and after addition of mercaptoethanol (7.0 mM). Temperature resistance was monitored recording the activity of the same sample at various temperatures (37–70°C).

2.6. Determination of free cysteamine in tissues

Cysteamine determination in kidney and liver from wild type and Vanin-1 deficient mice was performed on fresh organs immediately homogenized in $HClO_4$ [19] and stored at −80°C. Crude extracts were reduced using 10 mM mercaptopropionic acid and passed through Dowex-1 as previously reported. Two different procedures were used for cysteamine detection: an enzymatic assay [19] and a high performance liquid chromatography (HPLC) detection [20]. For the last procedure, an electrochemical detector (ESA) was used: the potentials of oxidizing electrodes were 450 and 475 mV. The lower detection limits were 4 nmol/g and 0.5 nmol/g for the enzymatic and HPLC methods, respectively.

3. Results

3.1. Expression pattern of the Vanin-1 mRNAs

A previous study of the cDNA origin of expressed sequence tag (EST) sequences has shown that Vanin-1 ESTs were found in various libraries (heart, liver, kidney, uterus, etc.) [14]. In Northern blot analysis (Fig. 1A), the 2.3 kb Vanin-1 transcript is detectable in kidney, intestine and liver. However, when this analysis was completed by RT-PCR experiments on a larger panel of mouse tissues, Vanin-1 transcripts were detected in almost all organs tested in variable amounts (Fig. 1B). Furthermore, the Vanin-1 mRNAs are found in lymphoid organs such as thymus and spleen whereas no Vanin-1 transcript was ever detected in circulating hematopoietic cells. To define the cell types expressing Vanin transcripts in kidney, an in situ hybridization experiment was performed. As shown in Fig. 1C, epithelial cells of cortical tubules and not glomeruli specifically express Vanin-1.

3.2. The Vanin-1 molecule is a membrane-bound pantetheinase

The available pig pantetheinase peptide [11] sequences were aligned with all known Vanin-like sequences and a phylogenetic tree was built using the ClustalX program. As shown in Fig. 2, pig pantetheinase segregates with mouse and human Vanin-1, suggesting that they are homologous genes in different species. To further document Vanin-1 pantetheinase activity, the enzymatic reaction was analyzed on the Vanin-1-transfected mouse epithelial cell line MTE1D/VH. As shown in Table 1, a specific activity of 2 mU/10^6 cells was detected on live Vanin-1-transfected but not parental cells. After extraction, the total amount of enzymatic activity could be recovered mainly from the DOC-solubilized membrane proteins (22 U) and less from the cytosol (4.5 U). Furthermore, the 407 anti-Vanin-1 but not the control 106 mAb could immunodeplete the enzymatic activity from the solubilized membrane fraction and this activity was recovered on the mAb 407-coated beads (Table 1). The Vanin-1 molecule contains a C-terminus consensus sequence for coupling a GPI moiety

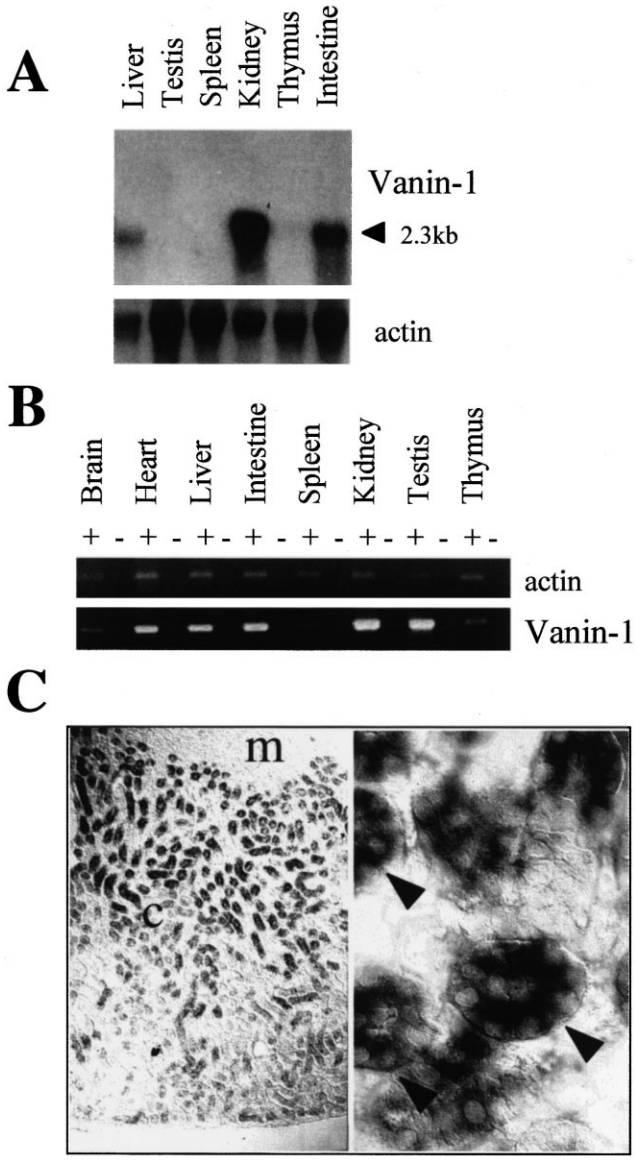

Fig. 1. Tissue distribution of the Vanin-1 mRNAs. A: Northern blot analysis: a unique 2.3 kb Vanin-1 transcript is found expressed in mouse tissues. B: RT-PCR analysis of the Vanin-1 gene expression in a larger panel mouse tissues: PCR fragments specific for Vanin-1 are found in all tissues tested. Amplification of a mouse actin PCR fragment is shown as a positive control and was used to approximately standardize the amount of cDNA templates used. For each RT-PCR experiment (+) a negative PCR control on non-reverse transcribed RNA (−) is shown. C: In situ hybridization detection of the epithelial cell expression of the Vanin-1 mRNA in the cortical tubules of mouse kidney. Left panel, c: cortex; m: medulla. Right panel, black arrow-heads indicate cortical tubule sections presenting Vanin-1 positive epithelial cells (magnification ×8 and ×32).

(S487) anchoring it at the membrane [12]. Thus, Vanin-1 is a membrane-bound pantetheinase.

3.3. Physicochemical properties of Vanin-1 pantetheinase activity

Pig pantetheine hydrolyzing activity is very resistant to temperature: its specific activity increases with temperature up to 75°C [9]. Mouse pantetheinase activity triples at 70°C compared to 37°C. Pig pantetheinase activity is regulated by disulfide [8]. Pantetheine and 4,4′-dithiodipyridine reversibly inhibit the activity to different extents and a detailed analysis suggested that inhibition by disulfide involved a thiol disulfide exchange between those molecules and catalytic -SH group(s). As expected, mouse pantetheinase activity is reduced by 60–70% in the presence of pantetheine (2.0 μM) and dithiodipyridine (8.0 μM), full activity was restored in the presence of mercaptoethanol (7.0 mM). Alkylating agents, such as iodoacetate and iodoacetamide, irreversibly inhibit pig pantetheinase by reacting with the two -SH groups essential for the activity [21]. Similarly, mouse pantetheine hydrolyzing activity was rapidly and irreversibly abolished by iodoacetate (10.0 μM) and iodoacetamide (10.0 μM), showing that the enzymatic activity depends upon thiol group(s). The pig pantetheinase K_m for pantetheine S-pyruvate as substrate was reported to be 28 μM at pH 8.0 and 30°C [18]. The calculated apparent K_m for mouse Vanin-1 pantetheinase activity (16 μM) was comparable to that calculated for pig pantetheinase added to solubilized membrane proteins of non-transfected cells (18 μM). Thus, the Vanin-1 and pig pantetheinase activities are similar.

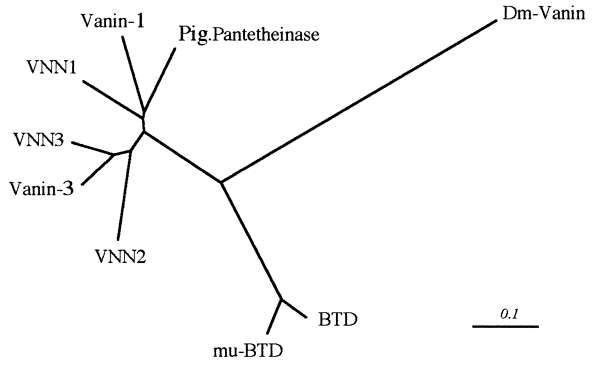

Fig. 2. Phylogenetic analysis of the Vanin family of proteins. All the partial peptide sequences of pig pantetheinase were fused to give a polypeptide sequence of 316 amino acid residues which was aligned with all available Vanin-like peptide sequences. A distance-base phylogenetic tree was generated using ClustalX (PHYLIP format) and edited using TreeView 1.5. Mouse Vanin genes: Vanin-1, Vanin-3; human Vanin genes: VNN1, VNN2, VNN3; BTD and mu-BTD: human and mouse biotinidase gene, respectively; Dm-Vanin: *Drosophila melanogaster* Vanin-like gene.

3.4. Generation of Vanin-1 deficient mice

A genomic clone (GV3) containing most of the promoter

Table 1
Determination of pantetheinase activity on Vanin-1-transfected cells

Cell samples	Live cells	Protein extracts	
		membranes (mU/mg)	cytosol
MTE1D	<0.1	<0.1	<0.1
MTE1D/VH	2 mU/10^6 cells	22	4.5 mU/mg
MTE1D/VH+407 mAb	2 mU/10^6 cells	<0.1	n.t.
MTE1D/VH+106 mAb	2 mU/10^6 cells	18	n.t.

Results are presented in mU/10^6 cells or mg protein as determined by the Lowry method. The lower limit of detection is 0.1 mU/mg protein. MTE1D is the parental cell line; MTE1D/VH are cells transfected with a native form of Vanin-1. Immunodepletion was performed using anti-Vanin-1 407 or 106 mAb-coated protein G-Sepharose beads (Pharmacia). n.t.: not tested. The 106 anti-JAM mAb served as an isotype-matched control [32].

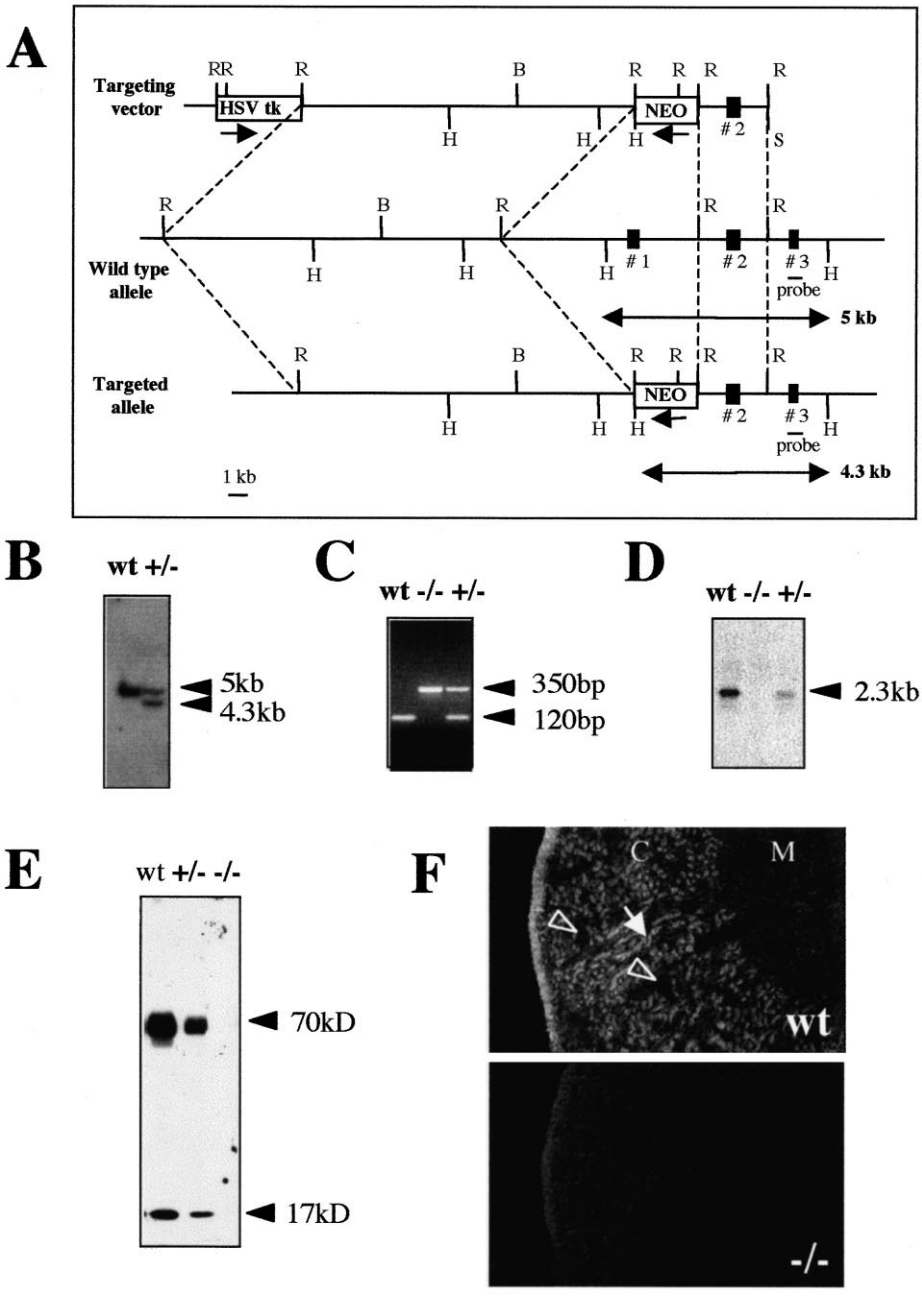

Fig. 3. Generation of Vanin-1 deficient mice. A: Map of the construct used for the homologous recombination. Arrows indicate the transcriptional orientation of the neomycin and HSV-tk genes. Double-head arrows indicated the size of the restriction fragments obtained by Southern blot analysis on a *Hin*dIII digest of genomic DNA from wild type or targeted alleles. The position of the 3′ probe is underlined. The result of the recombination event was checked by Southern blot (B), PCR (C) and Northern blot on kidney RNA (D). The Vanin-1 gene deficiency was confirmed by the loss of the Vanin-1 protein in kidney visualized by Western blot (E) or immunochemistry (F). C: cortex; M: medulla; the arrow indicates a Vanin-1 positive cortical tubule whereas arrow-heads show Vanin-1 negative glomeruli.

region and the three first Vanin-1 exons was used to design the knock-out construct. In this strategy, the first exon containing the ATG initiation codon and 2 kb of 5′ upstream regulatory sequences are replaced by the neomycin gene in the opposite transcriptional orientation preventing all possibilities of transcription of the Vanin-1 gene (Fig. 3A). As shown by Southern blot (Fig. 3B) and PCR analysis (Fig. 3C) on genomic DNA, the recombination event leads to the appearance of a 4.3 kb *Hin*dIII fragment detected by an exon 3 probe and a longer PCR fragment (350 bp) identifying the 5′ junction of the recombination. Northern blot from kidney (Fig. 3D) and RT-PCR analysis (not shown) confirmed the complete absence of Vanin-1 transcript in the Vanin-1 mutant mice. The absence of the Vanin-1 protein was confirmed by Western blot and immunohistochemistry on mouse kidney (Fig. 3D,F). A 17 kDa band which is often associated to Vanin-1 immunoprecipitates also disappeared in the null mutant, indicating that it corresponds to a degradation product of Vanin-1. Interestingly, heterozygous mice showed a 50% reduction of both Vanin-1 transcript and protein (Fig. 3D,E). The Vanin-1 null mice develop normally and do not show any major alteration in the maturation of lymphoid organs.

Table 2
Determination of pantetheinase activity and free cysteamine in tissues from wild type and Vanin-1 deficient mice

Organs	Genotypes	Pantetheinase activity (mU/mg)		Free cysteamine (nmol/g)
		Membrane fractions	Soluble fractions	
Kidney	+/+	10	<0.1	15
	−/−	<0.1	<0.1	n.d.
Liver	+/+	0.8	0.9	24 +/− 2
	−/−	<0.1	0.9	n.d.

For the pantetheinase activity, results are presented in mU/mg protein and <0.1 represents the limit of detection of the enzymatic activity. For the cysteamine determination, results are presented in nmol/g of fresh tissue. The results obtained on wild type kidney were variable from mouse to mouse ranging from undetectable to physiological amounts of cysteamine. +/+: wild type animals; −/−: Vanin-1 null mice; n.d.: not detectable.

3.5. Lack of cysteamine production in tissues from Vanin-1 −/− mice

We comparatively analyzed the enzymatic activity in organs from wild type and Vanin-1 deficient mice. As expected, in wild type animals, a mouse pantetheinase activity could be extracted from kidney membrane fractions using butanol (Table 2). A specific activity of 8–12 mU/mg protein was observed and found to be similar to that previously reported for the pig pantetheinase [3]. Interestingly, no pantetheinase activity was found in the kidney extracts from Vanin-1 deficient animals. In liver from wild type animals, a pantetheinase activity was found in both the membrane (0.8 mU/mg) and soluble (0.9 mU/mg) fractions. In Vanin-1 −/− livers, only the cytosolic (0.92 mU/mg) and not the membrane-bound pantetheinase activity was detected. All these results fit well with the membrane-bound distribution of the Vanin-1 molecule and confirm its enzymatic properties in vivo.

Hydrolysis of pantetheine by pantetheinase recycles the pantothenate moiety and produces cysteamine in vitro [2,3]. Since Vanin-1 deficient animals lack any detectable membrane-bound pantetheinase activity in kidney and liver, we evaluated free cysteamine levels in these organs by different methods (Table 2). Whereas cysteamine was present in wild type liver (24 ± 2.3 nmol/g), it was undetectable in liver from Vanin-1 −/− mice. Although variable results were obtained in kidney from control mice, we were unable to detect cysteamine in kidney from mutant mice. This is the first evidence that directly correlates the absence of a pantetheinase activity to an impaired production of free cysteamine in tissues in vivo.

4. Discussion

This report demonstrates that the Vanin-1 molecule is pantetheinase. Furthermore, the Vanin-1 mutant mouse, which develops normally, fails to produce detectable amounts of free cysteamine, a major metabolite of pantetheinase in tissues, under physiological conditions.

The Vanin molecules show significant sequence similarities with human biotinidase (about 42% amino acid identities) but Vanin-1 at least has no biotinidase activity. Biotinidase (EC 3.5.1.12) is an enzyme which participates in the recycling of biotin (vitamin B8) in the body by amidohydrolyzing biocytin [23,24]. Furthermore, Vanin molecules share with other amidohydrolases a protein domain containing one cysteine and one aspartic acid residue [14]. Mutations of the cysteine residue have been reported to impair the nitrilase activity in plants [25,26]. A recent report based on partial sequence comparisons between mouse Vanin-1 and pig pantetheinase has suggested that Vanin molecules might have a pantetheinase activity. The results presented in this report definitively demonstrate that Vanin-1 is pantetheinase based on two main arguments: Vanin transfectants acquire pantetheinase activity whereas Vanin-1 mutant mice are devoid of membrane-bound pantetheinase activity in kidney and liver.

Vanin-1 is the prototypic member of a larger family, which includes orthologous human and mouse Vanin genes and biotinidase [14]. In human, VNN gene transcripts have a broad tissue distribution and show partially overlapping expression patterns. Here, we report that Vanin-1 transcripts are ubiquitously expressed in mouse organs. The highest levels of Vanin-1 mRNAs are found in kidney where the tubular epithelial cells selectively express the Vanin-1 transcripts. This expression pattern was confirmed using the anti-Vanin-1 antibody, which detected the molecule at the brush border of kidney tubular cells, and enterocytes (not shown). Furthermore, a recent report documented the expression of Vanin-1 transcripts in developing testis [33].

In the literature, a pantetheinase activity has been found in several tissues in both soluble and membrane-associated forms [5,27]. The mouse Vanin-1 gene encodes a glypiated membrane-bound molecule [12]. Here we show that transfection of cells with the Vanin-1 cDNA leads to the detection of a specific pantetheinase activity in a membrane-bound form. In vivo, Vanin-1 deficient mice have no detectable pantetheinase activity in kidney and selectively lose the membrane-associated enzymatic activity in liver. These data are in complete agreement with the expression of the Vanin-1 molecule and strongly suggest the existence of soluble isoforms of pantetheinase, which might likely correspond to other Vanin family members (i.e. Vanin-3 in mouse).

Pantetheinase hydrolyzes one of the amide bonds of pantetheine recycling pantothenic acid (vitamin B5) and releasing cysteamine. Pantothenate is present in food mostly as CoA, which cannot be directly absorbed through enterocytes, whereas pantothenate freely diffuses across the epithelial barrier. Thus, one might speculate that conversion of CoA into pantothenate requires an extracellular, membrane-bound pantetheinase activity capable of recycling pantothenate in gut. Similarly, on the excretion pathway, the presence of a pantetheinase activity at the brush border of tubular epithelial cells might allow a salvage of vitamin B5. The other product of pantetheinase hydrolyzing activity is cysteamine, an anti-oxidant metabolite, which is one of the precursors of hypotaurine and taurine [28]. Levels of cysteamine in vivo were reported to range in rat liver from 7 pmol/g to 267 nmol/g tissue, demonstrating obvious problems with published methods [20,29,30]. Furthermore, levels of cysteamine in rat liver removed imme-

diately post-mortem or after 1 h are strongly different [20], demonstrating that all levels reported are not unequivocally accurate. Using two methods for cysteamine determination from fresh tissues, we obtained comparable results showing the absence of free cysteamine in liver and kidney from Vanin-1 deficient mice. Thus membrane-bound pantetheinase is a main source of free cysteamine in vivo.

A recent report documents the induction of Vanin-3 gene expression in metallothionein mutant mice, suggesting a possible link with the regulation of detoxification processes in vivo [31]. The human VNN2/GPI-80 molecule has been found to be involved in the migratory function of neutrophils [22], cells that have the potential of releasing reactive oxygenated species during inflammatory processes. The Vanin-1 putative function was discovered in the context of thymic reconstitution following sublethal irradiation, which generates an important source of free radicals. Preliminary results on Vanin-1 deficient mice revealed no major general or lymphoid developmental defects under physiological conditions. The importance of Vanin molecules might be revealed under stress such as irradiation. These results suggest that the Vanin gene family represents a novel class of ectoenzymes that might play a more general role in the regulation of the response to oxidative or inflammatory stress.

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