EXPRESSION PATTERN OF LYSOSOMAL PROTECTIVE PROTEIN/CATHEPSIN A:

Implications for the analysis of human galactosialidosis

Expressie patroon van het lysosomale "protective protein/cathepsin A":
Implicaties voor de analyse van patienten met galactosialidosis

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The front and back covers show the distribution of PPCA mRNA expression in the brain of an adult mouse (for details see chapter 5)

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Men give me credit for some genius. All the genius I have is this. When I have a subject in mind. I study it profoundly. Day and night it is before me. My mind becomes pervaded with it... the effort which I have made is what people are pleased to call the fruit of genius. It is the fruit of labor and thought.

Alexander Hamilton

Voor Esther

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List of abbreviations

BM	Bone Marrow
BMT	Bone Marrow Transplantation
(c)DNA	(complementary) Deoxyribonucleic Acid
CNS	Central Nervous System
ERT	Enzyme Replacement Therapy
GRE	Glucocorticoid Responsive Element
GS	Galactosialidosis
GTF	General Transcription Factor
Inr	Initiator
kb	Kilo base
kDa	Kilo Dalton
LCR	Locus Control Region
MPS	Mucopolysaccharidosis
(m)RNA	(messenger) Ribonucleic Acid
PIC	Pre-Initiation Complex
PPCA	Protective Protein/Cathepsin A
TAF	TBP-Associating Factor
TBP	TATA-Binding Protein
UTR	Untranslated Region

SCOPE OF THIS THESIS

The lysosome represents a well characterized, membrane-contained intracellular digestive system. In this important organelle a battery of lysosomal hydrolases and accessory proteins work in concert on the step-wise conversion of macromolecular substrates into small biological building blocks, which are either reutilized by the cell or discarded. A failure of any of these enzymes to properly exert their hydrolytic activity results in the progressive accumulation of partially degraded metabolites that are retained ('stored') in the lysosome. The genetic disorders caused by a malfunction of the lysosomal system are collectively known as lysosomal storage disorders, and are normally associated with a single enzyme deficiency. One known exception is the disease galactosialidosis which is due to partial or complete loss of activity of two glycosidases, acid β-D-galactosidase and N-acetyl-α-neuraminidase, because of a primary defect in the carboxypeptidase protective protein/cathepsin A (PPCA). The latter enzyme associates with both glycosidases soon after synthesis, and is essential for their proper intracellular routing, lysosomal stability and activity. Aside from the protective function, PPCA has cathepsin A/deamidase activity on a selected number of neuropeptides.

The aim of the experimental work presented in this thesis was to gain insights into the transcription regulation of the PPCA gene and the expression of PPCA mRNA and protein in mouse tissues. These studies have contributed to the understanding of the phenotypic abnormalities in the murine model of galactosialidosis, which reflected to a large extent the distribution pattern of the protein in normal tissues. Given the fact that the observed pathology in galactosialidosis is in part caused by the secondary neuraminidase deficiency, the isolation and characterization of the murine neuraminidase was instrumental to better understand in which way the two enzymes depend on each other and cooperate *in vivo*.

Chapter 1

General introduction

1.1 Eukaryotic transcription and its regulation

One of the requirements of the cell to function properly, is the tight control over the switching on, or off, of genes involved in various cellular activities. A diversity of internal and/or external signals results in an interplay between activating and repressing signals, which ultimately determines the transcriptional activity of a gene (42, 109, 123). Transcription is the complex enzymatic process of activating a gene and subsequently generating an identical RNA copy from the template DNA. These series of events is executed by a plethora of proteins and is subjected to tight regulation (132).

In eukaryotes, transcription is mediated by one of the three DNA-dependent RNA polymerases, ultimately resulting in the generation of small rRNAs (Pol. I), amino acid carrying tRNAs (Pol. III) or protein encoding mRNAs (Pol. II). The transcriptional process can be divided into several closely linked and often overlapping steps (see 167). 1) Recruitment entails the binding of promoter sequences by activators and/or repressors, leading to the rearrangement or disruption of the chromatin structure. This in turn will allow the preinitiation complex (PIC) to bind to the sequence specific site in the promoter of the gene. 2) PIC activation includes the melting, or unwinding, of the two DNA strands to make the promoter accessible for the transcriptional machinery. At least two groups of proteins are involved in this process: DNA helicases and single-stranded DNA binding proteins. 3) Initiation involves the production of the first phosphodiester bond in the nascent RNA molecule. 4) Promoter clearance defines the transition in the transcriptional process from the initiation to the elongation phase. This step probably involves the release of the transcriptional machinery from the general initiation factors present on the promoter. 5) Elongation is the continuous addition of ribonucleotides to the growing RNA molecule, which is in a stable ternary complex with the DNA and the RNA polymerase (review see 223). 6) Finally, termination is the process whereby the RNA polymerase reaches the termination specific regions at the 3' end of the gene, leading to the release of the nascent RNA molecule. Since the topic of this thesis is restricted to protein encoding genes, I will focuss on transcription mediated by RNA pol II, and refer to literature for RNA pols I and III dependent transcription processes (45, 53).

RNA polymerase II transcribed genes usually consist of a core DNA domain, to which the general transcriptional initiation factors bind, and other upstream or downstream gene specific DNA elements that are important for the correct regulation of the gene. The expression of the gene is regulated and controlled by specific interactions between complexes bound to the core promoter and the gene-specific factors bound to the regulatory elements. The promoter of a gene is required for the basal level of transcription and contains the site where the RNA polymerase holoenzyme, including its accessory factors, interacts with the DNA (definition: see 45, 132). The classical eukaryotic core promoter consists of a TATA-box located 25-30 nucleotides upstream of

the transcriptional start site. This box will tether the TATA-Binding Protein (TBP) and its interacting TBP-Associating Factors (TAFIIs , 209, 236), resulting in a complex, $TF_{II}D$, mandatory for the precise positioning of the DNA-dependent RNA polymerase II, and at least five accessory proteins TF_{II} A, B, E, F and H, the General Transcription Factors (GTFs), to the DNA (167). *In vitro* experiments have shown a sequential order for the general transcription factors and the pol II to associate with the $TF_{II}D$-DNA complex. However, recent data challenge the multistep recruitment of factors to the core promoter and suggest that the RNA polymerase II holoenzyme, or part of it, exists as a complex before its association with $TF_{II}D$-DNA complex (87, 155, 245). Aside from the TATA-box, another important DNA core element has been described that may initiate transcription either alone or in combination with the TATA-box (17, 194). This element, called the initiator (Inr), overlaps with the transcriptional start site and is sufficient to correctly position the transcriptional machinery in the absence of the TATA-box (81, 194). Various Inr elements have been identified and several factors, including TF_{II}-I, USF, YY1 and HIP1, have been shown to bind to Inr elements with different sequences resulting in the induction of the transcriptional initiation process (81). In addition, another conserved motif has been described which is involved in the transcription of TATA-less promoters and explains the multiple start sites of many of these types of promoters. This motif is downstream of the transcriptional start site and is referred to as MED-1 for Multiple start site Element Downstream (76). Although the exact mechanism of the initial binding and activation through the Inr has not yet been elucidated, both the TATA-box directed and the Inr regulated expression use the same factors and mechanisms to complete the transcriptional process.

The binding of the RNA polymerase holoenzyme to the core promoter and its activity are influenced by the properties of sequence specific activators. These types of molecules bind to a specific DNA sequence and subsequently recruit other factors and/or the RNA polymerase holoenzyme, positioning the originating complex on the DNA in the vicinity of the transcriptional start site (reviewed in 155). The specificity of a transcriptional activator is solely determined by its DNA binding domain, whereas its activating domain provides an interacting surface for other proteins to access the promoter, suggesting a general role for their recruitment abilities (154). Multiple activators act in a cooperative manner, leading to synergism between the actions of separate molecules. Aside from the recruitment of additional factors needed for stabilization and positioning of the basic transcriptional machinery to the promoter of a gene, activators also attract proteins that disrupt the nucleosome structures to free the DNA from its physical limitations (for review see 101). Eukaryotic DNA is condensed through its complex formation with histones and packaged into nucleosomes, which are structures that limit the availability of DNA for interacting proteins (88). Acetylation of the core histones by coactivators that possess histone acetyl transferase activity reduces the compressed state of the DNA and makes it accessibile for other transcriptional

factors. In addition to the acetylase activity of coactivators, the holoenzyme probably competes with histones for access to the DNA (89, 101, 143, 155, 197, 221, 240, 248). There is a close connection between the physical structure of the DNA and the transcriptional machinery, since chromatin remodelling complexes facilitate the binding of sequence-specific transcription factors to the appropriate promoter and the DNA-bound transcription factors possess the ability to recruit the chromatin remodelling complexes (83, 198). Apart from the DNA sequence elements that are located relatively close to the transcriptional start site, there are more distant regions that play a role in the activation and level of transcripiton, like enhancers, locus control regions and silencers (60, 84, 207).

1.2 *Transcriptional regulation of lysosomal genes*

After the early identification of promoter regions containing the canonical TATA – and CAAT boxes, an increasing number of genes have been characterized lacking these two domains in their transcriptional initiating site (194). Among these is a family of genes that encode proteins with essential metabolic functions and a wide tissue distribution, the housekeeping genes (41). The first two well characterized housekeeping genes were the murine hypoxanthine phosphoribosyl transferase (HPRT) and dihydrofolate reductase (DHFR) genes, soon followed by others, like adenosine deaminase (ADA), phosphoglycerate kinase (PGK) and adenine phosphoribosyl transferase (APRT, Refs. in 41). Members of this family are characterized by multiple transcriptional start sites and a high G/C content in the promoter region. Since the majority of the lysosomal enzymes have an essential function in the homeostasis of cells, it is not surprising that many of the isolated and characterized lysosomal genes have some features in common with housekeeping genes in their promoter (chapter 3 of this thesis, 54, 58, 70, 90, 91, 106, 107, 112, 120, 127, 130, 149, 150, 157, 160, 166, 173, 188, 200, 219, 241, 255). However, a number of human and mouse genes encoding lysosomal enzymes bear characteristics in their promoters, which suggest additional modes of regulation in comparison to housekeeping genes (chapter 3 of this thesis, 9, 28, 38, 68, 71, 75, 118, 136, 142, 158, 161, 162, 184, 215, 224, 244).

The identification of cis-acting elements in promoter regions is merely an indication that putative binding sites for specific transcription factors are present, but their functionality has to be shown *in vivo*. Some of the lysosomal genes have been characterized in more detail, and I will discuss just a few examples. For the human aspartylglucosaminidase promoter, for instance, footprinting analysis and electophoretic mobility shift assays showed that two SP1 binding sites in the core promoter region are protected from DNAse I digestion. Whether or not the TATA-like sequence, present in this promoter, is functional remains to be seen, since it was not protected in the assay

(224). Analysis of the human glucocerebrosidase gene revealed that the ubiquitous expression levels of the gene depend on the availability of transcription factors, like AP-1, PEA3 and a CAAT binding protein (118). Cathepsin B is presumably regulated by more than one promoter, which are responsible for a non-constitutive expression in spite of the presence of a housekeeping-like promoter (156, 157, 176). Expression of the gene encoding the human aspartyl proteinase cathepsin D is regulated by a composite promoter, leading to an estrogen-inducible, TATA-box dependent transcript and a constitutively expressed transcript (2, 21, 114, 116). The human β-glucuronidase gene appeared also to have two separate promoters that drive its expression, but this could only be demonstrated in transformed cells (188). In chapter 3 of this thesis I will discuss our findings on the expression of the murine PPCA gene that is regulated by the activity of two functional promoters.

1.3 Additional functions of lysosomal proteins

Detailed analyses of the promoter regions of several lysosomal genes have suggested that they may be subjected to a level of regulation much tighter than anticipated by their overal degradative function. In addition to the effect of estrogen on the transcription of the human cathepsin D promoter, other lysosomal enzymes are influenced by hormones. For example, β-hexosaminidase, β-glucuronidase and β-galactosidase are stimulated by testosterone, which leads to an elevated secretion in the mouse kidney (204). However, it was never demonstrated that the hormone directly induces the secretion, which could represent the secondary effect of an upstream, testosterone-dependent process. Nevertheless, it remains suggestive that the promoter of the β-hexosaminidase A gene contains two putative hormone binding sites, which could be responsible for the effect observed in the kidney (130). Similarily, we found that the murine PPCA gene carries a hormone responsive element, a GRE site, in the distal promoter, although it remains to be tested whether this element is functional and relates to the catalytic activity of PPCA to inactivate specific hormones (chapter 3 of this thesis). Other cathepsins, like cathepsins B and D, have also been implicated in the processing, secretion, activation and catabolism of hormones and other proteolytic enzymes (4, 46, 47, 129, 139, 157, 187, 199). The inducibility of a number of lysosomal genes may reflect the need for the encoded protein to be active under certain physiological conditions in order for a cell to cope with a defined substrate load.

Several lysosomal enzymes, including members of the cathepsin family of proteases, acid phosphatase, α-mannosidase, arylsulfatase, β-galactosidase, β-glucuronidase and β-N-acetylglucosaminidase have been implicated in cell proliferation and tumor growth (see table I in 15). The increase in specific activity of these lysosomal proteins in tumor tissues apparently correlates with the metastatic and invasive potential

(40, 57, 98, 108, 168, 191, 193, 251, 256). However, it is not proven yet that the observed increase in enzyme activity is the actual cause for the growth or malignancy of the tumor. Glucocerebrosidase, for instance, was shown to be upregulated in transformed cells, but these cells divide more frequently, are metabolically more active and hence may require higher levels of degradative enzymes. Therefore, the induced expression of lysosomal enzymes could be merely a consequence of the transformation, rather than the cause (118). Nevertheless, a change in subcellular localization, from lysosome to plasma membrane, of cathepsins B, D and L, as well as β-N-acetylglucosaminidase was observed in tumors with high metastatic potential (168, 193, 256). Furthermore, it was shown that a number of lysosomal enzymes, including PPCA, cathepsins B and D, have an intrinsic catalytic activity at neutral pH, supporting the idea for a functional role of these enzymes outside the acidic environment of the lysosomes (18, 79, 93, 256). Together, these observations support the idea that lysosomal enzymes facilitate the invasiveness of tumors by degrading the extracellular matrix (6, 102, 146, 168, 192, 220, 256). Two of the major constituents of the extracellular matrix are laminin and collagen type IV, both of which can be degraded by cathepsin B *in vitro* (16, 93). Degradation of these two structural proteins has been related to tumor invasion and metastasis as well as a poor clinical prognosis (65, 97). These processes are thought to be controlled by multiple proteases (256). In this pathway, cathepsin D can activate procathepsin B, elastase, cathepsin G and urokinase-type - and tissue-type plasminogen activator. Cathepsin B, in turn, may directly or indirectly act on other proteases and subsequently activates pro-urokinase-type plasminogen activator, which then catalyzes the conversion of plasminogen into plasmin. Plasmin can degrade components of the tumor stroma and may activate gelatinases and various matrix metalloproteinases (see also 251). Active proteolytic enzymes in the extracellular space may cause severe damage to tissues and lead to serious pathologic conditions by irreversibly hampering the normal physiological functions.

Different hypotheses have been postulated to explain the apparent correlation between tumorigenic potential and a higher lysosomal enzyme activity (15), which include: 1) Proliferating - and dividing cells generally have a higher rate of metabolism. 2) Certain types of tumors have been shown to contain an increased rate of pinocytosis. 3) Differences between intralysosomal pH of tumor cells and normal cells. 4) Both apoptotic and necrotic cells in tumorigenic tissue may secrete or expel lysosomal enzymes in the interstitium of the tumor, resulting in an overal increase in extracellular enzyme activity. Other physiological, cell proliferative processes are also accompanied by an increase in the activity of lysosomal enzymes, such as tissue regeneration (119), bone resorption and remodelling (13, 33, 165) and the involution of the uterus after partus (43).

Finally, lysosomal enzymes, and in particular lysosomal proteases, have been implicated in different immunological reactions. Cathepsin D apparently plays a role in

T-cell cytotoxicity (61, 144), antigen processing (126, 191, 231) and inflammation (4), while cathepsin B is secreted by macrophages during an inflammatory response (159). Cathepsin L and, most likely, cathepsin S control the processing of the invariant chain (Ii) in cortical thymic epithelium and bone marrow-derived antigen presenting cells, respectively (27, 125, 164, 237). In contrast to these *in vitro* obtained results (111, 164, 237), it was shown recently that, although required, both cathepsin B and D are not essential for the MHC class II mediated antigen processing (34). In the case of PPCA, thrombin-activated human platelets release the lysosomal enzyme into the extracellular space. This secreted form, active as deamidase at pH 7.0 could participate in the local inactivation of peptides implicated in inflammatory processes, like substance P and some of the bradykinins (this thesis, 79). Lysosomal N-acetyl-α-neuraminidase modulates immunological reactions by controlling the sialylation status of specific cell surface molecules. These studies will be discussed in depth in chapter 6.

In conclusion, although the majority of the lysosomal enzymes are encoded by genes containing promoters with typical features of housekeeping genes, a number of them have characteristics and activities, which suggests a more specialized mode of regulation: like (in) activation of bioactive peptides and pro-enzymes, immunological reactions and cell-proliferative mechanisms. In spite of the fact that some lysosomal enzymes are implicated in additional non-lysosomal functions, no clear phenotypic manifestations related to these functions have been described yet in patients deficient for one of these enzymes. These phenotypic abnormalities may be difficult to recognize, unless they can be mimicked and studied in animal models.

1.4 Lysosomal storage disorders

Lysosomal storage diseases comprise a group of over 40 human disorders of the metabolism caused by genetic lesions in one of the genes encoding lysosomal proteins. With the exception of two X-linked disorders, Fabry disease (α-galactosidase) and Hunter syndrome (α-L-iduronate-2-sulfatase), all lysosomal storage disorders are inherited as autosomal recessive traits. Although individual diseases can be very rare, as a group they have an incidence in the human population of 1:1500 life birth (1, 55, 128, 163). Some disorders are found at higher frequency in specific ethnic groups, because of their genetic isolation, like the Ashkenazi Jews (Gaucher disease, Tay-Sachs disease and Niemann Pick disease, 55), the Japanese (adult forms of galactosialidosis and GM1 gangliosidosis, 29, 186, 203) and the Finns (aspartylglucosaminuria, 216). Lysosomal diseases are subdivided into glycolipidoses, glycoproteinoses and mucopolysaccharidoses, depending on the major type of substrate that is accumulated. In addition, the routing of normal enzymes towards the lysosomal compartment or the export of the hydrolytic products to the cytosol can be affected (reviewed in 1, 55, 128).

The substrate(s) in the pathway of the affected enzyme is (are) progressively accumulated in the lysosome. In time, numerous and enlarged lysosomes will populate a cell, leading to cellular malfunction and eventually to deterioration of the organ/tissue. Lysosomal hydrolases are active towards a variety of substrates, because they recognize linkages between sugar moieties, rather than a single compound. Therefore, the storage products in a diseased cell or tissue can be very heterogeneous.

The genetic basis of lysosomal disorders can be relatively simple, but the presence of lysosomes in virtually every cell type makes the clinical phenotype of affected individuals rather complex. In general, these diseases display a wide range of clinical manifestations that include CNS involvement, skeletal malformations, eye abnormalities, cardiovascular problems and organomegaly. Patients can be grouped into clinical subtypes depending on the age of onset of the disease and the severity of the symptoms: early infantile, late infantile and juvenile/adult (55, 128). The discrimination between the three subtypes is not absolute and they often overlap, making the exact clinical outcome difficult to predict. In general, however, one can apply the rule that the earlier the onset of the disease, the more severe the symptoms and the shorter the life expectancy (55). Moreover, the increasing knowledge of affected genes and the screening of mutations allow for a careful correlation between genetic mutations and clinical phenotypes, and in some instances a better diagnosis (56, 99, 185, 206, 259).

Table: Overview of mouse models for lysosomal storage disorders

Part A: Naturally occuring mouse mutants

Affected gene	*Human disease*	*References*
Galactosylceramidase (Twitcher mouse)	Krabbe	(85, 86, 174)
β-glucuronidase	Sly-syndrome (MPS VII)	(8, 100, 178, 238)
Npc 1	Niemann-Pick type C	(105)
N-acetyl-α-neuraminidase (SM/J)	mild sialidosis	(this thesis chapter 5, 20)
Cathepsin L (furless)	?	(125)

Part B: Mouse models generated by targeted gene disruption

Affected gene	*Human disease*	*References*
β-hexosaminidase A	Tay-Sachs disease	(24, 145, 254)
β-hexosaminidase B	Sandhoff disease	(145, 182)
G_{M2} activator	G_{M2} activator deficiency	(103)
Protective protein/ Cathepsin A (PPCA)	Galactosialidosis	(chapter 3)
β-galactosidase	G_{M1} gangliosidosis	(62, 113)
α-galactosidase A	Fabry disease	(137)
acid sphingomyelinase	Niemann-Pick type A/B disease	(74, 141)
Arylsulfatase A	Metachromatic leukodystrophy	(67)
Glucocerebrosidase	Gaucher disease	(222)
Glycosylasparaginase	Aspartylglucosaminuria	(80, 82)
α-L-iduronidase	Hurler/Scheie syndrome (MPS I)	(23)
Arylsulfatase B	Maroteaux-Lamy syndrome (MPS VI)	(44)
α-glucosidase	Pompe disease	(7)
acid lipase	Wolman's disease/cholesteryl ester storage disease	(39)
sphingolipid activator (prosaposin)	total sphingolipid activator deficiency	(48)
α-N-acetylgalactosaminidase	Schindler disease	(243)
acid phosphatase	?	(171)
cathepsin D	?	(172)

1.5 Mouse models for lysosomal storage disorders

Both the low incidence of the individual diseases and the ethical implications limit the access to patients' specimens and material. Therefore, animal models that closely resemble the corresponding human conditions can be extremely useful to study the pathogenesis of the diseases in more detail, which is crucial for the development of therapeutic strategies. Furthermore, these models could answer important biological questions about the different pathways in which the affected enzyme is involved. The mouse is the most widely used animal model, since its biology and genetics are close enough to humans, with short reproduction cycles and multiple, large litters. Only a few naturally occuring murine models for lysosomal storage disorders have been identified (see table, part A 8, 85, 86, 105, 174, 178), but the possibility to generate targeted disruptions in the mouse genome has given scientists a powerful tool to manipulate and develop models of virtually every disease (19, 37, 110, 195, 217, 218). In addition, mouse mutants can be generated and studied that are either not paralelled by a human disease because of a high lethality, or are unlikely to exist, like double knockout mice. In general, the majority of these knockout mice resemble the most severe forms of the human diseases. Several mouse models for lysosomal storage disorders have been generated, primarily to test the feasability of different clinical treatments, like bone marrow transplantation and enzyme replacement (see table, part B). I will briefly discuss a number of the murine models that have led to surprising observations and unveiled discrepancies with the human disease.

The β-hexosamindase isoenzymes A, B and the minor form S are heterodimers of the α and β subunits, or homodimers of the β - or α subunits, respectively. These isoenzymes degrade various glycolipids, but the catabolism of G_{M2} ganglioside in humans is controlled exclusively by β-Hexosaminidase A and the G_{M2} activator protein, which promotes the solubilization of the substrate (59). Deficiencies in the genes encoding either the α subunit, β subunit or the activator protein result in the impaired degradation of G_{M2} ganglioside and other glycolipids, which form the molecular basis of three diseases known as G_{M2} gangliosidoses: Tay-Sachs (α subunit deficiency), Sandhoff (β subunit deficiency) and G_{M2} activator deficiency (*gm2a*). The G_{M2} gangliosidoses share common clinical manifestations, a massive accumulation of G_{M2} ganglioside and other glycolipids in neurons, and are characterized by a rapidly progressing neurological deterioration (55, 59). Mouse models have been generated for Tay-Sachs (*hexa*$^{-/-}$, 24, 145, 208, 254), Sandhoff (*hexb*$^{-/-}$, 145, 182) and the activator protein deficiency (*gm2a*$^{-/-}$, 103), but, unlike the human diseases, their phenotypes differ considerably. Despite the detected storage of G_{M2} ganglioside in some of the cortical neurons, the *hexa*$^{-/-}$ mice do not show neurologic disturbances in their behaviour, are fertile and have a normal life span. In contrast, the *hexb*$^{-/-}$ mouse resembles the human phenotype and is severely affected displaying progressive

neurodegeneration, tremor, spasticity, hind limb rigidity, ataxia and muscle wasting, leading to paralysis and death. An alternative metabolic pathway solely present in the mouse causes the different phenotypic appearance in comparison with human. The *hexa*$^{-/-}$ and *hexb*$^{-/-}$ mice both accumulate G_{M2} ganglioside through the block in hexosaminidase activity, although the latter stores four times more of this substrate as well as an asialo derivative of G_{M2} ganglioside, G_{A2} glycolipid, which is generated by a sialidase activity. This G_{A2} glycolipid can further be degraded by β-hexA (α and β subunit heterodimer) and β-hexB (β subunit homodimer) isoenzymes, but not by β-hexS (α subunit homodimer). So, the *hexa*$^{-/-}$ mouse, which still has β-hexB activity, is able to catabolize considerable levels of G_{M2} ganglioside through the de-sialylation pathway, whereas the *hexb*$^{-/-}$ mouse, which solely has β-hexS activity, intracellularly stores both gangliosides (103, 182, 201). The *gm2a*$^{-/-}$ mouse has a subtle neurological dysfunction, because it accumulates G_{M2} ganglioside at comparable levels as the *hexa*$^{-/-}$ mouse, as well as low levels of G_{A2} ganglioside. The catalysis of G_{A2} is hampered, but not completely blocked, which results in a more severe phenotype than the *hexa*$^{-/-}$ mouse, but less severe than the *hexb*$^{-/-}$ mouse. In conclusion, only the *hexb*$^{-/-}$ mice resemble the early-onset Sandhoff disease patients, whereas the *hexa*$^{-/-}$ and *gm2a*$^{-/-}$ mice display a milder phenotype in comparison with the human disorders. The analysis of the three different G_{M2} gangliosidosis mice unexpectedly unraveled the importance of an alternative pathway in mice which catalyzes G_{M2} ganglioside using sialidase activity.

Crossbreeding of the *hexa*$^{-/-}$ and the *hexb*$^{-/-}$ mice resulted in a double knockout mouse that completely lacked β-hexosaminidase activity, resulting in a neurovisceral storage disorder (181, 202). The phenotype of this double knockout mouse was more severe and extensive than that of the individual, single knockout mice and manifest at an earlier age. Interestingly, biochemical, clinical and pathological features of the double knockout mouse paralelled the mucopolysaccharidosis phenotype of the β-glucuronidase deficient mouse (MPS VII or Sly syndrome). This finding suggested that the lack of all three hexosaminidase isoenzymes, A, B and S, interferes with the catabolism of glycosaminoglycans as well. Although no patients have been described who completely lack all three isoforms, the double knockout mouse turned out to be a valuable model for studying the biological processes involved in the degradation of glycosaminoglycans and the consequences of excessive accumulation of these compounds.

Similarly to what was observed in the mouse models for the G_{M2} gangliosidoses, knockout mice lacking lysosomal acid β-D-galactosidase show phenotypic differences with the human disease. This disorder is characterized by the excessive accumulation of G_{M1} ganglioside, especially in the CNS. Patients with the early infantile type I form of G_{M1} gangliosidosis present with developmental arrest, progressive neurologic deterioration, facial dysmorphism, hepatosplenomegaly and generalized skeletal dysplasia (203). Disruption of the β-galactosidase gene to generate a model for G_{M1}-gangliosidosis results in mice with a severe neuropathological phenotype, which

resemble the early infantile patients (62, 113). Neurons throughout the cerebrum, cerebellum, brainstem and spinal cord store excessive amounts of G_{M1} ganglioside as well as its asialo derivative, G_{A1}. The latter feature is not observed in patients and results from the activity of a sialidase that converts G_{M1} ganglioside into G_{A1}. This is likely the same sialidase that desialylates G_{M2} ganglioside into G_{A2} in the G_{M2} gangliosidoses mice. Patients with either G_{M1} - or G_{M2} gangliosidosis primarily store sialylated glycolipids, and hardly the desialylated forms, suggesting that the sialidase is less active towards these substrates in humans. In contrast to the human disease, a minimal involvement of the visceral organs was observed in the G_{M1}-gangliosidosis mouse, which could be due to either partial redundancy in the catabolism of β-galactosidase substrates in mice or a difference in the type of substrates converted (62, 113). Absence of hepatosplenomegaly was also observed in other mouse models for lysosomal storage disorders, like in one of the two Niemann-Pick A models (74), in the Gaucher mouse (222), in the MPS I mouse (23) and in the naturally occuring twitcher or MPS VII mouse, which only displays splenomegaly (100, 238). On the other hand, hepatosplenomegaly is observed in other murine models for lysosomal storage disorders, like in galactosialidosis (this thesis) and in the second Niemann-Pick A mouse (141).

Another unexpected finding emerged from the analysis of the Gaucher mouse model, which dies within 24 hours after birth, whether it completely lacks glucocerebrosidase activity through disruption of the gene (222), or it contains point mutations corresponding to mutations found in human patients (104). The extent of the storage in these mice was not sufficient to explain their rapid deterioration and short lifespan, but the mutant mice apparently succumbed through a compromised epidermal permeability barrier caused by defective glucosylceramide metabolism (104, 222, 246). In addition, the glucocerebrosidase locus turned out to be very complex, since a gene called *metaxin* overlaps with the 3' end of the lysosomal gene and is responsible for embryonal lethality when inactivated (12). Other genes that fall into this chromosomal region are *thrombospondin 3*, *clk2*, *propin 1*, *cote 1* and the pseudogenes for glucocerebrosidase and *metaxin* (247). So, the genetic complexity of the glucocerebrosidase locus may contribute or add to the phenotypic abnormalities of the mouse.

The model for metachromatic leukodystrophy, generated by disruption of the arylsulfatase A gene, closely parallels the human disease, although no widespread demyelination could be detected (67). The latter would explain the absence of devastating neurologic deficits in the mouse, which are present in the advanced stages of the human disease. Two possible explanations could explain this difference between man and mouse: first, the murine pathology is compared with the final stage of the human disease; secondly, an alternative metabolic pathway in the mouse, able to clear sulfatide, may result in a reduced accumulation of lipid compared to humans.

Two murine models have been made of which no human counterparts are known: lysosomal acid phosphatase (LAP, 171) and cathepsin D (172). In spite of the fact that acid phosphatase led De Duve to the discovery of lysosomes, neither the physiological substrates, nor the function of this protein is known (3, 31, 32). The LAP-deficient mouse displays progressive storage in podocytes and tubular epithelium of the kidney, conspicuous bone abnormalities and CNS storage in the microglia, epenedymal cells and astroglia. There is only one report on a patient who completely lacked acid phosphatase activity, but the molecular basis still remains speculative especially since no other cases have been described (124). The disruption of the tartrate-resistant acid phosphatase (TRAP, *Acp5*), an enzyme also found in the lysosomes, generated mice with defective enchondral ossification and delayed mineralization of the cartilage (66). The cross mating of the LAP and TRAP mice resulted in a severe phenotype, which displayed excessive lysosomal storage in cells that were devoid of storage in the individual knockouts, for instance Kupffer cells, suggesting that LAP and TRAP may have overlapping activities (171). Mice lacking the aspartic protease cathepsin D (172) have reduced locomotion, progressive ataxia and die within 4 weeks after birth as a result of intestinal necroses accompanied by thromboemboli. The phenotype suggest that cathepsin D is important for limited proteolysis of specific proteins involved in cell growth or homeostasis, while its activity in bulk proteolysis is non-critical. However, no human patients have been described lacking cathepsin D, or any other lysosomal protease, like PPCA, which suggests a crucial physiological role of this protease *in vivo*. It is also not known whether the absence of cathepsin D makes these mice less susceptible to metastatic types of cancer.

In general, mouse models for lysosomal storage disorders, either naturally occurring or generated by gene-specific targeted disruption, have improved our knowledge about the biology of these diseases. Most of these models have proven to parallel the human disorder, although few discrepancies were observed, which relate to specific differences in biochemical pathways between man and mouse. Mouse models are better accessible to examine phenotypic changes at any given time during progression of the disease. This is particularly true for studying brain pathology, which would only be possible, if at all, at the final stages of the disease in humans. Mouse models can also be used to generate and develop new therapeutic protocols and curative agents. For this purpose, knowledge of the distribution of affected cells in different organs will be instrumental to understand and explain the benefits of treatment.

1.6 *Treatments of lysosomal storage disorders*

Treatments to reduce or correct the clinical manifestations, like enzyme replacement therapy, allogeneic bone marrow transplantation and somatic cell therapy,

are based on the capacity of soluble lysosomal enzymes to be secreted by one cell type and recaptured by neighboring or distant cells (for review see 14). The process of internalization occurs through the interaction of the mannose-6-phosphate moiety (M6P), present on the precursor enzyme, and the cation-independent M6P-receptor at the cell surface (69). One can anticipate that if sufficient amounts of normal enzyme were administered to affected individuals, it would be endocytosed by deficient cells in different organs and ameliorate the phenotype. Treatment of CNS pathology remains, nonetheless, a real challenge and the availability of animal models closely resembling the human conditions has facilitated the experimental trials.

Different animal models have been exploited to develop and implement therapeutic approaches in order to revert or diminish the deleterious effects of lysosomal storage disorders. I will describe a number of examples of experimental treatments that appeared very promising. Bone marrow transplantation on fucosidosis dogs resulted in a gradual improvement of the CNS lesions, a slower progression of the neurological symptoms and a milder phenotype (210, 211, 213). Although bone marrow transplantation of mucopolysaccharidosis type I dogs gave only 1-3 % of control values of α-L-iduronidase activity in the brain, significant reductions of stored glycosaminoglycans in cerebrospinal fluid and meninges were noted, as well as improvements of ultrastructural lesions (189, 190). Treatment of α-mannosidosis cats with allogeneic bone marrow seemed to improve the CNS pathology, except for the Purkinje cells in the cerebellum, and showed the presence of α-mannosidase positive staining in neurons, glia and endothelial cells (242). However, the absence of a detailed map of the affected regions in the brain of these animal models, makes the interpretation of these results difficult. The twitcher mouse, a model for globoid cell leukodystrophy or Krabbe disease, had a prolonged survival and improvement of locomotor ability after receiving congenic bone marrow before the onset of clinical symptoms (72, 257). Donor-derived foamy macrophages were present in the CNS at identical sites where brain lesions and globoid cells occur in the untreated animals, which resulted in an increase of galactosylceramidase activity and an improvement of myelination (72). Bone marrow transplantation (131) or enzyme replacement (25) in feline mucopolysaccharidosis type VI resulted in an improvement of the skeletal development, in spite of the persistent presence of vacuolation in the cartilage. Newborn acid sphingomyelinase deficient mice, model for Niemann-Pick types A and B, received normal bone marrow, which resulted in a delayed onset of ataxia and a reduction of lipid storage in the CNS, aside from the complete correction of the reticuloendothelial system (117). In certain areas of the cerebellum the presence of Purkinje cells, which are completely absent in the non-treated animals, was noted, but eventually all the treated animals developed severe ataxia and died approximately four months older than the non-treated animals. Since it is difficult to assess precisely by histopathologic analysis what level of correction is necessary to regain or maintain CNS function, behavioral tests are

important to evaluate the effect of specific therapies. Although BMT of newborn MPS VII mice improved their biochemical, clinical and pathological abnormalities, it did not ameliorate their behavioral abnormalities (5). Recently, Sands *et al.* showed that BMT-treated MPS VII mice had less severe histologic alterations in the ear and auditory tests evoked normal brainstem responses (179). Weekly injections of recombinant β-glucuronidase initiated at birth had comparable results, showing a less severe phenotype and histopathological improvements in brain and ear, as well as a better performance in a spatial learning test (133). However, since minute amounts of enzyme may already lead to improvement, functional tests seem to be required to assess the success of a particular treatment. For instance, although no β-glucuronidase activity could be measured in osteocytes, BMT on MPS VII mice reduced many of the bone abnormalities (177). I will discuss in detail in chapter 4 our encouraging findings on the therapeutic approach we have chosen to treat our mouse model of galactosialidosis.

In spite of these somewhat promising data, it is still necessary to optimize the current protocols or develop new methods, in order to be able to treat human patients. The major problem encoutered in the treatment of lysosomal storage disorders is the involvement of the CNS. The search for cells or drugs that have the capacity to pass the blood-brain barrier, the temporal disruption of the blood-brain barrier or the use of brain invasive techniques for the delivery of the corrective enzyme to the appropriate site may be extremely valuable. Since complete bone marrow engraftment takes 4-6 weeks, substantial improvement of the long term therapeutic benefits of BMT is achieved by enzyme replacement both prior to and immediately after transplantation (180). To increase the efficiency of uptake by neuronal cells, the atoxic proteolytic fragment of tetanus toxin may be conjugated to the enzyme. This method has successfully been tested for β-hexosaminidase A *in vitro* (36). An alternative could be the insertion of overexpressing cells into a patient as a continuous source of corrective enzyme, which would act locally. Human β-glucuronidase overexpressing primary mouse skin fibroblasts were engrafted into neo-organs and implanted in the peritoneal cavity of recipient mice, where they functioned for at least three months (122). Transplantation of either primary fibroblasts or a fibroblast cell line overexpressing β-glucuronidase into MPS VII brains only gave expression of the enzyme in the vicinity of the graft, and expression ceased after six months (214). Injection of murine neuronal progenitor cells, overexpressing either β-glucuronidase or β-hexosaminidase A, into the cerebral ventricles of newborn mucopolysaccharidosis type VII mice (196) or Tay-Sachs mice (92), respectively, resulted in the presence of donor cells throughout the brain and the subsequent reduction of storage in neurons and glia cells. The application of this method is limited, because these neuronal cells were shown to engraft in a safe and efficient manner in the cerebrum when injected into the ventricles, but no migration of donor cells occurred into the cerebellum or brain stem (212). Other methods are also

tested, like the use of adenovirus-based therapies, but succes so far is limited (35, 134, 135).

It is uncertain whether damage to the brain is reversible or not, but therapies to treat or prevent CNS lesions are more successful when applied at early stages of the developing brain, while bone abnormalities can be prevented and, if necessary, corrected through surgery. The main challenge remains the treatment of the CNS and multiple approaches are currently being tested to target any therapeutic protein at those sites, provided of course that the protein is secreted, is stable in the interstitial space, and is efficiently internalized by different deficient cells.

1.7 Introduction to the experimental work

Lysosomal storage disorders can be classified into several groups, glycoproteinoses, glycolipidoses and mucopolysaccharidoses, depending on the substrates that are accumulated. Furthermore, few diseases comprise defects in protein biosynthesis (I-cell disease, pseudo-Hurler polydystrophy) and membrane transport (cystinosis, Salla disease). Galactosialidosis is a member of the glycoproteinoses and is biochemically characterized by a combined deficiency of acid β-D-galactosidase and N-acetyl-α-neuraminidase, resulting in the accumulation and/or excretion of primarily sialylated oligosaccharides and – glycopeptides in human tissues and body fluids (reviewed in 29). The primary deficiency is the serine carboxypeptidase PPCA, which forms an active complex in the lysosome with these two hydrolases (reviewed in 29). The clinical phenotypes can be classified into three subtypes: the early infantile, late infantile and the juvenile/adult form. In addition to the typical features of lysosomal storage disorders, such as coarse facies, cherry-red spots, vertebral changes, foam cells in the bone marrow and vacuolated lymphocytes, the early infantile form is characterized by hepatosplenomegaly, kidney failure, edema, heart involvement, ascites, mental retardation, fetal hydrops and a short life span. The late infantile group has hepatosplenomegaly, growth retardation, cardiac problems and absence of mental retardation, whereas the juvenile/adult subtype has mental retardation, ataxia, angiokeratoma, myoclonus and absence of visceromegaly (29). Regardless of the clinical subtype, the residual β-D-galactosidase activity is between 5 and 15%, whereas the neuraminidase activity is usually reduced to less than 1%.

Cocultivation studies of patients' fibroblasts demonstrated that acid β-D-galactosidase and N-acetyl-α-neuraminidase activities could be restored by a corrective factor, secreted into the medium of normal cells (30, 73). The exact nature of the primary defect responsible for galactosialidosis was then elucidated and the human and mouse cDNAs were cloned and characterized. They encode a 452 and 454 aminoacid precursor molecule, respectively, that share 87% identity (30, 49, 51). The co-

translational removal of the signal sequence (239) yields an N-glycosylated precursor molecule of 54 kDa with an intramolecular "linker" domain of 2 kDa. The three dimensional structure of the human PPCA precursor homodimer has been elucidated by a combination of molecular replacement and two fold density averaging (169). The structure reveals an unusual inactivation mechanism of the zymogen. It is divided into a "core" domain, homing the active site, and a "cap" domain, encompassing the "linker" subdomain that covers the active site (169). The proteolytic removal of the "linker" in the endosomal/lysosomal compartment generates two subunits of 32 and 20 kDA, which remain associated through disulfide bridges (11, 51, 121). Each subunit contains one N-linked glycosylation site: the one on the 32 kDa subunit receives the mannose-6-phosphate marker necessary for correct routing to the lysosome, whereas the one on the 20 kDa subunit seems to be necessary for protein stability (121). The homodimeric precursor PPCA associates with the precursor β-galactosidase and neuraminidase shortly after their synthesis (121, 228, 258). The existence of this complex *in vivo* has been biochemically demonstrated previously by copurification studies using affinity matrices specific for either β-galactosidase or PPCA (147, 152, 183, 235, 253). It is likely that this association is mandatory for stabilization and correct routing of the glycosidases to the lysosomal compartment. The half-life of acid β-D-galactosidase in fibroblasts from galactosialidosis patients is significantly reduced as a result of enhanced proteolytic degradation (229, 230). This is only partially true for neuraminidase that is retained in an endosomal/prelysosomal compartment in the absence of PPCA. Recently, our group unravelled a new function of PPCA, that of a transport protein, which is required for neuraminidase to reach the lysosomal compartment (228).

The primary structure of the protein shares homologies with yeast and plant serine carboxypeptidases and has a conserved catalytic triad, Cys-Ser-His, in the active site (49, 51). Jackman *et al.*, and more recently Ostrowska *et al.*, showed that a protein isolated from thrombin-activated platelets resembled PPCA and had cathepsin A activity at acidic pH and esterase/deamidase activity at neutral pH (79, 140). The carboxypeptidase activity of PPCA was shown to inactivate a selected number of bioactive peptides, like substance P, oxytocin and endothelin I (63, 77-79). By site directed mutagenesis, we have demonstrated that the cathepsin A activity is fully separable from the protective function of the protein (51).

The isolation of the PPCA gene and cDNA has facilitated the identification of mutations present in patients with different clinical phenotypes (29, 55). We were able to correlate specific mutations with clinical phenotypes by analyzing the level of functionality of the mutant protein(s) in relation to its post-translational processing, subcellular localization and stability (259). Furthermore, a number of aminoacid substitutions found in defective PPCA from different galactosialidosis patients with varying clinical severity have been modelled into the three dimensional structure of the PPCA precursor (170). A remarkable correlation appeared to exist between the impact

of the mutations on the molecular integrity of PPCA structure and the severity of the disease caused by the same mutation. The availability of the three dimensional structure facilitates mutagenesis and biochemical studies to probe the function of PPCA alone or in the context of the multi-enzyme complex.

The already mentioned lysosomal storage disorder, sialidosis, also belongs to the group of glycoproteinoses. It is an autosomal recessive disease characterized by lesions in the N-acetyl-α-neuraminidase structural gene (216). There are two distinctive clinical types of sialidosis based on the age of onset and the severity of the symptoms. Type I is a mild form presenting in the teenage years, while the type II has severe symptoms with progressive CNS involvement and an early onset. Type I is characterized by cherry-red spots, progressive visual problems and generalized myoclonus. Type II is distinguished from type I by the presence of abnormal somatic features, like coarse facies and dysostosis multiplex, progressive neurologic deterioration and mental retardation. The residual activity of the neuraminidase in affected individuals is between 0-10%, leading to the progressive storage of sialylated compounds (216). Sialidosis has a number of clinical features in common with galactosialidosis and in order to discriminate between the two disorders, enzyme activity assays need to be done. As mentioned previously, neuraminidase is in complex with PPCA and β-galactosidase, which can be co-purified in a high molecular weight complex (151, 152, 235, 253). Although this complex contains only a small percentage of the total pool of β-galactosidase and PPCA, it consistently contains all the neuraminidase activity. This implies that active neuraminidase can not be isolated separately from the complex (225-227, 232-235). The dependency of neuraminidase activity on PPCA was already known from the analysis of galactosialidosis patients. Adding exogenous PPCA to galactosialidosis fibroblasts resulted in a correction of the lysosomal storage, suggesting that neuraminidase resides in a pre-lysosomal compartment in order to associate with PPCA (49, 50).

Recently, we were the first to isolate and characterize the cDNA encoding human lysosomal neuraminidase using the computer database search programs available, soon followed by others (10, 115, 153). The isolation and characterization of the human gene showed that it is expressed as a 1.9 kb mRNA, resulting in a 43 kDa protein after cleavage of the signal peptide, which contains a "FRIP"-domain and five Asp-boxes (10, 115, 153). The primary sequence showed extensive homology with other members of the sialidase superfamily, including bacteria, rodents, protozoa and influenza virus. In spite of the homology, mammalian neuraminidase requires PPCA to be active, whereas the bacterial proteins function without the need for accessory proteins. The question remains whether mammals acquired the need for additional proteins, or bacteria may have lost this restriction. Furthermore, the three-dimensional structure of bacterial and viral sialidases revealed a common catalytic core with a

characteristic six-bladed β-propeller fold (26, 52). Since a number of critical aminoacid residues are conserved, it may be anticipated that the human neuraminidase has a comparable structure. The availability of the human cDNA has enabled the identification of several mutations in patients with different clinical phenotypes (10, 153). The responsible gene is mapped to the HLA region on chromosome 6, which confirmed an earlier assignment done after a family study of a patient with a combined neuraminidase and 21-hydroxylase deficiency (10, 64, 138, 153).

A naturally occurring mouse strain, SM/J, was originally selected for its small body size, but biochemical analysis demonstrated an abnormal sialylation pattern of at least four lysosomal glycoproteins (148, 250). The responsible gene was designated *neu-1* and was mapped by linkage analysis to the H2 locus on chromosome 17, which is the syntenic region to the human chromosome 6 (175, 249). In addition, these mice appeared to have specific immunological abnormalities, which are thought to occur due to an altered processing of cell surface molecules present on a subset of T-cells. (22, 94-96, 205, 252). The lysosomal storage in these mice has no drastic impact on the phenotype, so this mouse could reveal interesting characteristics of neuraminidase, which would otherwise be overshadowed by storage in a full knockout.

We have isolated and characterized human and mouse PPCA genes with the intent to pinpoint regulatory elements that control the human and primarily the murine gene. The knowledge of the genomic organization of the murine gene was applied to develop a targeting vector to generate a mouse model for galactosialidosis. This model is a reliable phenocopy of the severe human condition and has been useful for studying the pathogenesis of the disease and the exploration of novel therapeutic approaches. Finally, given the dependence of neuraminidase on PPCA and the fact that galactosialidosis and sialidosis share similar features, we have isolated the murine neuraminidase gene. The cDNA enabled us to identify the mutation impairing the neuraminidase activity in the natural occuring SM/J strain. Our findings and main conclusions are given in chapters 2-7.

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Chapter 2

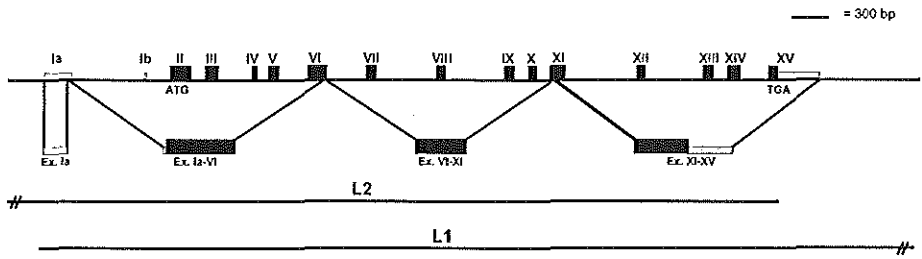
Genomic organization of the murine protective protein/cathepsin A (PPCA) gene

Genomic organization of the murine protective protein/cathepsinA (PPCA) gene

ABSTRACT

The primary defect in the human lysosomal storage disorder galactosialidosis is protective protein/cathepsin A (PPCA). PPCA associates with acid-β-D-galactosidase and N-acetyl-α-neuraminidase and protects these hydrolases from degradation in lysosomes. The lack of PPCA results in the combined deficiency of the two glycosidases, determining the nature of the storage products. Human and murine cDNAs have been cloned and the corresponding proteins extensively characterized. Recently, the genomic organization of the human gene has been reported. Here we describe the organization of the murine gene. The gene consists of sixteen exons, one more than the human, since the first two exons represent alternatively used 5'UTRs. Aside from exon Ia and 15, all exons are relatively small, ranging in size from 51 to 179 nucleotides, and the introns vary in length from 88 to 830 nucleotides. The gene has been mapped to chromosome 2H4 and all exons are located in a 6.5 kb region. The characterization of the murine PPCA gene has been important for studying its expression pattern in different tissues and for the generation of the mouse model for galactosialidosis.

The human protective protein/cathepsinA (PPCA) is a lysosomal carboxypeptidase involved in the inherited metabolic storage disorder galactosialidosis (for review see 1). The clinical phenotypes of the disease can be grouped into three subtypes, depending on the age of onset and the severity of the symptoms: early infantile, late infantile and juvenile/adult. Galactosialidosis is biochemically characterized by the combined deficiency of acid-β-D-galactosidase and N-acetyl-α-neuraminidase, which are in complex with PPCA in the lysosome. The interaction with PPCA protects the two glycosidases from degradation in the acidic environment of the lysosome. Aside from the protective function and fully separable from it is the cathepsin A activity of PPCA which is able to cleave bioactive peptides, like substance P, endothelin I and oxytocin (6, 7, 4, 5). The human and murine cDNAs have been cloned and characterized (2, 3) and the chromosomal localization of the respective genes have been mapped to chromosome 20q13.1 and 2H4 (10, 11). Recently the genomic organization of the human gene was published (9), and we now report on the murine counterpart.



Figure

Genomic organization of the mouse PPCA gene, showing the position of the exons and introns. The two alternatively used 5'UTR-encoding exons and the 3'UTR part of exon XV are represented as gray boxes. The two lambda phage inserts, L1 and L2, and the cDNA probes used to characterize these inserts are shown below the genomic organization. Each probe is designated on the basis of the cDNA sequence they span.

A mouse genomic library, derived from inbred strain 129, was screened with a full length mouse cDNA probe. Two independent phage inserts were isolated and analyzed with restriction enzymes, followed by hybridization of Southern blots with different PPCA specific cDNA probes (Fig. 1). The inserts, with estimated sizes of about 13 kb, appeared to partially overlap and together encompassed the 5' and 3' ends of the cDNA, indicating that we had isolated the complete mouse PPCA gene. The inserts were subcloned using the unique flanking *SalI* sites and sequenced with exon specific oligonucleotides.

The murine gene consists of 16 exons (Fig. 1), one more than the human gene, since it is transcribed from two different promoters. The distal promoter contains a typical TATA-box, while the proximal one is GC-rich. The two promoters give rise to the alternative usage of the first two exons, exon Ia and Ib, representing different 5'UTRs (8). The exons of the murine gene are localized in a 6.5 kb region, which excludes the promoter region and other putative upstream and/or downstream regulatory sequences. The sizes of exon Ia and Ib have been deduced from RNase

protection assays. Exon Ia has a well-defined length of 231 bp, being initiated from the TATA-box containing promoter. In contrast, exon Ib has multiple start sites varying in size between 25 and 45 bp, a feature common to other GC-rich, TATA-less promoters (8). In spite of the high degree of sequence similarity, the mouse exon Ia has no functional human counterpart. In contrast, exon Ib is the murine homolog of the human exon I, which is at least 26-27 nucleotide long (8, 9). The variation in size of murine exons 7, 8, 9 and 11 compared to their human counterparts is the result of a calculation error by Shimmoto (9). The differences in exon 2 and 10 represent genuine changes: the murine exon 2 is 15 nucleotides shorter than the human resulting in a predicted signal peptide of only 23 amino acids compared to 28 in the human precursor protein; exon 10 is 3 nucleotides shorter in the mouse, explaining the absence of the lysine 265 present in the human protein sequence.

As shown in Table 1, all exon/intron boundaries comply to the gt/ag rule, and the splice sites have the consensus for correct splicing. The exon/intron boundaries share a considerable degree of homology between the human and mouse genes, although the intron sizes are not entirely conserved. However, the overall organization of the two genes is identical, with introns 7, 8, 11 and 12 being relatively large compared to the other introns. Intron 11 is the only one that has a significant size difference, being 1.84 kb in the human and only 0.58 kb in the mouse sequence. Comparison of a number of the murine introns with the corresponding human introns, revealed that some of them share homologous sequences, which may imply important regulatory features of these introns in transcriptional regulation or splicing. Knowledge of the organization of the murine gene has been important for the creation and analysis of the PPCA knockout mouse (12). The structural organization is facilitating the analysis of the endogenous expression of the PPCA gene in different tissues of the adult mouse. Future research may enable us to localize intronic regions that are important for the regulation of the two genes.

Sizes and locations of exons and introns and the sequences at the exon-intron boundaries

Exons			Introns			
number	and size	cDNA position	5' splice site	3' splice site	intron size	
Ia	231	-231 - -1	CAAGAC gtaggtgccc....	cctcctgcag ATGCCC	830	(1a)
Ib	22-40	-22 - -1	GCAGAG gtacggagat....	cctcctgcag ATGCCC	179	(1b)
II	179	1-179	CTACTG gtgcttggct....	tccttcccag GTTTGT	112	(2)
III	112	180-291	TTTCTG gtgagttgac....	tcctccccag ATCCAG	259	(3)
IV	51	292-342	AACCTG gtactcctgg....	cctcccccag ATTGCC	88	(4)
V	87	343-429	ACAGAG gtgagtctgt....	gcatcctcag GTGGCG	222	(5)
VI	156	430-585	CTTCAG gtgcagggca....	tgtttcgcag GGGCTG	316	(6)
VII	92	586-677	GAACAG gtaaaggaag....	tgatccacag ACTTTG	488	(7)
VIII	85	678-762	AACAAT gtgagctcct....	gtcttcacag CTCCTG	474	(8)
IX	92	763-854	ACATAG gtaggtgctg....	tttctgacag ATATGA	104	(9)
X	76	855-930	CCTGAG gtatgcgggg....	ccccccccag GCACTG	101	(10)
XI	140	931-1070	GTGCAA gtgagcttcc....	tccccggcag CTTCTT	580	(11)
XII	76	1071-1146	TCACAG gtatgtgtgc....	cctattccag AAATAC	488	(12)
XIII	90	1147-1236	CAGAAG gtaaagttgc....	gatgggacag ATGGAG	115	(13)
XIV	105	1237-1341	ATCAAG gtaggacttt....	ttgctcccag GGTGCC	199	(14)
XV	433	1342-1774	CTTCCA			

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Chapter 3

Identification of the promoters for the human and murine protective protein/cathepsin A genes

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Identification of the Promoters for the Human and Murine Protective Protein/Cathepsin A Genes

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ABSTRACT

Protective protein/cathepsin A (PPCA) is a lysosomal serine carboxypeptidase that forms a complex with β-galactosidase and neuraminidase. Its deficiency in humans leads to the lysosomal storage disorder galactosialidosis (GS). The pathologic manifestations in patients relate primarily to the severe deficiency of neuraminidase, and the physiological significance of cathepsin A activity remains unclear. The mouse model of GS, which closely resembles the human phenotype, shows that cells from numerous tissues, especially the central nervous system (CNS), are affected by this disease. To study the site and level of expression of PPCA mRNA in murine and human tissues, we analyzed the promoter regions of the corresponding genes. Their 5′ genomic regions were strikingly similar in both organization and sequence. A single 1.8-kb PPCA transcript is present in humans, whereas mouse tissues have a major 1.8-kb and a minor 2.0-kb transcript, both of which are differentially expressed. These two mouse mRNA species differ only in their 5′ untranslated region (UTR). The larger mRNA, unique to mouse, is transcribed from an upstream TATA-box-containing promoter, which is absent in the human gene. The downstream promoter, which transcribes the 1.8-kb mRNA common to human and mouse, has characteristics of housekeeping gene promoters and contains putative Sp1 binding sites and three USF/MLTF sequences. *In vitro* studies demonstrated that expression from the downstream promoter is higher than that from the upstream murine-specific promoter. *In situ* hybridization of mouse tissue sections identified regions of the brain that preferentially express the 2.0-kb transcript. Our results imply that PPCA mRNA distribution and regulation in murine tissues differs from that in human tissues.

INTRODUCTION

HUMAN PROTECTIVE PROTEIN/CATHEPSIN A (PPCA) is a lysosomal carboxypeptidase whose deficiency in humans results in the lysosomal storage disorder galactosialidosis (GS; for review, see d'Azzo *et al.*, 1995). Three clinical phenotypes are distinguished, depending on the age of onset and severity of the symptoms: early infantile, late infantile, and juvenile/adult. The biochemical hallmark of this autosomal recessive disease is a secondary combined deficiency of two glycosidases: β-D-galactosidase (EC 3.2.1.23) and *N*-acetyl-α-neuraminidase (EC 3.2.1.18). These deficiencies cause excessive accumulation and excretion of sialylated oligosaccharides in patients' tissues and body fluids. PPCA associates with the two glycosidases, forming a stable and active three-enzyme complex in the lysosomes. In addition to its protective function, PPCA exerts carboxypeptidase activity at acidic pH and esterase/carboxyl terminal deamidase activity at neutral pH; both activities are absent in galactosialidosis patients (Galjart *et al.*, 1991; Itoh *et al.*, 1993). The catalytic activity of PPCA is thought to be responsible for the local inactivation of selected bioactive peptides, including substances P, endothelin I, and oxytocin (Jackman *et al.*, 1990, 1992; Hanna *et al.*, 1994; Itoh *et al.*, 1995).

The human PPCA mRNA and its mouse homolog share 85% identity in their coding regions and 72% identity in their 3′ untranslated region (UTRs) (Galjart *et al.*, 1988, 1990, 1991). The human gene localizes to chromosome 20 and the murine locus is found on the synthenic chromosome 2 (Wiegant *et al.*, 1991; Williamson *et al.*, 1994). The genomic organization of the human gene was recently reported (Shimmoto *et al.*, 1996). Previously, we showed that the major PPCA mRNA species in

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mouse is ubiquitously, but differentially, expressed (Galjart *et al.*, 1990). Here, to gain further insights into the regulation of human and mouse PPCA expression, we characterized and compared the promoter regions of these genes. We found two different promoter regions within a 1.5-kb fragment of the mouse PPCA gene: one proximal, with characteristics of a "housekeeping" gene promoter, which gives rise to a 1.8-kb transcript, and one distal, which contains a consensus TATA box and generates a longer 2.0-kb transcript. These mouse PPCA mRNAs have different 5′ UTRs, which are spliced to yield the same final protein product. The short 1.8-kb transcript is expressed more abundantly than the 2.0-kb species, which *in vivo* appears only in specific regions of the brain. Despite the high degree of homology between the murine and human 5′ genomic sequences, the human gene has apparently lost its distal promoter activity, and, therefore, may not be subject to the same type of transcriptional regulation as the murine gene.

MATERIALS AND METHODS

Materials

A MEG-01 cDNA library was a gift of Dr. Hans C. Clevers (University Hospital, Utrecht, The Netherlands). Restriction endonucleases and DNA modifying enzymes were purchased from Bethesda Research Laboratories, Inc., Boehringer Mannheim, New England Biolabs, Inc., and Promega Biotec. The RACE-5′-amplifinder kit was supplied by Clontech, Inc., the BCA protein assay reagents by Pierce Chemical Co., and the fmol sequencing kit by Promega Biotec. All enzymes and kits were used according to the directions provided by the manufacturers. [α-^{32}P]dATP (3,000 Ci/mmole), [α-^{32}P]UTP (800 Ci/mmole), [γ-^{32}P]ATP (3,000 Ci/mmole), [α-^{35}S]dATP (>1,000 Ci/mmole), [α-^{35}S]UTP (>1,000 Ci/mmole), and [^{14}C]chloramphenicol (55 mCi/mmole) were provided by Amersham Corp. and Dupont NEN Research Products. All other reagents were obtained from standard commercial suppliers.

RNA isolation and Northern blotting

Total human RNA from MEG-01 and XP-2 cell lines and mouse tissue RNA were isolated with the LiCl/urea method as previously described (Auffray and Rougeon, 1980). Poly(A)$^+$RNA was isolated according to Promega's polyAtract system. A total of 1–2 μg of poly(A)$^+$RNA was run on a 1% agarose gel containing 0.66 *M* formaldehyde, transferred onto a Zeta-Probe membrane (Bio-Rad), and hybridized under standard conditions (Sambrook *et al.*, 1989). Human multiple tissue Northern blots were purchased from Clontech and hybridized according to the manufacturer's protocol.

Cell culture and transfections

The megakaryoblast cell line MEG-01, kindly provided by Dr. Hans C. Clevers (University Hospital, Utrecht, The Netherlands), was obtained from Dr. Saito (Nagoia University, Japan). The XP-2 SV40-transformed cell line was from a Xeroderma pigmentosum patient (European Cell Bank, Rotterdam, The Netherlands, Dr. W.J. Kleijer). MEG-01 cell line was cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and antibiotics. XP-2 and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, glutamine, and antibiotics. COS-1 cells were cultured under the same conditions except the medium was supplemented with 5% FBS. Both cell lines were seeded at 5×10^5 cells/10-cm dish 12–24 hr prior to transfection as described previously (Graham and Eb, 1973). Protein extracts of transfected cells were prepared 36 hr post transfection and were tested for chloramphenicol acetyl transferase (CAT) activity (Gorman *et al.*, 1982) and β-galactosidase (β-Gal) activity (An *et al.*, 1982). The protein concentration in each extract was also determined (Pierce Chemical Co.). The ratio of converted [^{14}C]chloramphenicol to the total input [^{14}C]chloramphenicol was determined with a Phosphorimager (Applied Biosystems).

Rapid amplification of cDNA ends polymerase chain reaction and reverse transcriptase polymerase chain reaction

First-strand PPCA cDNA was prepared using total RNA from several mouse tissues and human fibroblasts under standard conditions. We used an antisense oligonucleotide, primer R, located at human cDNA position 286–305 (5′-GGGCCATGCTCTGTGAGGAG-3′) or at mouse cDNA position 501–520 (5′-CACCATCTGGCTGGATCAGA-3′). Rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) of this cDNA was performed according to the manufacturer's direction using a PPCA-specific, antisense oligonucleotide containing an *Eco* RI site (primer 1, human cDNA position 200–181, 5′-GGAATTCAGTAGTGGAGGTGCTTGGA-3′; mouse cDNA position 412–393, 5′-GGAATTCGTTCTGCGACTCCACAAAC-3′) and the RACE amplifinder, sense primer provided by the manufacturer.

The reverse transcription (RT)-PCR mix contained the antisense primer 1 and a transcript specific, sense primer, the sequence of which comes from exon 1a (primer 2, human genomic region 6–25, 5′-GGAATTCTGCTCAATGCGCAGATATGG-3′; mouse exon Ia position 11–30, GGAATTCGATGCGCAGATAGGGTTCAA-3′). The other primer sequence comes from exon 1b (primer 3, human, 5′-GGAATTCGGCAAGGACGCGGGGGAGCAG-3′, mouse, 5′-GGAATTCAAGGAAGACGCAAGGAAGCAG-3′). The PCR products were electrophoretically separated on 1.5–2% agarose gels, blotted, and hybridized. If we omitted the first-strand cDNA synthesis step, we got no PCR products, which indicates that our RNA samples did not contain genomic DNA.

Construction of CAT vectors

The backbone of all CAT vectors was the pBLCAT2, which had been slightly modified to create pBLCAT6 (Schöler *et al.*, 1989). Most constructs were made by subcloning PCR-amplified genomic fragments, which contained a 5′ Sal I restriction site and a 3′ *Bam* HI restriction site, upstream of the cat reporter gene. Cloned DNA fragments were sequenced to exclude mutations. Antisense clones were created by subcloning the PCR-amplified genomic promoter fragments into the *Bam* HI

and *Xho* I restriction sites of the pBLCAT6 vector. Each promoter construct (10 μg) was transiently transfected with a Rous sarcoma virus (RSV) promoter-driven *lacZ* reporter gene construct (2 μg) to check transfection efficiency.

RNase protection and in situ *hybridization*

The RNase protection assays were performed using the RPA II kit from Ambion by following the manufacturer's protocol. *In situ* hybridization and signal detection was performed according to the method of Simmons *et al.* (1989). Eight- to 12-week-old FVB mice were perfused with 4% paraformaldehyde in phosphate buffer pH 7.2, and brain specimens were isolated and saturated in 25% sucrose. Cryosections from these brains were hybridized for 16–20 hr at 50°C. The washed slides were then dipped in Kodak NTB-2 autoradiography emulsion and exposed for 8 days (cDNA probe) or 21 days (Exla probe), at which time they were processed by using Kodak developer D-19 and fixer. The slides were counterstained with 0.1% toluidine blue.

RESULTS

Identification of a new 5′ exon in the human and murine PPCA gene

The human PPCA cDNA, isolated from a testis library, and its murine homolog, isolated from a brain and testis library, shared close homology throughout their coding regions (85%) and 3′ UTRs (72%) (Galjart *et al.*, 1988, 1990). However, comparisons of the murine and human 5′ UTRs could not be done because the human clone lacked a 5′ UTR. Therefore, we cloned a new full-length PPCA cDNA from a human megakaryocyte library. This cDNA was identical to the clone derived from the human testis library (Galjart *et al.*, 1990) with two exceptions. It extended 5′ into a short UTR of 22 bp that shared no homology with the murine 5′ UTR. And, instead of the original 8 CTG codons (Leu) in the 5′ sequence coding for the signal peptide, the new clone only contained 7 leucine codons. This difference appeared to be a polymorphism, because a group of 50 normal individuals had either 7, 8, or 9 CTG triplets in this region (unpublished data).

To ascertain whether the human and murine sequences do indeed diverge in their 5′ UTRs, we amplified additional mouse 5′ sequences from brain, kidney, and liver total RNA by using RACE-PCR. We isolated novel murine cDNA clones containing short 5′ UTRs that were homologous to the 22-bp UTR of the human cDNA. Curiously, all of our amplified clones contained this short 5′ UTR, although some differed slightly in the length of their 5′ extension. None of the clones included the 213-bp-long 5′ UTR of the original murine PPCA cDNA clone that was isolated from a mouse brain library (Galjart *et al.*, 1990). This could be attributed to either tissue-specific expression or low expression of this transcript in different tissues (see below). In contrast, RACE-PCR on human fibroblast RNA generated cDNA clones that exclusively contained the 22-bp 5′ UTR. Thus, both human and mouse PPCA mRNAs contain the short 22-bp 5′ UTR, but only certain mouse tissues contain a small pool of transcripts with the longer 213-bp 5′ UTR.

Sequence analysis of the human and mouse PPCA promoter regions

To define the origin of the two 5′ UTR sequences, we cloned and analyzed these regions from the human and mouse genomes. The two PPCA genes shared striking similarities in the organization and sequence of their 5′ genomic regions (Fig. 1). Both the human and mouse 5′ UTR sequences were encoded by a single exon, called exon I in human and Ib in mouse. This exon was located 0.18 kb upstream of the first coding exon (exon II in both species). The long 213-bp murine 5′ UTR sequence comprised an exon, located 0.8 kb upstream of exon II and shared 78% sequence homology with the human gene sequence at the corresponding positions. Our results strongly suggested the presence of two promoters in the mouse gene.

Direct sequence analysis of the putative promoter regions of both the human and mouse PPCA genes revealed that the mouse distal promoter contained a TATA box and two imperfect CAAT boxes: one at position −95 (CCAAG) and one at −125 (CCCAT). A putative glucocorticoid-responsive element (TGTCCT; Karin *et al.*, 1984) was found at position −150, and two AP-2 boxes (CCCA/CNG/CG/CG/C; Williams *et al.*, 1988) at positions −90 and −174, respectively. Although the human sequence was highly homologous in this area (Fig. 1), it did not encode either the TATA box or the imperfect CAAT boxes.

The proximal promoter had all the features of a typical housekeeping gene promoter (Dynan, 1986): it lacked TATA and CAAT boxes, was G/C rich, and had two Sp1-binding sites (Fig. 1). This promoter also contained three USF/MLTF motifs (Hen *et al.*, 1982; Carthew *et al.*, 1985; Sawadogo and Roeder, 1985), which were conserved between human and mouse.

PPCA transcripts in mouse and human

The identification of two different 5′ UTRs in murine PPCA cDNAs led us to analyze the extent to which the mRNAs derived from these cDNAs is expressed in different tissues. Previous studies of total RNA from human fibroblasts identified only a single PPCA transcript of 1.8 kb when a human PPCA cDNA lacking a 5′ UTR was used as a probe (Galjart *et al.*, 1988). Here, we analyzed multiple human tissues on Northern blots hybridized with a full-length cDNA probe that included the 22-bp 5′ UTR. A single, ubiquitously expressed transcript of 1.8 kb was detected in all tissues (Fig. 2A). Because the mouse exon Ia-containing cDNA was isolated from a brain library, we also analyzed different human brain regions; however, only a single PPCA mRNA species was detected (Fig. 2B). A human genomic probe containing the mouse exon Ia-homologous region did not hybridize to any tissues tested (data not shown).

Although every mouse tissue we tested contained the 1.8-kb PPCA mRNA, the levels of this transcript varied considerably from tissue to tissue, suggesting that PPCA expression is regulated either at the level of transcription or by mRNA turnover. In addition, the probe recognized a 2-kb transcript that was present in variable amounts and only in some tissues (*e.g.*, brain, spleen, heart, liver and kidney).

To explain these results, we hybridized a mouse multiple tissue, poly(A)$^+$RNA blot with an exon Ia-specific probe. This probe detected exclusively the larger of the two transcripts (Fig.

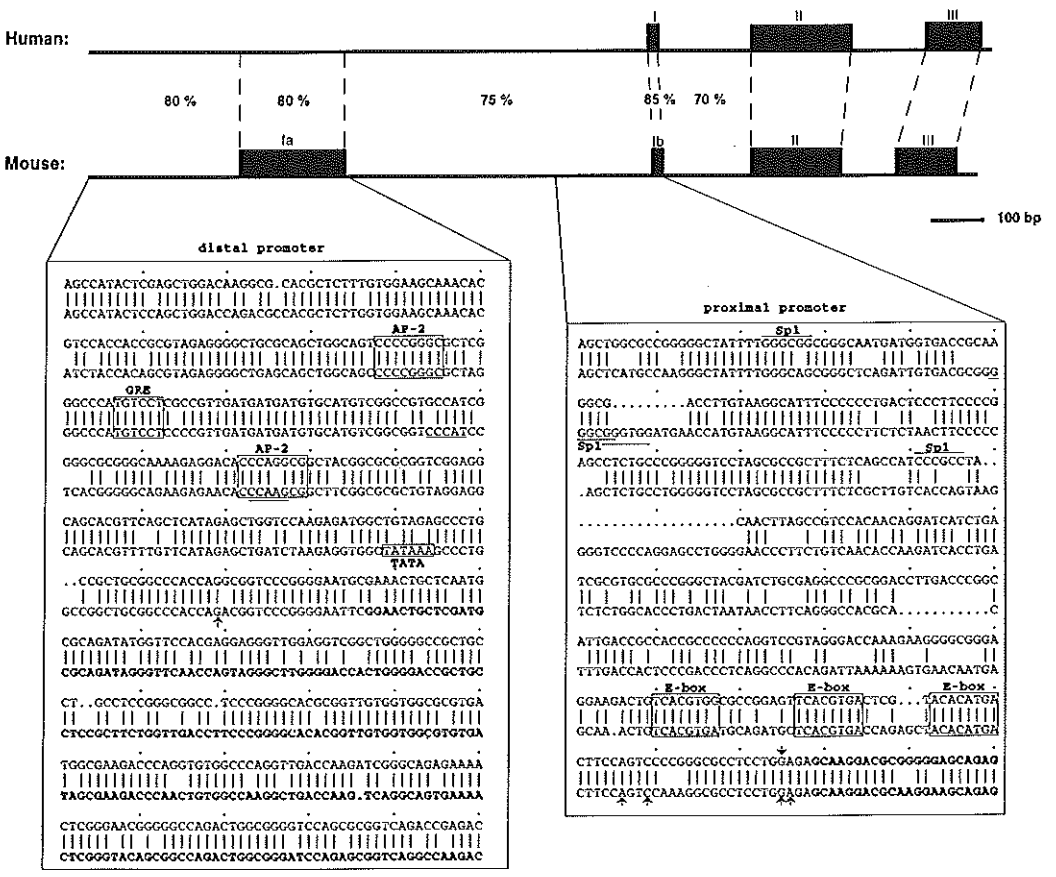

FIG. 1. Schematic representation of the human and mouse 5′ genomic regions (top), and sequence comparison of their 5′ UTRs and adjacent promoter regions (bottom). Percentage of homology is indicated. The 5′ UTR exons are in boldface and putative transcription factor binding sites are indicated. Two imperfect CAAT boxes are underlined in the murine distal promoter (bottom, left). The transcriptional start sites, found by using RNase protection assays, are marked with an arrow above (human) or below (mouse) the sequence.

2C), and identified it as the exon Ia-containing mRNA. An exon Ib-specific oligonucleotide probe recognized only the ubiquitous 1.8-kb transcript (Fig. 2D). These experiments unequivocally demonstrated that exon Ia and exon Ib do not occur together in the same molecule, but specify different PPCA transcripts. Rehybridization of the blot with a murine cDNA probe to part of the coding region (bp 777–1,180) confirmed that both transcripts were genuine PPCA mRNAs (Fig. 2E). The exon Ia-specific probe also detected a 1.0-kb transcript in murine heart poly(A)$^+$RNA (Fig. 2C). This species did not represent an alternatively spliced PPCA mRNA, because it was not recognized by a cDNA probe devoid of exon 1a (Fig. 2E). Hybridization of the human multitissue Northern blot with the homologous human probe also identified a 1.0-kb RNA transcript in heart and skeletal muscle. The nature of this 1.0-kb transcript is currently under investigation.

The apparent lack of the 2.0-kb mRNA in some mouse tissues could represent the limited sensitivity of Northern blot analysis. Therefore, we reassessed the distribution of this transcript by using the more sensitive method of RT-PCR (Fig. 3A). Analysis of the amplified products by Southern blot hybridization with human or murine cDNA probes, confirmed the Northern blot results: The two human cell lines expressed exclusively exon I-containing mRNA (Fig. 3B), whereas the mouse tissues expressed both exon Ia- and exon Ib-containing mRNAs (Fig. 3C). Sequence analysis of these hybridizing products confirmed their identity and further revealed RT-PCR products that were longer than expected (Fig. 3C), being derived from unspliced pre-mRNA.

Transcription of human and mouse PPCA genes

The transcriptional start sites of the three mRNAs were identified by using RNase protection assays. The human start site was mapped with a riboprobe containing 412 bp of the promoter, exon I, and 30 bp of intron I (Fig. 4A). Using total RNA

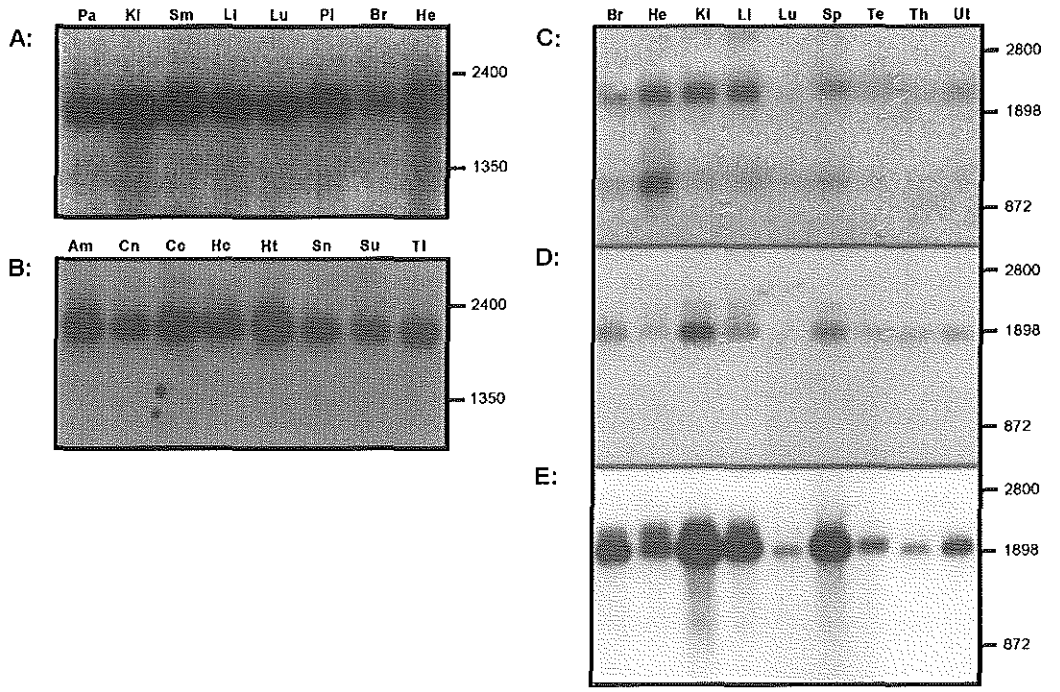

FIG. 2. Expression of PPCA mRNAs in human and mouse tissues. Northern blots containing 1–2 μg of poly(A)$^+$RNA from several human tissues (A) and from different brain regions (B) were hybridized with a human cDNA probe. A Northern blot containing 1–2 μg of poly(A)$^+$RNA from different mouse tissues was hybridized with a 194-bp exon Ia-specific probe (C; exposure 14 days), a 20-bp oligonucleotide-spanning exon Ib probe (D; exposure 7 days), and a mouse 400-bp cDNA probe (E; exposure 1 day). Am, Amygdala; Br, brain; Cc, corpus callosum; Cn, caudate nucleus; Hc, hippocampus; He, heart; Ht, hypothalamus; Ki, kidney; Li, liver; Lu, lung; Pa, pancreas; Pl, placenta; Sm, smooth muscle; Sn, substantia nigra; Sp, spleen; Su, subthalamic nucleus; Te, testis; Th, thymus; Tl, thalamus; Ut, uterus.

isolated from a human megakaryocyte cell line, a single protected fragment of 26 bp was detected (Figs. 1 and 4B).

The mouse distal promoter start site was identified by using a riboprobe spanning 226 bp of the promoter and 190 bp of exon Ia (Fig. 4A). We found one major protected fragment of 211 bp in both kidney and liver poly(A)$^+$RNA, indicating a single site, 25 bp downstream of the TATA box (Figs. 1 and 4C). Primer extensions with two different primers located in exon Ia confirmed the position of the start site (data not shown). To map the start site of the proximal promoter, we used a riboprobe containing 140 bp of the promoter linked to exon Ib (Fig. 4A). Total mouse kidney RNA or kidney and liver poly(A)$^+$RNA showed two major protected fragments of 42 and 45 bp, and two minor fragments of 25 and 26 bp, indicating two major start sites, 3 bp apart, and two minor ones, 20 bp further downstream (Figs. 1 and 4D).

To assay the transcriptional activity of the two mouse promoters and the human promoter, different fragments of their sequences were linked to a chloramphenicol acetyl transferase (CAT) reporter gene (*cat*). Figure 5A shows a schematic representation of the various CAT constructs used for this analysis. A 7-kb genomic fragment from the murine gene (Mo-CAT), which included exons Ia and Ib, had very high promoter activity in both NIH-3T3 and COS-1 cells (Fig. 5C). Truncation of this fragment to an 1.7-kb fragment that contained only the distal promoter region and 17 bp of exon Ia (Mo-CAT5.1, Fig. 5B), severely reduced but did not suppress promoter activity. To minimize further the region required for distal promoter activity, we generated progressive 5′ deletions of the 1.7-kb fragment, which resulted in constructs Mo-CAT5.2, 5.3, 5.4, and 5.5 whose fragment sizes were 0.9, 0.7, 0.5, and 0.25 kb, respectively (Fig. 5A). Reducing the distal promoter fragment to 0.9 kb did not significantly alter its activity (Fig. 5B). Further 5′ deletions appeared to up-regulate transcriptional activity four- to five-fold for fragments of 0.7 and 0.5 kb and an additional four- to five-fold for the 0.25-kb fragment (Fig. 5B). These results suggest that two transcription suppressing sequences lie in the distal promoter region, one located between -0.9 and -0.5 kb, and the other between -0.5 and 0.25 kb of the cap site.

The proximal promoter was analyzed using a genomic fragment that included exon Ib and 600 bp of 5′ sequences linked to the *cat* reporter gene (Mo-CAT 3.1; Fig. 5A). This fragment had a high, unidirectional promoter activity identical to that of construct Mo-CAT, which suggests that Mo-CAT activity was mainly derived from the proximal promoter (Fig. 5C). Pro-

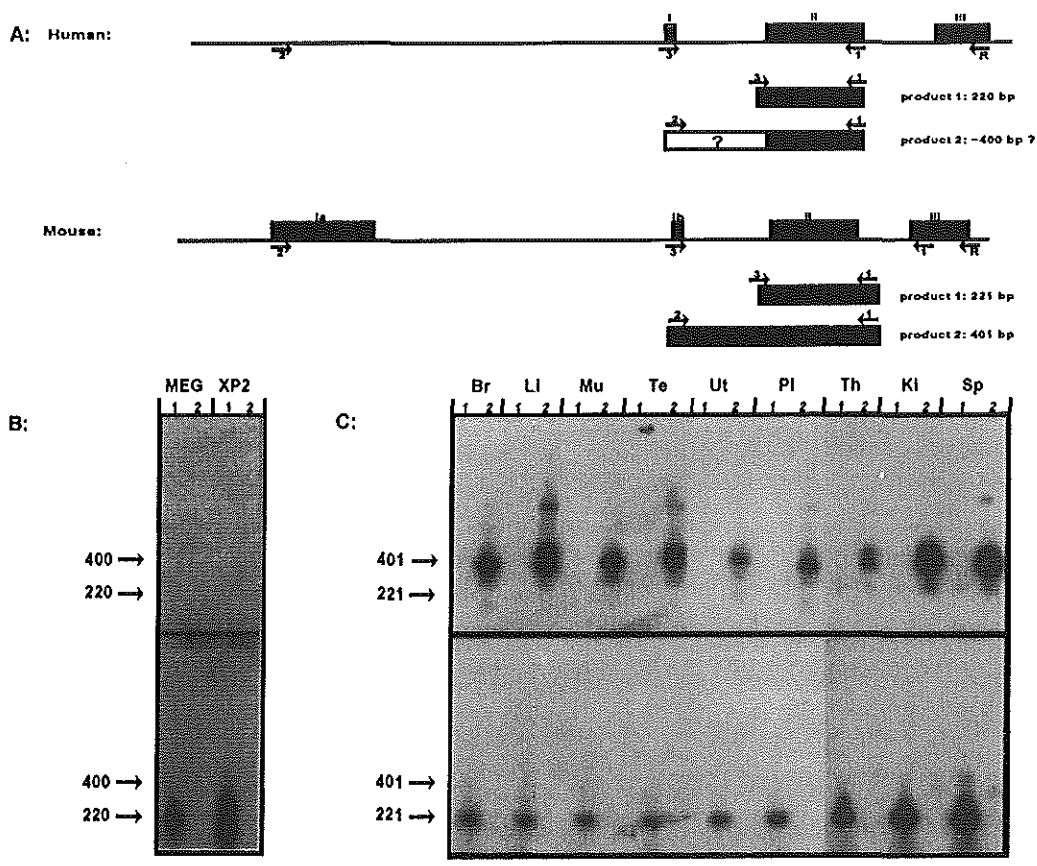

FIG. 3. RT-PCR showing the presence of different PPCA transcripts in human and mouse. A. Outline of the RT-PCR strategy, indicating the location of the different primers used to generate the first-strand cDNA and the PCR products. B. Southern blot analysis of the human derived RT-PCR samples run on a 2% agarose gel and hybridized with a probe homologous to the murine exon Ia (top) or an exon I probe (bottom). C. Southern blot analysis of the mouse-derived RT-PCR samples hybridized with an exon Ia probe (top) or an exon Ib probe (bottom). Lanes marked 1 contained PCR products amplified with primers 1 and 3, and lanes marked 2 contained products amplified with primers 1 and 2. Br, brain; Ki, kidney; Li, liver; Mu, skeletal muscle; Pl, placenta, at day 18 of gestation; Ut, uterus, at day 18 of gestation; Te, testis; Th, thymus; MEG, megakaryocyte cell line; XP2, Xeroderma pigmentosa fibroblast cell line.

gressive 5′ deletions of Mo-CAT3.1 demonstrated that the sequences necessary for maximal promoter activity were located within a 298-bp fragment (Mo-CAT3.3; Fig. 5A,C), which excluded the two SP1 binding sites. Further 5′ truncation of this 298-bp fragment to 125 bp resulted in a dramatic loss of promoter activity (Mo-CAT3.4; Fig. 5A,C), despite the fact that the three conserved USF/MLTF motifs were retained in this fragment. Deletion of exon Ib from the 3′ side of Mo-CAT3.1 did not diminish the promoter activity of this fragment (Mo-CAT3.5; Fig. 5A,C); however, deletion of an additional 105 bp from the 3′ side of Mo-CAT3.5, which eliminated the three USF/MLTF motifs, completely abolished activity (Mo-CAT3.6; Fig. 5A,C). Taken together, these results indicate that the promoter elements necessary to drive transient expression of the mouse exon Ib PPCA transcript are located in two regions: one between −298 and −125, and one between −105, and −1.

To compare the activity of the mouse distal promoter with that of the corresponding region in the human gene, two human genomic fragments homologous to the mouse distal promoter, were subcloned into the *cat* reporter plasmid and transfected into COS-1 and NIH-3T3 cells (Fig. 5A; Hu-CAT5 and Hu-CAT5.4). As expected, these two constructs completely lacked promoter activity (Fig. 5B). In contrast, a human proximal promoter fragment, Hu-CAT3.5, displayed a very high, unidirectional activity, comparable to that of its mouse counterpart, Mo-CAT3.5 (Fig. 5C). Overall the activity of these promoters reflect the mRNA levels detected on mouse and human Northern blots.

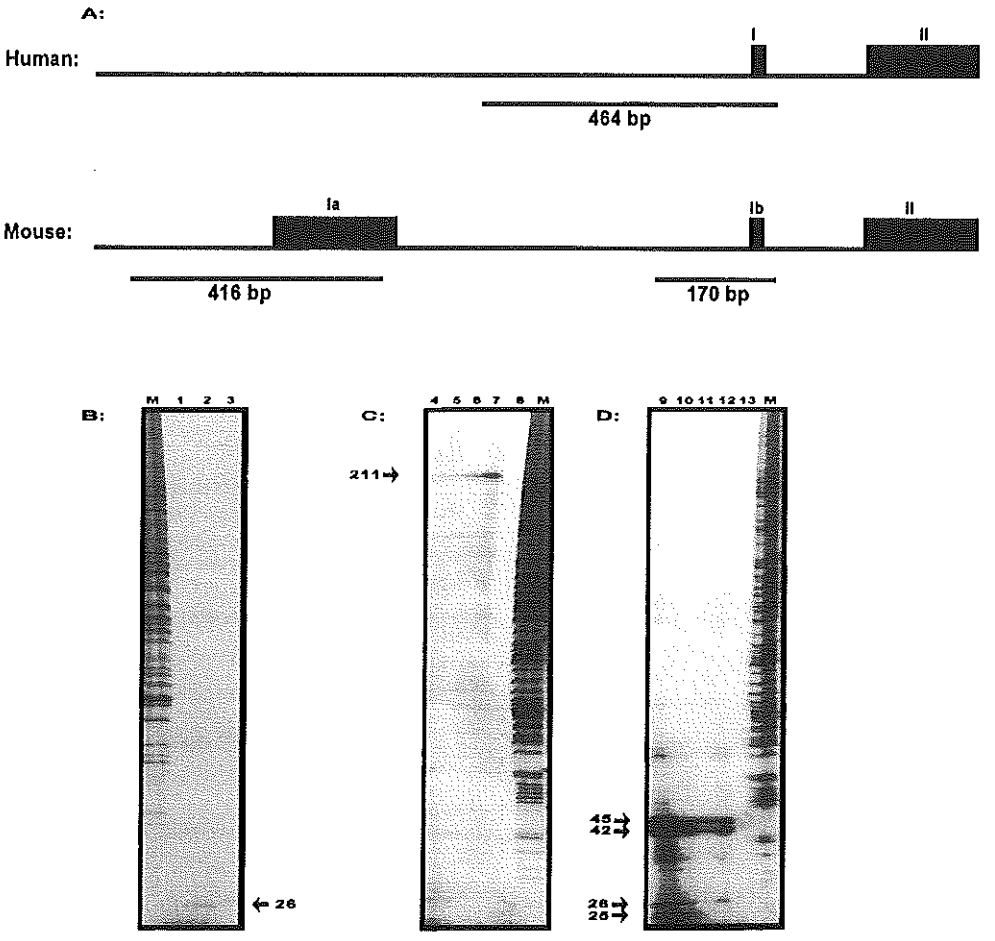

FIG. 4. RNase protection analysis of the human and mouse PPCA promoter. A. Genomic location of the fragments used to generate the riboprobes. The indicated fragments were subcloned into pBluescript, linearized, and *in vitro*-transcribed using either T3 or T7 RNA polymerase. B. The human promoter was analyzed using 10 μg (lane 1) or 30 μg (lane 2) of total RNA from a human megakaryocyte cell line. C. Mapping of the mouse distal promoter, using 15 μg (lane 4) or 30 μg (lane 5) of total kidney RNA, and 2 μg of poly(A)$^+$RNA from kidney (lane 6) or liver (lane 7). D. Mapping of the mouse proximal promoter using 15 μg (lane 9) or 30 μg (lane 10) of total kidney RNA, and 2 μg of poly(A)$^+$from kidney (lane 11) and liver (lane 12). For each probe, a negative control containing 15 μg of yeast tRNA was used (lanes 3, 8, and 13). A sequence marker lane was run next to the samples (lane M). The arrows indicate fragments of interest.

The murine exon Ia transcript is expressed in vivo

To validate the *in vivo* occurrence of the two murine mRNAs, we performed *in situ* hybridizations on mouse brain cryosections using two PPCA cDNA fragments to generate ^{35}S-labeled antisense riboprobes: ExIa, specific for exon Ia-containing transcripts and cDNA, which recognizes both exon Ia and Ib transcripts (Fig. 6, bottom panel). The cDNA antisense probe showed high expression of PPCA mRNAs in the choroid plexus, the hippocampus, and the dentate gyrus (Fig. 6A,D,G). Subsequent sections hybridized with ExIa showed specific and discrete expression of the exon Ia transcript in only the hippocampus and the dentate gyrus (Fig. 6B,E,H). This finding suggests that the two murine transcripts may be differentially distributed *in vivo* and that their regional expression may not overlap. Moreover, these data indicate that the exon Ia mRNA is genuinely transcribed *in vivo* and is not an artifact of the *in vitro* systems used. Hybridization of similar mouse brain regions using the sense control of either riboprobe is shown for the choroid plexus, the hippocampus, and the dentate gyrus (Fig. 6C,F,I).

DISCUSSION

To understand better the transcriptional regulation of the PPCA gene, we have analyzed the human and mouse PPCA 5′

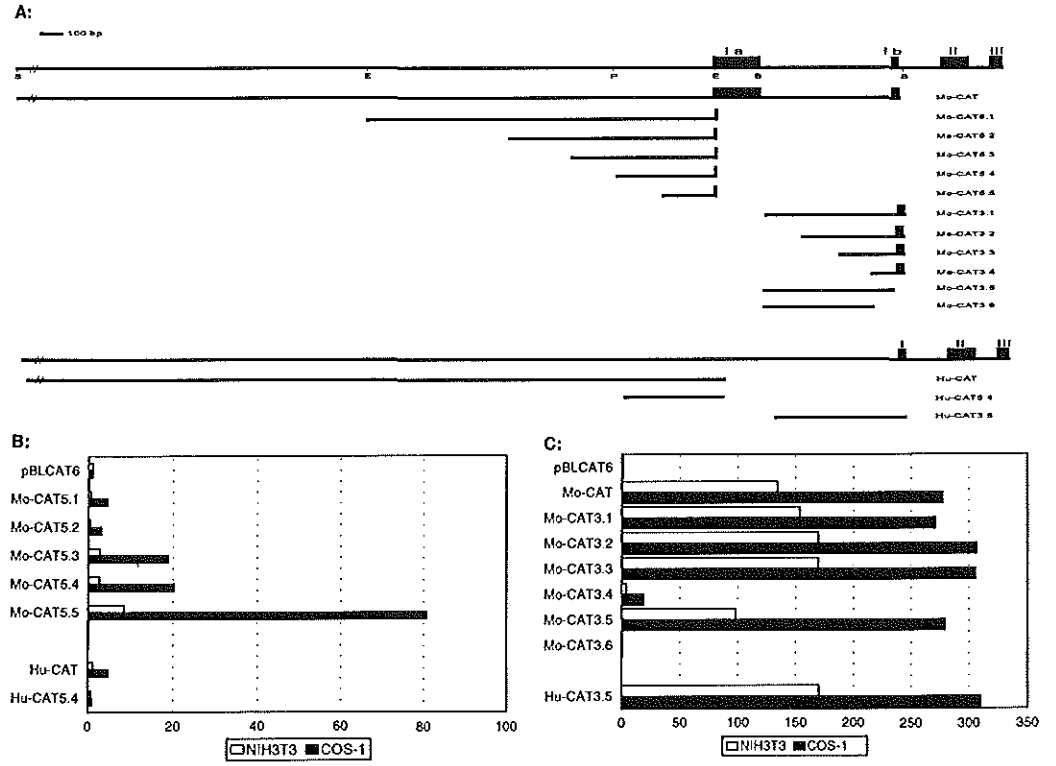

FIG. 5. *In vitro* promoter activity associated with the human and mouse promoter regions. A. Schematic representations of the human and mouse constructs. B. Relative CAT activity of the distal promoter fragments driving the *cat* gene. C. Relative CAT activity of the proximal promoter fragments driving the *cat* gene. All activities are relative to the pBLCAT6 empty vector.

genomic regions and have identified their promoter elements. Although transcription of the human gene is controlled by a single promoter, there are two promoter elements in the murine gene that generate two PPCA transcripts with alternative 5′ exons. Multiple promoters occur in a number of genes from different species, ranging from bacteria to humans (for review, see Schibler and Sierra, 1987). Such genes can be grouped in four different classes. Classes I and II contain the most common types of gene, which either have one constitutive and one tissue-specific promoter (Izzo *et al.*, 1988; Stauffer and Ciejek-Baez, 1992; Strähle *et al.*, 1992; Saitta and Chu, 1994; Chotani *et al.*, 1995; Salminen *et al.*, 1996), or they have two tissue-specific promoters (Fraser *et al.*, 1989; Visvader and Verma, 1989; Neuhaus *et al.*, 1995; Jahng *et al.*, 1996; Mutoh *et al.*, 1996). Class III genes use differential promoters to create proteins with alternative amino termini (Libri *et al.*, 1990; Rathjen *et al.*, 1990; Snijders *et al.*, 1993; Banville *et al.*, 1995), and class IV consists of genes that use differential promoters to control developmental switches (Cartwright, 1987; Tautz *et al.*, 1987; Corbin and Maniatis, 1989; Podgorski *et al.*, 1989; Takadera *et al.*, 1989; Allen *et al.*, 1992). Besides PPCA, two other lysosomal genes have dual promoters. The human β-glucuronidase gene is driven by a constitutive and a tissue-specific promoter (Shipley *et al.*, 1991). However, the expression from the latter promoter has only be shown in transformed cells. In the human lysosomal cathepsin D gene, the same promoter seems to have dual activity—driving constitutive expression of the mRNA, and sensitivity to estrogen in breast cancer cells (Cavaillès *et al.*, 1993; May *et al.*, 1993; Augereau *et al.*, 1994). Our data suggest that, although developmental regulation cannot be excluded at present, the murine lysosomal PPCA gene is a class I multiple promoter gene. The function of the additional, tissue-specific promoter is, as yet, unclear. One explanation could be that the distal promoter is substrate sensitive and also induced by high local substrate concentration. For instance, this promoter could regulate PPCA transcription in specialized cells that require the enzyme to inactivate and clear bioactive peptides (Jackman *et al.*, 1990, 1992); at high bioactive peptide levels, more PPCA would be expressed.

The two alternative 5′ exons might also generate mRNAs that differ in their stability or translation efficiency (Kozak, 1989), which, in turn, may influence the PPCA protein concentration in specific cells. We have not analyzed other mammalian species and, therefore, we are unable to determine whether the mouse has gained the distal promoter or the human has lost it. In either situations, the presence of the distal promoter in the mouse could indicate differences in local metabolic needs between the two species. Alternatively, the mouse

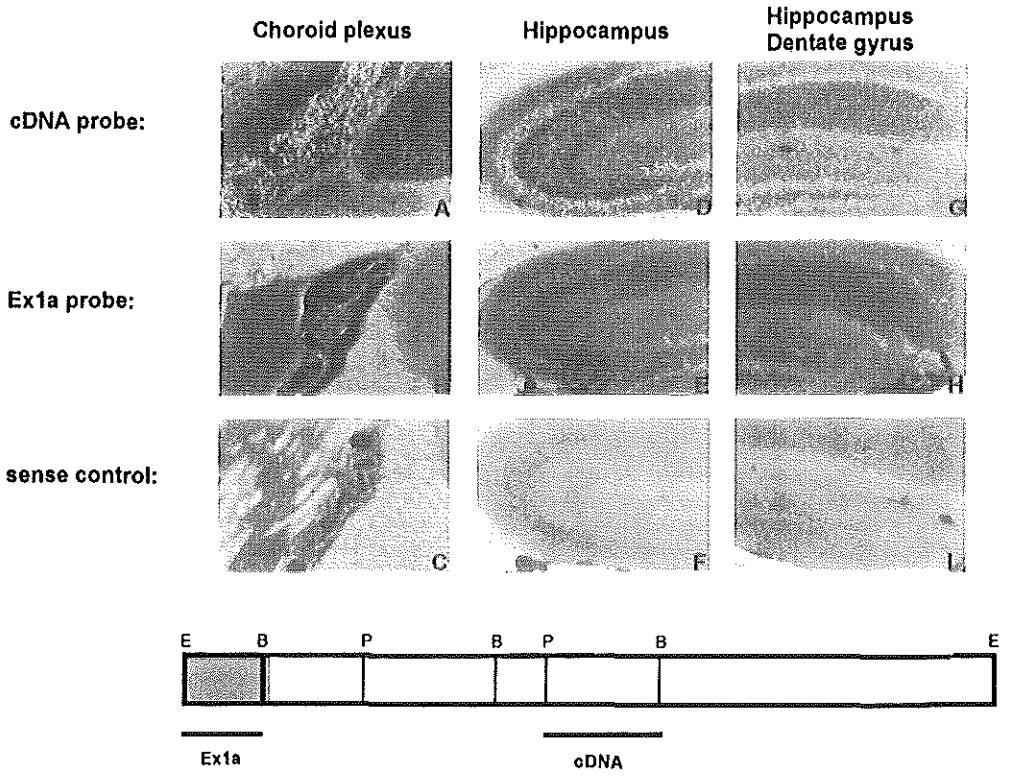

FIG. 6. *In vivo* expression of the murine PPCA transcripts in adult mouse brain. An outline of the two riboprobes used for the *in situ* hybridizations is shown at the bottom of the figure. The indicated fragments were subcloned into pBluescript, linearized, and subsequently *in vitro*-transcribed using either the T7 or T3 RNA polymerase. A–C. The choroid plexus hybridized with the cDNA (A), the Ex1a (B), and the sense control (C) riboprobes. D–I. The hippocampus and the dentate gyrus hybridized with the cDNA (D,G), Ex1a (E,H), and the sense control (F,I) riboprobes. The hippocampus and the dentate gyrus are clearly positive with both probes, whereas the choroid plexus is only recognized by the cDNA probe.

may have kept this nonessential remnant promoter that is nonetheless still active, in an evolutionary step analogous to that involving the human δ-globin gene promoter (Wood *et al.*, 1978).

Despite the high level of homology between the human upstream sequences and the mouse distal promoter, the human region completely lacks transcriptional activity. It has lost the TATA box (TGTAGA vs. TATAAA) and the two imperfect CAAT boxes (CCAGG vs. CCAAG and GCCAT vs. CCCAT). Site-directed mutagenesis of these sites in either gene could verify their involvement in transcription regulation. Our preliminary data suggest that mutating the mouse TATA box to the human counterpart (TATAAA into TGTAGA) increases rather than decreases promoter activity *in vitro*, which implies that the TATA box is not the principal transcriptional activator of the upstream murine promoter (data not shown). It is evident from our Northern blot analyses that expression of human PPCA mRNA is less variable than that of the murine exon Ib transcript. This could represent a compensatory effect for the loss of the second human promoter.

The sequence of the human promoter has several features in common with that of the murine proximal promoter. Both lack conventional TATA and CAAT boxes, and both are G/C rich. In addition, the mouse gene has multiple transcriptional start sites. These characteristics are shared by housekeeping genes, which encode proteins that perform essential metabolic functions and have a wide tissue distribution (Dynan, 1986). Several other lysosomal genes are members of this gene family (Hoefsloot *et al.*, 1988; Neote *et al.*, 1988; Proia, 1988; Geier *et al.*, 1989; Kreysing *et al.*, 1990; Martiniuk *et al.*, 1991; Morreau *et al.*, 1991; Redecker *et al.*, 1991; Shipley *et al.*, 1991). We demonstrate that the human and mouse proximal promoter fragments possess maximal transcriptional activity in transient transfections, and deletion analysis of the mouse promoter confined the necessary regulatory elements for maximal transcription *in vitro* to a 298-bp fragment. These findings correlate well with what has been described for the human β-galactosidase (Morreau *et al.*, 1991), the human β-hexosaminidase A and B (Norflus *et al.*, 1996), the mouse hexosaminidase A (Wakamatsu *et al.*, 1994), and the human β-glucuronidase (Shipley *et al.*, 1991) genes.

The most striking homology between the human promoter and the mouse proximal promoter is the presence of USF/MLTF

sequence motifs (Hen *et al.*, 1982; Carthew *et al.*, 1985; Sawadogo and Roeder, 1985), also known as E boxes (Murre *et al.*, 1989; Blackwell *et al.*, 1990) or adenovirus 2 major late promoter upstream element factors (Ad2 MLP UEF; Hen *et al.*, 1982). Transcription factors like MyoD, kE2-binding protein, and c-Myc are known to bind to these elements (Murre *et al.*, 1989; Blackwell *et al.*, 1990; Blackwell and Weintraub, 1990). Moreover, a number of cellular genes involve UEF in their activation (Carthew *et al.*, 1987; Chodosh *et al.*, 1987; Peritz *et al.*, 1988). This sequence motif is also found in the promoter of the lysosomal human and mouse cathepsin D (Augereau *et al.*, 1994; Hetman *et al.*, 1994) and β-hexosaminidase A genes (Proia and Soravia, 1987; Wakamatsu *et al.*, 1994), where it is thought to regulate transcription. However, the importance of the USF/MLTF motifs in regulation transcription of the murine PPCA gene is not yet clear. Deletions from the 3′ side of the proximal promoter identified sequences between −100 and +1 that are necessary for transcription.

In GS patients, the activities of lysosomal β-galactosidase and neuraminidase are reduced because the enzymes are unable to complex with PPCA. This leads to lysosomal storage of primarily sialylated oligosaccharides and oligopeptides. Pathologic manifestations in GS patients include the vacuolation of predominately reticuloendothelial cells in most organs. We have recently generated a GS mouse model (Zhou *et al.*, 1995) that closely resembles the human phenotype. The storage pattern observed in these mice may reflect the differential expression of PPCA mRNAs in specific cells of affected organs. Indeed, our *in situ* hybridization experiments clearly demonstrated a local distribution of the two mRNAs in the adult mouse brain. It will be of interest to analyze further the need for differential expression of the PPCA transcripts in mouse tissues and to correlate these findings with the pleiotopic functions of the protein in both mice and humans.

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Chapter 4

Mouse model for the lysosomal storage disorder galactosialidosis and correction of the phenotype with overexpressing erythroid cells

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Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells

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The lysosomal storage disorder galactosialidosis results from a primary deficiency of the protective protein/cathepsin A (PPCA), which in turn affects the activities of β-galactosidase and neuraminidase. Mice homozygous for a null mutation at the PPCA locus present with signs of the disease shortly after birth and develop a phenotype closely resembling human patients with galactosialidosis. Most of their tissues show characteristic vacuolation of specific cells, attributable to lysosomal storage. Excessive excretion of sialyloligosaccharides in urine is diagnostic of the disease. Affected mice progressively deteriorate as a consequence of severe organ dysfunction, especially of the kidney. The deficient phenotype can be corrected by transplanting null mutants with bone marrow from a transgenic line overexpressing human PPCA in erythroid precursor cells. The transgenic bone marrow gives a more efficient and complete correction of the visceral organs than normal bone marrow. Our data demonstrate the usefulness of this animal model, very similar to the human disease, for experimenting therapeutic strategies aimed to deliver the functional protein or gene to affected organs. Furthermore, they suggest the feasibility of gene therapy for galactosialidosis and other disorders, using bone marrow cells engineered to overexpress and secrete the correcting lysosomal protein.

[*Key Words*: Mouse model; galactosialidosis; lysosomal disease; protective protein; erythroid expression; BMT]

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Galactosialidosis (for review, see d'Azzo et al. 1995) is a lysosomal storage disease, inherited as an autosomal recessive trait and characterized by a combined deficiency of β-D-galactosidase and *N*-acetyl-α-neuraminidase, secondary to a defect of the protective protein/cathepsin A (PPCA) (Wenger et al. 1978; d'Azzo et al. 1982). This serine carboxypeptidase (Galjart et al. 1988; Rawlings and Barrett 1994) has at least two distinct functions. First, as demonstrated by copurification experiments (Verheijen et al. 1982; Yamamoto and Nishimura 1987; Potier et al. 1990; Scheibe et al. 1990; Hubbes et al. 1992), it associates with the two glycosidases, thereby modulating their intralysosomal stability and activity (Hoogeveen et al. 1983; van der Horst et al. 1989; Galjart et al. 1991). Second, the protein is catalytically active as a lysosomal cathepsin at acidic pH, as well as a deamidase/esterase at neutral pH, and has been shown to participate in the inactivation of selected neuropeptides like substance P, oxytocin, and endothelin I (Jackman et al. 1990; Galjart et al. 1991; Jackman et al. 1992; Hanna et al. 1994). Mammalian and avian protective proteins are highly conserved and share homology to yeast and plant serine carboxypeptidases (Galjart et al. 1988; Galjart et al. 1991). The murine and avian enzymes can even substitute for their human homolog to stabilize and activate human β-galactosidase and neuraminidase (Galjart et al. 1991).

Patients with galactosialidosis have clinical manifestations indicative of a lysosomal disorder, including coarse facies, ocular cherry red spots, vertebral changes, foamy cells in the bone marrow, and vacuolated lymphocytes in peripheral blood. Based on the age of onset of the symptoms, three clinical phenotypes are distinguished: early infantile, late infantile, and juvenile/adult variants. The most severe early infantile form is associated

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with fetal hydrops, diffuse edema, ascites, hepatosplenomegaly, psychomotor delay, bone deformity, and severe heart and kidney involvement. Death occurs within the first year of age, likely as a result of renal failure. Late infantile and juvenile/adult patients have a later onset and slower progression of the disease; however, whereas adult patients have central nervous system (CNS) involvement, the late infantile types are not mentally retarded or only mildly so. In spite of the triple enzyme deficiency, the storage products isolated from tissues and urine of galactosialidosis patients are primarily sialylated oligosaccharides (Okada et al. 1978; van Pelt et al. 1988a,b, 1989). The characteristic pathology of the disease includes vacuolation of cells in the central, peripheral, and autonomic nervous systems, retina, liver, kidney, skin, and peripheral blood (d'Azzo et al. 1995).

There is currently no effective therapy for galactosialidosis or for other lysosomal disorders with CNS involvement. Transplantation of allogeneic bone marrow as a source of normal enzyme has proven of limited benefit in human studies with other lysosomal diseases (for review, see Krivit et al. 1992; Hoogerbrugge et al. 1995), but has never been tried for galactosialidosis. Also in naturally occurring animal models with these disorders, the effect of bone marrow transplantation (BMT) has been variable (for review, see Haskins et al. 1991). Improvement of the pathology was observed after BMT in cats with α-mannosidosis (Walkley et al. 1994a), but only partial correction was obtained with this procedure in mice with mucopolysaccharidosis VII (Birkenmeier et al. 1991) and no amelioration was detected in treated G_{M2}-gangliosidosis cats (Walkley et al. 1994b). This difference may depend on the number of perivascular monocytes/macrophages originating from the graft but also on differential secretion of the correcting enzyme by donor-derived cells, as seems to be the case for α-mannosidase and β-hexosaminidase, at least in in vitro studies (Dobrenis et al. 1994). Other novel approaches, like intraperitoneal implant of fibroblasts organoids (Moullier et al. 1995) or direct transfer of neuronal progenitor cells (Snyder et al. 1995), have been employed recently for the local delivery of exogenous functional enzyme to the liver and the CNS, respectively. Overall, these studies establish the validity of suitable animal models, which closely resemble the human disease, to evaluate therapeutic strategies for the correction of disorders like galactosialidosis. Although sheep with a combined β-galactosidase and neuraminidase deficiency have been identified (Ahern-Rindell et al. 1988), both the localization of severe storage only to neurons and the enzymatic characteristics differentiate this model from the human disease. Recently, a disease with pathology similar to the adult form of galactosialidosis was described in a Schipperke dog (Knowles et al. 1993), but the biochemical data were insufficient to verify the diagnosis. To produce an animal model for galactosialidosis we have generated mice carrying a null mutation at the PPCA locus. Deficient animals are viable and fertile but exhibit severe abnormalities soon after birth that closely resemble those found in galactosialidosis patients. They also develop the characteristic histopathology of the human disease. Thus, this mouse model provides an attractive opportunity to study the pathogenesis and pathophysiology of the disease and to evaluate experimental therapies, including gene therapy. In this report we have tested the hypothesis that substantial secretion of PPCA by genetically modified bone marrow cells could translate into a more timely and effective correction of the lesions in affected mice. We demonstrate that treatment of the (−/−) phenotype with transgenic bone marrow overexpressing the human PPCA in erythroid precursors, which also secrete the protein at high levels, can improve the pathological conditions better than normal bone marrow cells. These results give the first indication of the potential of overproducing and secreting hematopoietic cells for the treatment of galactosialidosis and possibly other similar disorders.

Results

Generation of PPCA-deficient mice

A targeting vector for homologous recombination at the murine PPCA locus was constructed as outlined in Figure 1A. The position of the positive selectable marker prevents translation of the polypeptide and thus the targeted replacement constitutes a genuine null mutation. Targeted E14 embryonic stem (ES) cells were obtained at a frequency of 13%–15%. Chimeric males derived from two independent clones (28 and 139) yielded germ-line transmission of the null allele. Interbreeding of heterozygotes (+/−) from both clones readily generated homozygous (−/−) mice. Offspring were genotyped by Southern blotting and hybridization with a 5′ external probe (Fig. 1B). Combined data from crosses between clone 28 and/or clone 139 heterozygotes showed a distribution of 24% (+/+), 20% (−/−), and 56% (+/−) in 292 offspring analyzed, suggesting that an intact PPCA gene is not required for normal embryonic development of homozygous null mice. Northern blot analysis (Fig. 1C) of total RNA isolated from PPCA (−/−) tissues confirmed the lack of a PPCA transcript in deficient mice.

Biochemical analysis of PPCA-deficient mice

The engineered mutation rendered (−/−) mice unable to encode the PPCA protein, as established by assaying cathepsin A activity in cultured fibroblasts and tissues from (+/+), (+/−), and (−/−) mutant animals of different age (Table 1). This catalytic activity either was absent or reduced severely in tissues of homozygous mutant mice, with the levels ranging from nondetectable in bone marrow to ~8% of normal in liver. The small, residual activity measured in some of the samples may be attributable to the presence of catalytically related proteins that have a low affinity for the Z-Phe-Ala substrate. Heterozygous mice showed clear intermediate values (Table 1). Because direct biochemical evidence of galactosialidosis in human patients is the combined deficiency of neuraminidase and β-galactosidase in fibro-

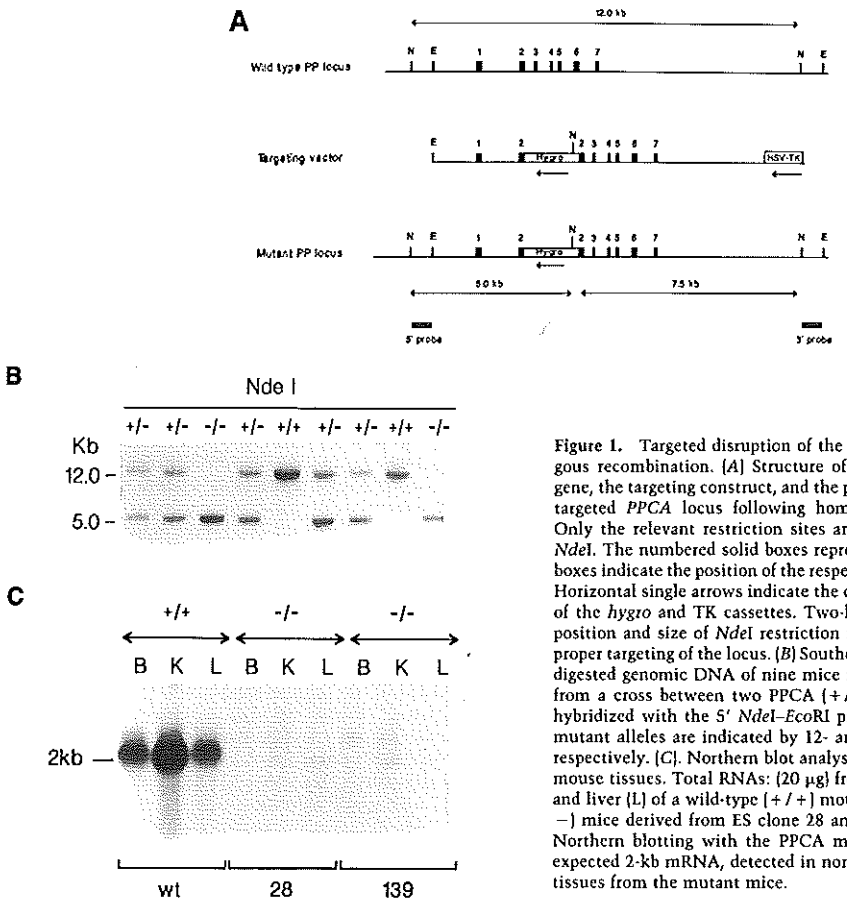

Figure 1. Targeted disruption of the *PPCA* locus by homologous recombination. (*A*) Structure of a portion of the *PPCA* gene, the targeting construct, and the predicted structure of the targeted *PPCA* locus following homologous recombination. Only the relevant restriction sites are shown: (E) *Eco*RI; (N) *Nde*I. The numbered solid boxes represent exons. The labeled boxes indicate the position of the respective 5' and 3' *hy* probes. Horizontal single arrows indicate the direction of transcription of the *hygro* and TK cassettes. Two-headed arrows show the position and size of *Nde*I restriction fragments diagnostic for proper targeting of the locus. (*B*) Southern blot analysis of *Nde*I-digested genomic DNA of nine mice from one litter resulting from a cross between two PPCA (+/−) mice. The blot was hybridized with the 5' *Nde*I–*Eco*RI probe. The wild-type and mutant alleles are indicated by 12- and 5-kb *Nde*I fragments, respectively. (*C*). Northern blot analysis of PPCA mRNA from mouse tissues. Total RNAs: (20 μg) from brain (B), kidney (K), and liver (L) of a wild-type (+/+) mouse and two mutant (−/−) mice derived from ES clone 28 and 139 were analyzed by Northern blotting with the PPCA mouse cDNA probe. The expected 2-kb mRNA, detected in normal tissues, is absent in tissues from the mutant mice.

blasts and leukocytes (d'Azzo et al. 1995), we assessed these parameters in the mutant mice. The deficiency of cathepsin A in (−/−) tissues directly influenced the activity of neuraminidase, which was severely reduced, especially in fibroblasts and kidney samples (Table 1). However, β-galactosidase levels varied considerably for the different tissues from deficient mice, being ~20% of normal levels in fibroblasts, as found in human galactosialidosis (10%–15%), but even higher than normal in liver. These results suggest that murine β-galactosidase may be less dependent for its stability and activity on complex formation with PPCA than is murine neuraminidase.

A second parameter used in clinical diagnosis is the overexcretion of undegraded sialyloligosaccharides in the urine of affected individuals (van Pelt et al. 1989). The total content of these metabolites in PPCA (−/−) mice thus was determined for urine from PPCA (−/−), (+/−), and (+/+) mice. Twenty-one affected mice, tested between the age of 2 and 5 months excreted 3–10 times more sialylated oligosaccharides in urine than their normal and heterozygous littermates (Table 2). The amount of accumulated products increased progressively with time, eventually reaching levels 25 times higher than controls and heterozygotes in seven (−/−) mice tested at the age of 6–8 months. These biochemical data clearly mirror the situation for humans with galactosialidosis and establish the validity of this model as an experimental substrate.

Phenotypic characterization and histopathology of deficient mice

PPCA (−/−) mice are viable and fertile, and die at ~12 months of age. At birth and during the first 6 weeks of life their gross appearance was normal, except for an ap-

Table 1. *Lysosomal enzyme activities in cultured fibroblasts and tissues from PPCA (−/−) mice*

	Cathepsin A (mmole/min/mg)			Neuraminidase (nmole/hr/mg)		β-galactosidase (nmole/hr/mg)	
	+/+	+/−	−/−	+/+	−/−	+/+	−/−
Fibroblast	159 ± 9	98 ± 17	1.3 ± 0.9	23.00 ± 0.10	0.43 ± 0.17	812 ± 82	263 ± 50
Kidney	135 ± 14	61 ± 8	1.8 ± 0.9	40.00 ± 6.40	0.57 ± 0.09	275 ± 72	176 ± 25
Brain	20 ± 3	10 ± 2	0.7 ± 0.3	3.06 ± 0.36	0.98 ± 0.19	68 ± 21	59 ± 9
Spleen	114 ± 16	43 ± 18	5.5 ± 2.3	4.00 ± 1.92	0.94 ± 0.21	291 ± 51	240 ± 46
Liver	77 ± 19	31 ± 13	3.8 ± 1.1	3.78 ± 0.66	0.42 ± 0.07	68 ± 8	144 ± 22
Bone marrow	163 ± 27	N.D.	0.0 ± 0.0	3.66 ± 0.36	0.19 ± 0.02	119 ± 11	106 ± 25

Fibroblast cell lysates and total tissue homogenates in H_2O from PP normal (+/+), heterozygous (+/−), and homozygous (−/−) mice were assayed for cathepsin A activity, using the acylated dipeptide Z-Phe-Ala, and for neuraminidase and β-galactosidase activities, using synthetic 4-methylumbelliferyl substrates. Values represent means ± S.E.M. of measurements done on 4 (+/+), 2 (+/−), and 7 (−/−) mice. All mice were between 2 and 8 weeks of age and were derived from ES cell clones 28 and 139. (N.D.) Not done.

parent flattening of the face. However, the majority of young (−/−) mice weighed 25%–40% less than (+/+) and (+/−) littermates and were identified readily by size. Two of the smallest mice died spontaneously at 3 weeks of age and were found to have hepatic and splenic enlargement. No distinguishable skeletal deformities were observed in the young (−/−) mice, but their physical condition overall worsened with age. Progressive and diffuse edema was accompanied by apparent ataxic movements and tremor. The characteristic broad face, rough coat, and extensive swelling of subcutaneous tissues, limbs, and eyelids are obvious in the 10-month-old (−/−) mouse shown in Figure 2.

Visceromegaly is a specific clinical abnormality associated with the early and sometimes late infantile forms with galactosialidosis. This feature was also found in PPCA (−/−) mice with enlargement of both the liver and the spleen being prominent at autopsy (not shown). Morphological studies of tissues from (−/−) mice were carried out on either paraffin, cryosections, or epoxy-resin sections examined both by light and electron microscopy. Affected mice were sacrificed at the age of 14 days, 1 month, 3–4 months, 6 months, and 10 months. Signs of metabolic storage were found primarily in the reticuloendothelial system of most organs, already noticeable in the first mouse analyzed 14 days after birth. Thirty percent of the lymphocytes in peripheral blood had translucent vacuoles in their cytoplasm. In the skin, foamy macrophages, sometimes clearly perivascular, were visible between collagen bundles of the dermis. The presence of stored material was confirmed by positive cytoplasmic staining with periodic acid–Schiff (PAS) and diastase treatment (PAS+). The kidney appeared to be the earliest and most severely affected organ in young mutant mice, analogous to the situation for severe human disease. Electron microscopy of ultrathin sections of kidney from a 14-day-old (−/−) mouse demonstrated vacuolation of the tubular epithelium, most evident in the proximal tubules (Fig. 3A), whereas distal tubules and glomeruli were less or minimally affected at this stage (Fig. 3B). The numerous membrane-bound vacuoles representing secondary lysosomes appeared either empty or filled with sparse fibrillar structures, reflecting lysosomal accumulation of low molecular weight compounds (e.g., oligosaccharides or glycopeptides).

This spectrum of pathological changes worsened pro-

Table 2. *Total urinary sialyloligosaccharides in affected and BM-transplanted mice*

Mice	Number	Age	nmole NANA/mg creatinine
Controls	4	2–8 months	1906 ± 155 (range 1663–2310)
Heterozygotes	14	2–8 months	1905 ± 117 (range 993–2923)
Homozygotes	21	2–5 months	7215 ± 647 (range 5305–19362)
	7	6–8 months	27368 ± 4845 (range (11200–45763)
BMT-N	1	5 months	1812 (5 weeks; 3months p.t.)
BMT-TG	4	5 months	2114 (5 weeks; 8 weeks p.t.)
		5 months	2106 (8 weeks 3 months p.t.)
		5 months	1799 (8 weeks; 9 weeks; 3 months p.t.)
		2.5 months	2011 (8 weeks; 9 weeks; 3 months p.t.)

Total sialyloligosaccharides were measured after acid hydroloysis of urine samples with thiobatinturic acid and were expressed in nmoles of released neuraminic acids per milligram of creatinine. (BMT-N) PPCA (−/−) mice transplanted with bone marrow from a normal donor. (BMT-TG) PPCA (−/−) mice transplanted with bone marrow from a transgenic mouse, overexpressing human protective protein in the erythroid lineage. Mice were transplanted at either 2.5 or 5 months of age. Sialyloligosaccharide determinations were performed at various time points post-transplantation (p.t.), as specified in parentheses. The values represent the mean of independent measurements.

Figure 2. Gross phenotypic appearance of a PPCA (−/−) mouse (*middle*) at 10 months of age, compared to a wild-type (+/+) littermate (*right*), and a (−/−) littermate (*left*) transplanted 5 months earlier with transgenic BM. The affected mouse has a broad face, disheveled coat, and swollen limbs and eyelids. In contrast, the BMT mouse clearly has improved in appearance. Evidence of edema has disappeared, and the coat is shiny and full.

gressively with age. The toluidine blue- and PAS-stained histological sections shown in Figure 4 were derived from a (−/−) mouse at 6 month of age. In the affected kidney the epithelial cells of the proximal tubules were filled with swollen lysosomes. The glomeruli also were severely impaired by abundant vacuolation of parietal and visceral epithelium, endothelium and mesangial cells at this stage of the disease. Accumulation of undegraded material again was not apparent in the distal tubules. These abnormalities were not observed in control specimens, except for small resorption vesicles present normally at the apical side of the tubular epithelial cell surface (Fig. 4A). No evidence of storage was detected in the adrenal glands (not shown). In the (−/−) liver, hepatocytes contained numerous, small membrane-bound vacuoles, whereas the Kupffer cells in the sinusoids were more affected and acquired the characteristic foamy appearance (Fig. 4A). Both hepatocytes and Kupffer cells stained positive with PAS+ (not shown). The spleen of the diseased mouse showed extensive congestion of the sinuses and the marginal zones with foamy macrophages, which were also slightly PAS-positive. Furthermore, large cytoplasmic vacuoles were found in the lymphoid cells. Megakaryocytes were devoid of storage, despite their extensive lysosomal system (Fig. 4A).

The histopathology of the brain was examined in paraffin sections, and portions of the cerebrum and cerebellum were processed for frozen sections stained with PAS or for epoxy–resin sections stained with toluidine blue. Finely vacuolated storage cells similar to those found in the visceral organs also were observed in the brain. They often were located around neurons or blood vessels (Fig. 4B, a–c). Neuronal storage was rather inconspicuous on paraffin sections even in older mice. However, on frozen sections stained with PAS, affected neurons were identified easily by a brilliantly red storage material (Fig. 4B, d). Although frozen section study was carried out on limited portions of the brain, there was a significant regional variation in the distribution of PAS-positive neurons. Entorhinal cortex and hippocampus appeared to be more involved (Fig. 4B, d) than the somatosensory cortex (Fig. 4B, e). In the cerebellum, there was a loss of Purkinje cells and some of the remaining Purkinje cells contained storage material in their perikarya (not shown). The granular cells were well preserved. Extensive storage was apparent in the epithelial and stromal cells of the choroid plexi (Fig. 5B, below). Finally, vacuolated cells were present in the trigeminal ganglia and the pituitary gland. The pituicytes also showed fine vacuolation (Fig. 4C).

Examination of other organs (i.e., pancreas, small and large intestine, lymph nodes, testis, ovaries, uterus, heart, bone marrow, and eye) confirmed the presence of membrane-bound vacuoles primarily in macrophages and cells of epithelial/endothelial origin (not shown). No evidence of lysosomal storage was detected in the lungs and skeletal muscles. The distribution and type of accumulating cells remained similar in 10-month-old mutant mice.

Rescue of the galactosialidosis phenotype in the mouse model

The rationale for using allogeneic BMT to treat lysosomal storage disorders is that progenitor bone marrow (BM) cells will repopulate the transplanted host and supply the missing enzyme to deficient cells in the tissues of the recipient. Correction takes place when the therapeutic enzyme secreted from normal BM cells is reinternalized by deficient cells, with restoration of lysosomal

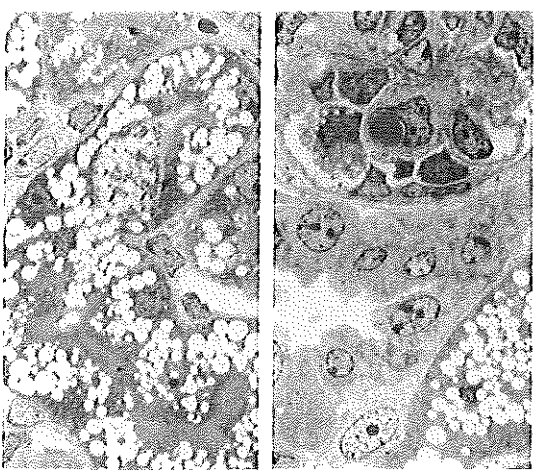

Figure 3. Electron microscopy of kidney from a 2-week-old (−/−) mouse. (*A*) Micro- and macrovacuolation of the proximal tubules is apparent. (*B*) Distal tubules and glomeruli are less, if not at all, affected at this stage of the disease. Magnification, 2000× (*A*) and 2500× for (*B*).

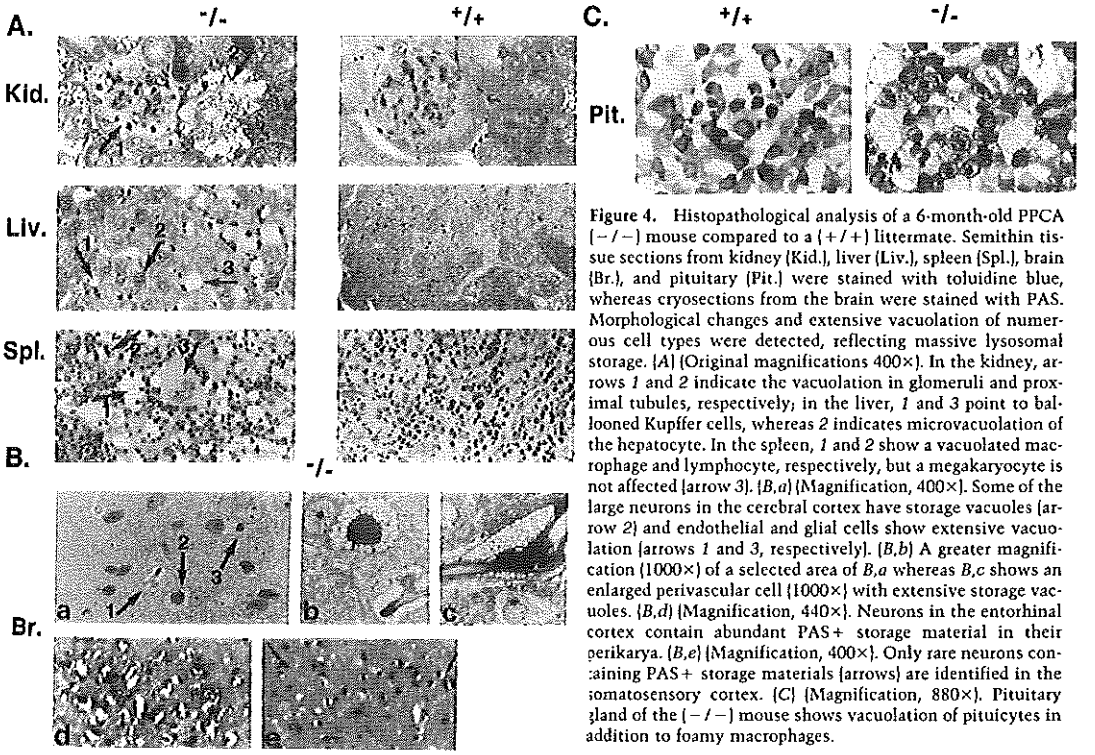

Figure 4. Histopathological analysis of a 6-month-old PPCA (−/−) mouse compared to a (+/+) littermate. Semithin tissue sections from kidney (Kid.), liver (Liv.), spleen (Spl.), brain (Br.), and pituitary (Pit.) were stained with toluidine blue, whereas cryosections from the brain were stained with PAS. Morphological changes and extensive vacuolation of numerous cell types were detected, reflecting massive lysosomal storage. (*A*) (Original magnifications 400×). In the kidney, arrows *1* and *2* indicate the vacuolation in glomeruli and proximal tubules, respectively; in the liver, *1* and *3* point to ballooned Kupffer cells, whereas *2* indicates microvacuolation of the hepatocyte. In the spleen, *1* and *2* show a vacuolated macrophage and lymphocyte, respectively, but a megakaryocyte is not affected (arrow 3). (*B,a*) (Magnification, 400×). Some of the large neurons in the cerebral cortex have storage vacuoles (arrow *2*) and endothelial and glial cells show extensive vacuolation (arrows *1* and *3*, respectively). (*B,b*) A greater magnification (1000×) of a selected area of *B,a* whereas *B,c* shows an enlarged perivascular cell (1000×) with extensive storage vacuoles. (*B,d*) (Magnification, 440×). Neurons in the entorhinal cortex contain abundant PAS+ storage material in their perikarya. (*B,e*) (Magnification, 400×). Only rare neurons containing PAS+ storage materials (arrows) are identified in the somatosensory cortex. (*C*) (Magnification, 880×). Pituitary gland of the (−/−) mouse shows vacuolation of pituicytes in addition to foamy macrophages.

function. It is conceivable that higher levels of the correcting protein will relate directly to more timely and effective treatment. We have shown previously that both human and murine PPCA precursors, selectively secreted by overexpressing cells, undergo endocytosis by cultured galactosialidosis fibroblasts via the mannose-6-phosphate receptor and correct both β-galactosidase and neuraminidase activities (Galjart et al. 1991). Having a suitable animal model for galactosialidosis, we now could test whether transplantation of affected mice with BM cells overexpressing and secreting a functional PPCA protein could correct the lysosomal storage better than normal bone marrow. Therefore, four transgenic mouse lines were generated in which expression of a human PPCA transgene was driven by the β-globin promoter and locus control region (LCR) of the β-globin gene (Grosveld et al. 1987). The transgenic mice synthesized the heterologous human protein at high levels in cells of the erythroid lineage. Prior to transplantation, we ascertained that the product of the transgene also was secreted by BM cells to the extent that the high uptake precursor form of the protein was detectable in plasma and would undergo endocytosis by recipient cells. For this experiment, total BM cells derived from either transgenic or normal mice were cultured in the presence of cytokines for 5 days. The conditioned medium from these cultures, as well as serum from the same animals, was first assayed for α-galactosidase, α-glucosidase, and β-hexosaminidase activities (not shown) to ensure that overproduction of PPCA did not lead to aspecific release of other lysosomal proteins. Similar values for the three enzyme activities were measured in normal and transgenic samples, indicating that oversecretion of human PPCA was selective. Neuraminidase, being membrane bound, was not secreted and extracellular PPCA activity could not be tested because the precursor form of the protein is a zymogen, activated only in lysosomes. Conditioned media and sera then were added separately to deficient galactosialidosis cells from an early infantile patient (Galjart et al. 1988). As shown in Table 3, cathepsin A, β-galactosidase, and neuraminidase activities were clearly enhanced upon uptake of the human PPCA precursor from either BM-conditioned medium or directly from transgenic mouse serum. No significant increase in activity was detected in deficient fibroblasts treated with serum or BM-conditioned medium from normal mice. These results suggested that transgenic BM cells might achieve similar correction in vivo in affected mice.

Homozygous (−/−) mice, ranging in age from 2.5 to 5

Table 3. *Uptake of BM-secreted human PPCA precursor by galactosialidosis fibroblasts and restoration of enzyme activities*

	Cathepsin A (nmole/min/mg)	β-galactosidase (nmole/hr/mg)	Neuraminidase (nmole/hr/mg)
No addition	2.1	66	0.05
Normal serum	3.3	78	0.1
Normal BM-CM	5.5	72	3.1
TG serum	31.2	186	14.5
TG BM-CM	51.2	216	13.5

Conditioned media (CM) from cultured BM cells and serum samples, both derived from either normal or transgenic (TG) mice, were added to PPCA (−/−) human galactosialidosis fibroblasts. After uptake, cell lysates were assayed for exogenous cathepsin A activitiy using the acylated dipeptide Z-Phe-Ala and for endogenous neuraminidase and β-galactosidase activities using synthetic 4-methylumbelliferyl substrates.

months, were lethally irradiated and transplanted, six with bone marrow from transgenic mice and six with bone marrow from normal mice. Both donor mouse lines were T cell depleted and were H-2B compatible with the affected recipients. The repopulated mice were monitored for the level of storage material excreted in urine (Table 2). At 5 weeks after transplantation the total excretion of sialyloligosaccharides already was reduced to normal values, indicating that the PPCA provided by both transgenic and normal BM cells was sufficient to correct the accumulation of these storage products in urine. Furthermore, gross examination of transplanted animals, starting at 3 months post-BMT, showed complete reversal of the diseased phenotype, as exemplified by the 10-month-old mouse shown in Fig. 2 (left), transplanted 5 months earlier with transgenic bone marrow.

Histopathology of transplanted mice

Tissue sections from kidney, liver, spleen, intestine, heart, lymph nodes, and brain of two (−/−) mice transplanted with bone marrow from either transgenic (BMT–Tg) or normal donors (BMT–N) were compared with similar sections from normal and untreated littermates 3 months after transplantation. As shown in Fig. 5A, the abnormalities in the proximal tubular epithelium in the kidney of the BMT–N mouse were resolved practically, whereas storage vacuoles were still abundant in the glomerular epithelium. These aberrant features still persisted in similarly treated mice examined 2 months later. In contrast, kidney specimens of the BMT–Tg mouse showed complete reversal of the diseased phenotype, even within the glomeruli, indicating that a higher dose of normal enzyme had a more beneficial and immediate effect on this severely affected tissue. Clearance of storage material and normalization of cellular morphology was apparent from the liver of mice treated either with normal or transgenic bone marrow (Fig. 5A). However, Kupffer cells with some vacuoles were encountered infrequently in liver sections of the BMT–N mouse, but not in corresponding sections of the BMT–Tg mouse. As expected, spleen (Fig. 5A) as well as bone marrow specimens appeared completely normal. Most of the abnormalities in the colon, jejunum, and duodenum were corrected, though the autonomic ganglia still showed a lesser degree of storage. In the heart vacuolated endothelial cells and foamy histiocytes were no longer encountered. The lymph nodes of both types of transplanted mice had a normal appearance with no evidence of accumulation of foamy macrophages, although storage in occasional lymphoid cells was seen only in the specimens from the BMT–N mice (not shown). In the brain of BM-transplanted mice storage neurons and perivascular as well as perineural vacuolated cells still could be identified (not shown). However, further quantitative assessment is necessary to evaluate the effect of the BM transplant in the brain. On the other hand, vacuolated cells in the choroid plexi (Fig. 5B), the trigeminal ganglia, and the pituitary had disappeared completely in both transplanted mice.

In conclusion, reversal of the diseased phenotype clearly was significant in BMT–N mice but was more complete in BMT–Tg animals, supporting the idea that the release of a higher level of enzyme by overproducing donor BM cells is likely to be more effective for treatment.

Discussion

Molecular and biochemical characteristics of PPCA (−/−) mice correlate well with the most severe form of galactosialidosis in humans. Although viable and fertile, deficient mice present signs of illness very early in life with clear evidence of metabolic storage in cells from different organs. The edema, visceromegaly, and coarse face noticed in (−/−) animals are abnormalities characteristic of the human disease phenotype. Nephropathy, which is a major complication and cause of death in early infantile patients (d'Azzo et al. 1995), is also the most apparent cause of physical deterioration in affected mice. Discrepancy in the severity of the disease between early infantile patients and young affected mice may be attributed simply to the much shorter gestation period of mice because the pathology of lysosomal disorders is largely associated with progressive accumulation of undegraded products in lysosomes, leading eventually to cellular dysfunction. The greater longevity of affected mice relative to human patients could also relate to the observed higher levels of β-galactosidase in some of the

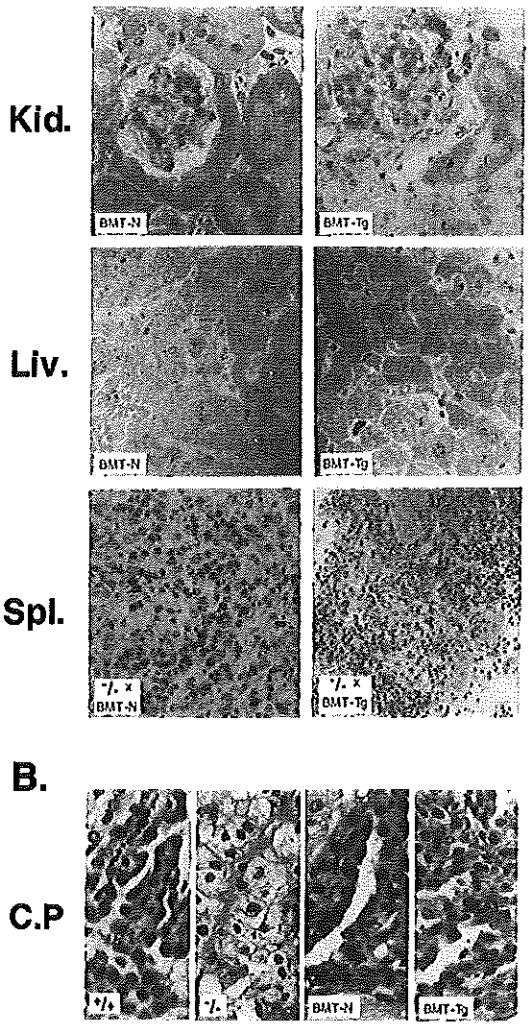

Figure 5. Histology of BMT mice. (*A*) Sections from the kidney (Kid.), liver (Liv.), and spleen (Spl.) of a (−/−) mouse transplanted with either BMT–N or BMT–Tg were stained with toluidine blue (magnification, 400×). In the BMT–N kidney, storage vacuoles still are apparent in the glomerular epithelium. In contrast, the BMT–Tg kidney shows complete correction of the lesions. In both BMT–N and BMT–Tg mice, the liver and the spleen show normal cellular morphology. However, in the BMT–N mouse, some of the Kupffer cells still are slightly vacuolated. (*B*) Haematoxylin/eosin-stained paraffin sections (magnification, 400×) of the choroid plexus from normal, affected, and transplanted mice. Storage in the epithelial cells of the (−/−) animal is signified by the dispersed staining of the cytoplasm. This aberration is reversed completely by transplantation with normal or transgenic BM.

affected mouse tissues. Alternatively, but not exclusive of the first two, the difference may be caused by species-specific variation in substrate metabolism. Similar explanations may account for divergence in time of development of the diseased phenotype for mice and humans with hexosaminidase A deficiency (Yamanaka et al. 1994). Thus, in spite of the longer survival, galactosialidosis mice are affected severely and can be considered a faithful model for the human disease.

The deficiency of PPCA in tissues of mutant mice is accompanied by an equivalent loss of lysosomal neuraminidase activity, whereas β-galactosidase values are variable. Although in murine (−/−) fibroblasts the residual β-galactosidase activity is consistent with that measured in patients' cells (d'Azzo et al. 1995), in other tissues the activity is either only slightly reduced or higher than normal levels. This finding may again reflect differences between species, although equivalent data for galactosialidosis patients are scarce, as autopsies are limited to a few cases from adult Japanese patients (Amano et al. 1983; Suzuki et al. 1984). The mouse results may be in line with earlier purification and coprecipitation studies, indicating that only a fraction of the β-galactosidase and PPCA in mammalian tissues exists in the associated mode, whereas all neuraminidase activity is detected in complex with the other two enzymes (Verheijen et al. 1985; Galjart et al. 1991; Hubbes et al. 1992). Thus, it is possible that in different murine cell types, β-galactosidase is stable whether or not associated with PPCA, whereas the interaction of neuraminidase with PPCA clearly is essential for its activity. The consequences of the severe neuraminidase, rather than β-galactosidase, deficiency are reflected in the type of storage products present in urine of the (−/−) mice, which are mainly sialylated oligosaccharides, as observed in human patients (Amano et al. 1983; van Pelt et al. 1989).

The pathological findings in the PPCA-deficient mice are consistent with those reported for galactosialidosis patients. Epithelial cells, endothelial cells, and macrophages are the first to show lesions in most organs, and the distribution of storage appears similar to that observed in the Gaucher and Nieman Pick mouse models (Tybulewicz et al. 1992; Horinouchi et al. 1995; Otterbach and Stoffel 1995). The type and number of vacuolated cells in galactosialidosis mice gradually increase with age. However, not all cells become affected and the extent of the damage is variable, even in older (−/−) animals. The profiles may depend on the metabolic state of the cells and the type of substrates to be catabolized, in particular by neuraminidase. Alternatively, it may reflect a microheterogeneity in the distribution of the normal protein in different cell types. We have shown previously that the protective and catalytic functions of PPCA are fully separable (Galjart et al. 1991), but the physiological significance of the cathepsin A activity is still unclear. We can speculate that in certain cells the protein exerts its activity outside the lysosomal complex. In this case, loss of catalytic activity may not result in lysosomal storage. Perhaps evidence of a functional lesion will be found when knockout mice are screened for symptoms associated with loss of cathepsin A activity on peptides (e.g., substance P, oxytocin, or endothelin I), which may be substrates of the protein in vivo.

The most striking results of this study are those obtained with BMT. The main aim for generating a mouse model for galactosialidosis was to evaluate therapeutic strategies that could be applicable to genetic correction of this and other similar disorders in humans. The possibility of manipulating autologous hematopoietic progenitor cells in vitro that will express constitutively the correcting enzyme at high levels in the repopulated recipient has important implications for somatic gene therapy of lysosomal disorders with CNS involvement. Transfer and expression of foreign genes into animal and human hematopoietic cells, as well as other cell types, has been achieved using different viral vectors (for review, see Kay and Woo 1994). In designing a therapeutic approach for our galactosialidosis mouse model, we started with the supposition that the amount of normal enzyme (supplied by BM cells) would be crucial for effective correction. Thus, we have analyzed the consequences of transplanting PPCA (−/−) mice with BM cells, derived from a transgenic line overexpressing the normal protein in a specific hematopoietic lineage. By analogy with other heterologous genes (van Assendelft et al. 1989; Needham et al. 1992), the LCR and β-globin cassette (used in this study) promote high expression of PPCA in erythroid precursor cells in a copy number-dependent, position-independent fashion. Overexpression of the transgene results in the extracellular release of PPCA precursor that is readily taken up by deficient human cells via a mannose-6-phosphate-mediated endocytosis. Hyper secretion is selective for the product of the transgene, as observed previously for other lysosomal enzymes overexpressed in various cell types (Ioannou et al. 1992; Grubb et al. 1993; Kakkis et al. 1994). BMT has proven to be very effective for improvement of the diseased phenotype. Treated PPCA (−/−) animals look completely normal and outlive the affected mice. Analysis of their tissues demonstrates that transplantation of the overexpressing bone marrow clearly is more effective than normal bone marrow for clearing storage in cells of visceral organs that undergo complete reversal of the lesions. The most plausible explanation of these data is that erythroid precursor cells secrete sufficient amounts of a stable PPCA precursor into the plasma to permit its ready uptake by cells in distant organs. Correction in the CNS, which mostly excludes plasma proteins because of the blood–brain barrier, is less evident but still noticeable, especially in the epithelial cells of the choroid plexi. This is likely attributable to internalization of the heterologous human protein from the plasma via the mannose-6-phosphate receptor (Nilsson et al. 1992) and/or the murine protein from donor-derived monocytes and perivascular macrophages. Detailed evaluation of the degree of CNS correction awaits fine mapping of the affected cells in different regions of the brain, which is currently in progress, and the analysis of a larger number of treated mice. Our findings conform in part with the results obtained with BMT in cats with the lysosomal storage disorder α-mannosidosis (Walkley et al. 1994a), another disorder of glycoprotein metabolism. These investigators report a clear improvement of the CNS lesions, but its extent is unclear, as no detailed spatial mapping of the storage neurons was presented. To improve the correction of the CNS, we currently are testing whether transgenic bone marrow with targeted expression of PPCA in the monocyte/macrophage lineage would be more effective. Transplantation of young animals with such transgenic bone marrow is expected to be more beneficial because donor-derived monocytes and macrophages can become part of the microglial population (Perry et al. 1985; Hickey and Kimura 1988).

Although galactosialidosis affects only a small proportion of the population, it has devastating consequences in children, with the early infantile form of the disease resulting in early death, multiple organ dysfunction, and mental retardation. There are, however, a limited number of patients, some of them diagnosed recently, in which the disease does not impair the CNS. Our findings indicate that somatic gene therapy approaches using autologous human BM cells, which continuously express the correcting enzyme at high levels, may be feasible in the future as a cure of galactosialidosis, especially in mildly affected children. Moreover, they set the stage for the use of this approach in other lysosomal storage diseases.

Materials and methods

Construction of the PPCA targeting vector

A mouse genomic clone encompassing the entire coding region of the *PPCA* gene was isolated from an EMBL-3 λ phage library constructed with DNA from the CCE ES cell line derived from mouse strain 129Sv. *PPCA* DNA sequences contained in a 14-kb *SalI* fragment were identified by restriction mapping and partial sequencing of exons. From this clone a 10-kb *Eco*RI–*Sal*I fragment was subcloned into pTZ18 and used to generate the targeting vector. The *hygro* cassette (van Deursen and Wieringa 1992) was inserted into exon 2 of the *PPCA* gene, 6 nucleotides downstream of the ATG start codon (see Fig. 1). In the resulting targeting vector, the *hygro* gene was flanked 5′ and 3′ by 3 and 7 kb of homologous sequences, respectively. A 2.0-kb *SalI* fragment of the herpes simplex virus thymidine kinase (HSV–TK) cassette was positioned 3′ of the construct, outside the region of homology. Both positive and negative selectable markers were inserted in the antisense orientation with respect to the transcriptional orientation of the *PPCA* gene and were driven by the TK promoter and PyF441 polyoma enhancer.

Gene targeting in ES cells

The 129/Ola-derived ES cell line E14 (a gift of Anton Berns, The Netherlands Cancer Institute, Amsterdam) was maintained in 60% Buffalo rat liver (BRL)-conditioned Dulbecco's modified Eagle medium (DMEM)/40% fresh DMEM, 15% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 2 mM glutamine, and 1000 U/ml of leukemia inhibiting factor (GIBCO). The cells were trypsinized, resuspended at a concentration of 10^7/ml in PBS, and electroporated at room temperature with 25 μg of *SalI* linearized vector DNA at 117 V and 1200 μF for 10 msec, using a Progenetor II gene pulser (Hoeffer). After electroporation, cells were kept on ice for 10 min and transferred onto 10-cm culture dishes coated with 0.1% gelatin in above medium. After recovery for 24 hr, cells were put on selection medium containing 188

μg/ml of hygromycin B (Calbiochem) and 0.2 μM FIAU (1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 iodouracil, a kind gift of Bristol Myers) for 7–10 days. Resistant colonies were expanded in 24-well plates; half of the cells in each well was cryopreserved, and the other half was expanded for genotype analysis. Positive clones were stored in liquid nitrogen and thawed at least 3 days before blastocyst injection.

Generation of mutant mice

Blastocysts were isolated at day 3.5 postcoitum by flushing the uterine horns of naturally mated C57BL/6 pregnant females with DMEM plus 10% FBS. Approximately 10–15 ES cells from each homologous recombinant clone carrying 40 chromosomes were microinjected into recipient blastocysts, and 9–14 embryos were transferred into the uterine horns of (C57BL/6×CBA/Ca) F_1 pseudopregnant fosters (Bradley 1987). Chimeric males were mated with C57BL/6 or FVB/J females, and germline transmission of the mutant allele was verified by Southern blot analysis of tail DNA from F_1 offspring with either agouti or gray coat color. F_2 offspring from interbred heterozygotes was genotyped by Southern blotting to identify homozygous null mutants. The phenotypic alterations in PPCA (−/−) mice, as presented in this paper, were uniform and showed complete penetrance, irrespective of their genetic background.

Southern and Northern Blot Analyses

Genomic DNA isolated from ES cells or mouse tails was digested with *NdeI*, resolved on a 0.8% agarose gel, and transferred onto Hybond-N+ membranes (Amersham). The 5′ junction was checked by hybridization with a 1-kb *EcoRI–NdeI* probe positioned immediately 5′ of the targeting construct, and the 3′ junction was checked with a 0.5-kb PCR probe positioned immediately 3′ of the targeting construct. Absence of additional random integrations of the targeting construct was checked with a *hygro* probe. Clones that contained the expected 5-kb *NdeI* fragment, diagnostic for homologous recombination, were obtained at a frequency of 1 in 7. For genotype analysis of tail DNA, only the 5′ probe was used. Total RNA was isolated from brain, kidney, and liver tissues of 4-to 6-week-old mice as described (Auffray and Rougeon 1980). RNA (15–20 μg) was separated on a 1% agarose gel containing 0.66 M formaldehyde. After electrophoresis, the RNA was blotted onto a Zeta-probe membrane (Bio-Rad) and hybridized with the mouse cDNA probe.

Enzyme activity assays and urine oligosaccharide determinations

For enzyme activity assays, primary cultures of skin fibroblasts and tissues from affected and normal mice were homogenized in double-distilled water. Cathepsin A activity was measured with the synthetic dipeptide substrate Z-Phe-Ala, according to the procedure described earlier (Galjart et al 1991). The activities of β-galactosidase and neuraminidase were assayed with artificial 4-methylumbelliferyl substrates (Galjaard 1980). Total protein concentration was determined using the method of Smith et al. (1985). Total sialic acid content in urine was measured in 10 μl samples after hydrolysis in 0.1 N H_2SO_4 by a modification of the method of Aminoff (1961), as described in Wenger and Williams (1991). *N*-acetylneuraminic acid was used as a standard. Creatinine was measured in a fraction of each sample by the central diagnostic laboratory of Thomas Jefferson University (Philadelphia, PA).

Histopathological analyses

Tissues isolated from affected and BM-treated mice as well as normal or heterozygous littermates were fixed with 2% glutaraldehyde in 0.2 M phosphate buffer, postfixed with 1% osmium tetroxide in 0.2 M phosphate buffer, dehydrated in graded ethanol (70% through absolute), en bloc stained with 4% uranyl acetate in absolute ethanol, and embedded in Spurr resin (Ladd Research Industries, Inc., Burlington, VT). Semithin sections were cut at 470 nm and stained with toluidine blue for light microscopy. Sections for electron microscopy were cut at 85 nm and stained with 2% uranyl acetate/lead citrate and screened with JEOL 1200 EX II (JEOL USA Inc., Peabody, MA). For preparation of paraffin sections, dissected tissues were fixed overnight in 10% formalin. After dehydration and paraffin embedding, 6-μm sections were made, mounted on slides, and stained with haematoxylin/eosin. For the analysis of the brain, affected and BM-treated mice were perfused through the left cardiac ventricle with 4% paraformaldehyde in PBS. The cerebrum was sectioned coronally at the level of the optic chiasm. The anterior half of the cerebrum, cerebellum, and brainstem were processed for paraffin embedding, sectioned 6 μm thick, and stained solochrome/eosin and luxol fast blue (LFB)/ PAS. The posterior half was cryosectioned serially at 10 μm thick and stained with PAS. The trigeminal ganglia and pituitary gland were postfixed with osmium and processed for epoxy–resin embedding as described above.

Uptake in galactosialidosis fibroblasts

BM cells from three normal and three transgenic mice were harvested by flushing the femurs with DMEM and 15% FBS. Cells were washed once with the same medium and seeded at a concentration of 1.5×10^6/ml in three 30 mm dishes. Then they were cultured for an additional 5 days in the above medium supplemented with 10 U/ml of erythropoietin (Amgen, Inc). The conditioned medium from each culture then was pooled and added to confluent galactosialidosis fibroblasts from an early infantile patient, seeded 5 days in advance in six-well plates. In parallel, serum samples from the same mice were also pooled, concentrated, and added to the deficient cells. Uptake was carried out for 4 days. Treated fibroblasts then were harvested by trypsinization and homogenized for enzymatic assays as above.

BM transplantation

The recipient galactosialidosis mice at 2.5–5 months of age were lethally irradiated with 9.25 Gy from a cesium irradiator, 24 hr before transplant. Normal BM was derived from two C57BL/6 Thy1.1 mice. Transgenic BM was derived from two mice of a (C57Bl/6×CBA) F_1 transgenic line in which the human protective protein precursor is overexpressed in the erythroid lineage under the control of the β-globin promoter and LCR. BM cells were harvested by flushing the femurs with Hanks medium with 5% FBS. Prior to BMT, donor cells were incubated with HO-22-1 and AT 83 antibodies for 15 min to deplete the T-cell population. A cell suspension (0.5 ml) containing 2×10^7 cells/ml was injected intravenously into the tail vein. Starting at 3 months post-transplantation, tissues from both normal and transgenic BM-treated mice were isolated and prepared for light microscopy, as described above.

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Chapter 5

Lack of PPCA expression only partially coincides with lysosomal storage in galactosialidosis mice: Indirect evidence for spatial requirement of the catalytic rather than the protective function of PPCA

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Lack of PPCA expression only partially coincides with lysosomal storage in galactosialidosis mice: indirect evidence for spatial requirement of the catalytic rather than the protective function of PPCA

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Protective protein/cathepsin A (PPCA) is a pleiotropic lysosomal enzyme that complexes with β-galactosidase and neuraminidase, and possesses serine carboxypeptidase activity. Its deficiency in man results in the neurodegenerative lysosomal storage disorder galactosialidosis (GS). The mouse model of this disease resembles the human early onset phenotype and results in severe nephropathy and ataxia. To understand better the pathophysiology of the disease, we compared the occurrence of lysosomal PPCA mRNA and protein in normal adult mouse tissues with the incidence of lysosomal storage in PPCA(-/-) mice. PPCA expression was markedly variable among different tissues. Most sites that produced both mRNA and protein at high levels in normal mice showed extensive and overt storage in the knockout mice. However, this correlation was not consistent as some cells that normally expressed high levels of PPCA were unaffected in their storage capability in the PPCA(-/-) mice. In addition, some normally low expressing cells accumulated large amounts of undegraded products in the GS mouse. This apparent discrepancy may reflect a requirement for the catalytic rather than the protective function of PPCA and/or the presence of cell-specific substrates in certain cell types. A detailed map showing the cellular distribution of PPCA in nomal mouse tissues as well as the sites of lysosomal storage in deficient mice is critical for accurate assessment of the effects of therapeutic interventions.

INTRODUCTION

Lysosomal protective protein/cathepsin A (PPCA) is a serine carboxypeptidase that forms a high molecular weight multienzyme complex with acid β-D-galactosidase and *N*-acetyl-α-neuraminidase (1–6). PPCA's role in this complex is to facilitate the intracellular routing, lysosomal localization and activation of neuraminidase (7,8), and to stabilize β-galactosidase in the lysosomal environment (5,9). Genetic lesions in the PPCA gene that abolish its protective function cause severe loss of neuraminidase activity, and render β-galactosidase susceptible to rapid intralysosomal proteolysis (10,11). The resulting combined enzyme deficiency is the basis of the lysosomal storage disorder galactosialidosis (GS), which is characterized by the primary accumulation of sialylated oligosaccharides and glycopeptides in patient tissues and body fluids (12–15). Patients with GS present clinically with one of three forms of the disease (early infantile, late infantile or juvenile/adult), depending on their age at onset and the severity of their symptoms (16). The early onset patients, who completely lack PPCA activity, die within the first months of life from heart and kidney failure. The mouse model of GS closely resembles the phenotype of early infantile patients (17). Extensive vacuolation of certain cells in most organs is caused by the abnormal accumulation of undigested metabolites that result primarily from the severe secondary deficiency of lysosomal neuraminidase.

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As a member of the serine protease family, PPCA exerts cathepsin A activity at acidic pH and esterase/C-terminal deamidase activity at neutral pH. The catalytic activity of PPCA, which is fully separable from its protective function (18), is apparently important for the initial catalysis of selected bioactive peptides, including substance P, oxytocin and endothelin I (19–22). However, the physiological significance of this enzyme activity has not yet been proven. Recently, we identified the minimal promoter regions of the human and mouse PPCA genes (23). Transcription of the murine gene, unlike that of the human gene, gives rise to two differentially expressed transcripts that contain the same protein-encoding region. The more abundant 1.8 kb species is expressed in all tissues and originates from a promoter with features characteristic of housekeeping gene promoters (23). A minor murine-specific 2.0 kb mRNA is transcribed from a TATA box-containing promoter and is expressed at lower levels in specific tissues.

Here, we studied the *in vivo* expression of PPCA in adult murine tissues by using *in situ* hybridization and immunocytochemistry. Our aim was to correlate the distribution pattern of PPCA mRNA and protein in normal mice with the disease pathology in the knockout mouse. Our findings demonstrate that PPCA expression is not ubiquitous in normal adult tissues, but rather is confined preferentially to specific cells, especially those that are metabolically active. Most regions that express PPCA at high levels develop overt morphologic changes in the knockout mouse. Interestingly, however, there are also cell types where the correlation between gene expression and the occurrence of lysosomal storage is not maintained. We discuss how this result could suggest a role for the catalytic rather than the protective function of PPCA.

RESULTS

The distribution of PPCA mRNA and protein in the tissues of normal adult mice was analyzed using *in situ* hybridization and immunocytochemistry, respectively. The presence of lysosomal storage material in defined cell populations of GS mice was evidenced by periodic acid Schiff (PAS) staining and lysosomal vacuolation.

Brain

Despite a generalized expression of PPCA in the brain, regional and cellular differences were obvious. The frontal, parietal and occipital lobes of the cortex displayed a moderate signal throughout the inner five layers, whereas the external layer, which contains unmyelinated axons, was devoid of PPCA-specific signal (Fig. 1A). The inferior colliculus and cells within the meningeal layers also contained PPCA transcripts and protein. The expression in the cortex and the diencephalon was confined primarily to neuronal cell bodies rather than to nerve fibers. Sustained expression of PPCA mRNA was detected throughout the olfactory bulb, being most prominent at the level of the mitral cell layer (Fig. 1B). This was paralleled by strong punctated PPCA staining in the large mitral cells (Fig. 1C). Weak expression was present in the granular cells and the periglomerular neurons. The axons of the mitral cells are in contact with the olfactory cortex, which consists of five defined regions: the anterior olfactory nuclei, the tuberculum olfactorium, the pyriform cortical neurons, the amygdala and the entorhinal cortex. All of these areas, which belong to the limbic system, and in particular the tuberculum olfactorium and the pyriform nuclear cells, expressed PPCA mRNA and protein at high levels (not shown). The pyramidal neurons of the hippocampus and the granular cells of the dentate gyrus were also highly positive for both mRNA and protein (Fig. 1E and F). Only basal expression levels were detected in the caudate putamen, the thalamic and hypothalamic regions, and the substantia nigra (not shown). In the cerebellum, a strong positive signal was confined to the Purkinje cells, whereas the molecular and granular layers had significantly lower expression (Fig. 1H and I). The large neurons of the cerebellar nucleus also stained strongly. Like the cerebrum and the cerebellum, the brain stem expressed PPCA primarily in gray matter nuclei, containing the cell bodies of the different neurons. Both PPCA transcripts and protein were very prominent in the epithelial cells of the choroid plexus, whereas expression in the ependymal cells that line the cerebral ventricles was low (Fig. 1K and L). Strong expression was detected in the perivascular and leptomeningeal macrophages.

In general, PPCA expression in the brain correlated well with the distribution of vacuolated cells with lysosomal storage in the PPCA(–/–) mouse. PAS-positive cells appeared early in life. At 2 months of age, they were detected only in the anterior olfactory nucleus, the amygdala and the entorhinal cortex (not shown). Occasional Purkinje cells were also positive. As the disease progressed, the number of accumulating cells became more conspicuous in these regions; other neurons also appeared positive, including mitral cells (Fig. 1D), neurons of the 3rd–4th cerebral cortical layer, scattered cells of the thalamus and hypothalamus, and the large neurons of the cerebellar nucleus and brain stem (not shown). Interestingly, PAS-positive staining differed in different vacuolated cell types, probably reflecting variation in the composition of storage products. The choroid plexus, for example, showed clear morphological changes without evident PAS staining (Fig. 1M). Contrary to what might be expected from the high PPCA expression in the pyramidal neurons of the hippocampus and the granular cells of the dentate gyrus, very few PAS-positive or vacuolated cells were detected in these areas (Fig. 1G). This apparent inconsistency could be attributed to differences in the metabolic state of these cells or to the type of macromolecular substrates. Furthermore, there appeared to be a variable effect of the storage products on cell viability. Purkinje cells display few large PAS-positive granules prior to cell death (Fig. 1J), whereas cerebellar basket cells, and cells of the amygdala and anterior olfactory nucleus, become filled with PAS-positive material prior to obvious signs of cellular deterioration (not shown). It should also be noted that the undegraded products accumulate first in the Purkinje cells of the more anterior lobules which also die earliest. As the disease progresses, however, Purkinje cells in more posterior regions store and die. Loss of these cells contributes to the progressive ataxia seen in these mice (17). Interestingly, a similar feature was observed in other mouse models of lysosomal storage disorders such as Niemann–Pick A and C (24–27).

Visceral organs

PPCA mRNA expression was distributed throughout the liver (Fig. 2A). Immunocytochemistry clearly demonstrated that the protein expression was moderate in hepatocytes and high in Kupffer cells (Fig. 2B), reflecting the storage pattern detected in

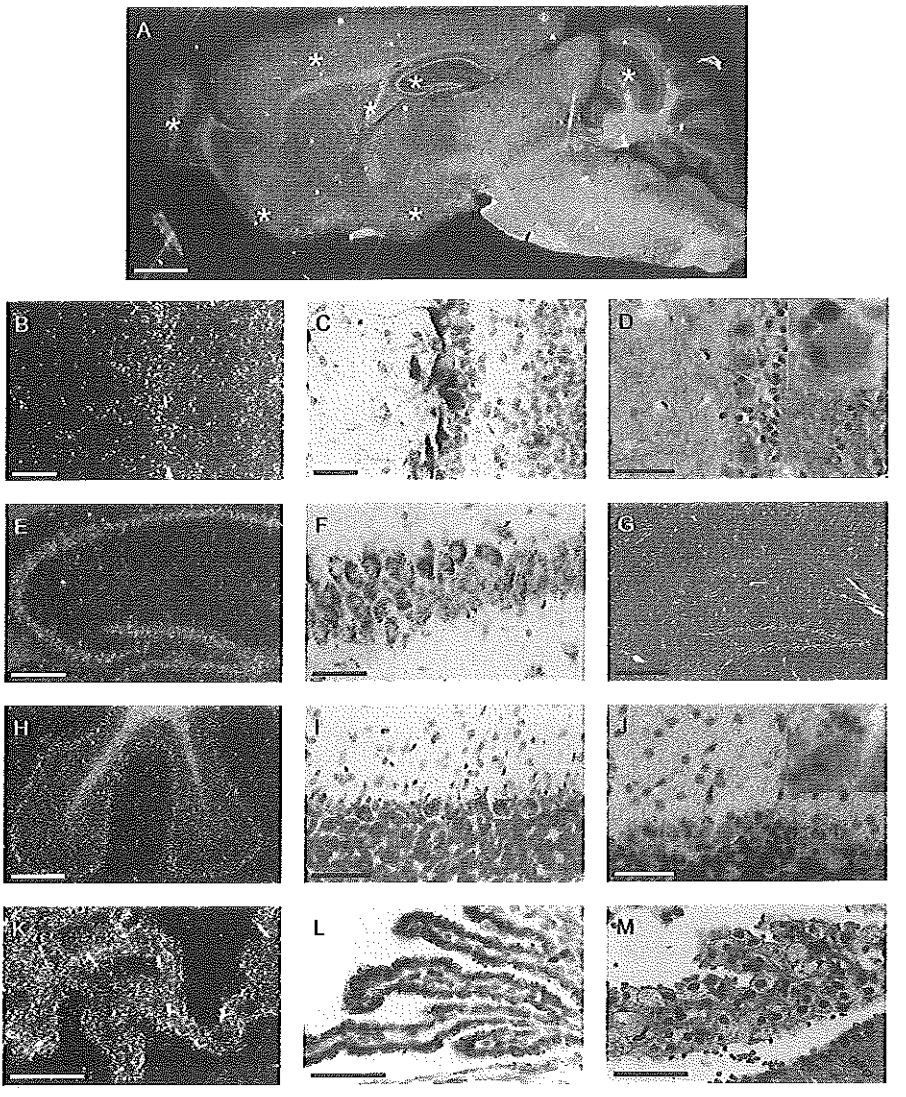

Figure 1. Comparison of PPCA mRNA and protein expression in wild-type mouse brain with lysosomal storage in PPCA(–/–) mouse brain. Sagittal brain sections demonstrate the localization of mouse PPCA mRNA expression by *in situ* RNA hybridization (A, B, E, H and K) and protein distribution by immunocytochemistry with PPCA antibodies (C, F, I and L) in relation to the accumulation of undegraded lysosomal products in the PPCA(–/–) mouse (D, G, J and M). (**A**) An overview of a dark-field image of a mouse brain; the asterisks mark the regions of high PPCA mRNA expression. Representative areas show PPCA expression and accumulated storage in the mitral cell layer of the olfactory bulb (**B–D**), the hippocampus and dentate gyrus (**E–G**), the Purkinje cell layer of the cerebellum (**H–J**) and the choroid plexus (**K–M**). Note the PAS-positive storage granules in the mitral (D) and Purkinje cells (J), the lack of storage in the hippocampus and dentate gyrus (G), and the PAS-negative storage vacuoles in the choroid plexus (M). Mice of age 2–6 months were used for *in situ* RNA hybridization and immunocytochemistry, and mice of 10–13 months were used for PAS staining although storage was visible in some cells earlier. All *in situ* RNA hybridization and PAS staining were performed on frozen sections, and immunocytochemistry was performed on paraffin sections. Scale bar = 1 mm (A), 250 μm (E, G and H), 50 μm (B–D, F and I–M).

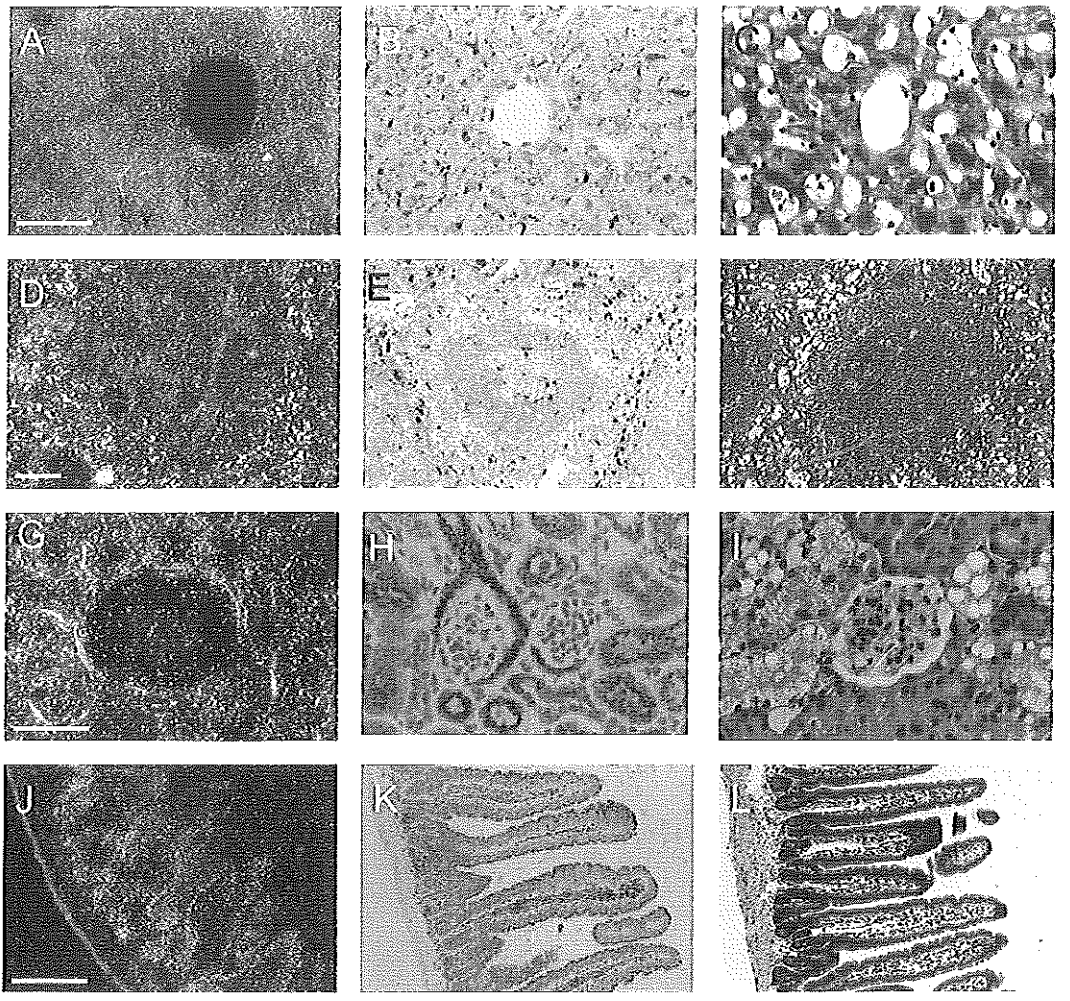

Figure 2. Comparison of PPCA mRNA and protein expression in wild-type mouse tissues with lysosomal storage in PPCA(–/–) mouse tissues. PPCA mRNA expression was analyzed in wild-type mice (2–4 months) by *in situ* RNA hybridization (A, D, G and J), the distribution of protein was detected by immunocytochemistry with PPCA antibodies (B, E, H and K) and lysosomal storage products were visualized in PPCA(–/–) mice (2–4 months) by PAS staining (C, F, I and L). Liver (**A–C**), spleen (**D–F**), kidney (**G–I**) and small intestine (**J–L**) are shown. Note that most visceral organ storage products are PAS-negative and appear as vacuoles. Scale bar = 50 μm (A–I), 150 μm (J–L).

the knockout mouse, where Kupffer cells are more affected than hepatocytes (Fig. 2C). The red pulp of the spleen showed higher protein and mRNA expression than the white pulp, probably because of the numerous macrophages that express high levels of PPCA (Fig. 2D and E). Consistent with this, the pathology of the spleen in the (–/–) mouse showed macrophages filled with vacuoles, whereas lymphocytes and megakaryocytes displayed little or no signs of storage. As a result of accumulating products in the liver and spleen, the affected mice develop hepatosplenomegaly, a clinical feature associated with the early and sometimes late infantile forms of the human disease (16).

In the kidney of wild-type mice, PPCA expression was high in the cuboidal epithelium of the proximal convoluted tubules and somewhat lower in the distal convoluted tubules of the nephron (Fig. 2G and H). The epithelial cells of Bowman's capsule were highly positive, whereas the glomerulus expressed very low levels that could be seen only weakly by immunocytochemistry (Fig. 2H). No expression was detected in the renal medulla. The epithelia of the proximal convoluted tubules and the Bowman's capsule were the first sites to store overtly in PPCA(–/–) mice (Fig. 2I). Interestingly, although the expression was very low in the glomerulus of the wild-type mouse, this region of the deficient

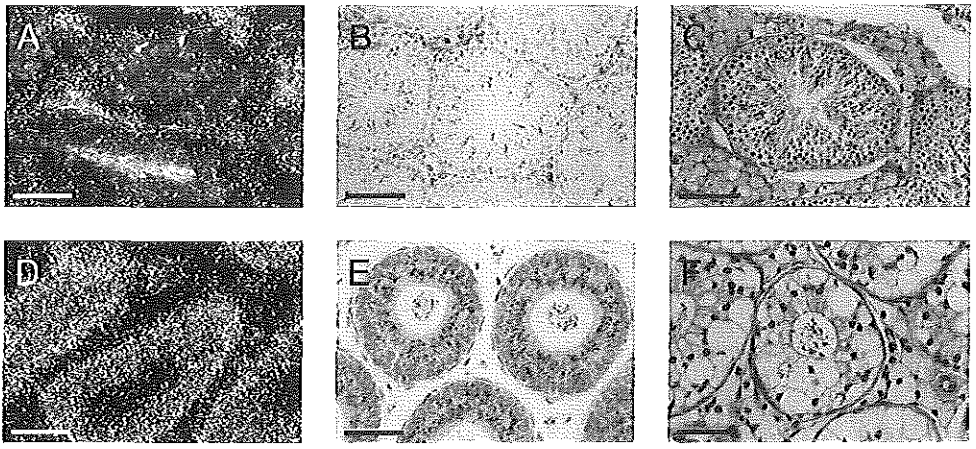

Figure 3. Comparison of PPCA mRNA and protein expression in wild-type mouse with lysosomal storage in PPCA(–/–) mouse male reproductive organs. PPCA mRNA expression was analyzed in wild-type mice by *in situ* RNA hybridization (A and D), the distribution of the protein was detected by immunocytochemistry with PPCA antibodies (B and E) and lysosomal storage products were visualized in the PPCA(–/–) mouse by PAS staining (C and F). Shown are the seminiferous tubules and interstitial tissue of the testis (A–C) and the caput of the epididymis (D–F). Mice ranged in age from 2 to 4 months. Scale bar = 90 μm (A–C), 35 μm (D–F).

mouse displayed high levels of storage (17). This severe renal pathology in knockout mice resulted in diffuse edema and closely paralleled the nephrotic syndrome seen in GS patients (16).

In the duodenum and jejunum of the small intestine, the crypts of Lieberkuhn expressed high levels of PPCA mRNA, but little protein, whereas the mucosal layer of the microvilli expressed moderately (Fig. 2J and K). In (–/–) mice, storage products or vacuolation were not detected in these cells (Fig. 2L). However, foamy macrophages were seen in the interstitial space of the mucosa, submucosa, muscular outer layer and the lymphoid Peyer's patches (not shown).

Heart and skeletal muscle appeared to have an overall, low PPCA expression. In muscle fibers, the knockout mouse displayed vacuolation only within endothelial cells and macrophages, but showed no signs of storage. The adrenal gland showed high expression in a specific rim of cells, probably the X zone, which is loosely defined between the cortical and the medullar cell layers (not shown). Cells in this layer secrete glucocorticoids and androgens, and are only present in sexually immature male and virgin female mice (28). PPCA(–/–) mice did not show detectable levels of storage products in the adrenal gland.

Reproductive system: testis and epididymis

Within the seminiferous tubules of the testis, PPCA expression was observed in the Sertoli cells, which provide nutrients to the developing spermatocytes. No expression was noted in the spermatogonia or spermatocytes (Fig. 3A and B). Moderate mRNA and protein levels were also detected in the interstitial cells of Leydig, which surround the seminiferous tubules and secrete testosterone under the influence of luteinizing hormone. Interestingly, the PPCA signal varied along the length of the epididymis. Most proximal to the testis, in the caput epididymis, PPCA levels were very high in the columnar epithelial cells of the ducts with little or no apparent expression in the supportive connective tissue (Fig. 3D and E). Further along through the corpus of the epididymis, the number of expressing cells decreased to the cauda, where only a few expressed high levels of the protein (data not shown).

In the (–/–) mice, extensive vacuolation occurred in all high expressing cells and, in addition, in the interstitial cells (Fig. 3C and F). Marked vacuolation of the epididymis and Leydig cells was already visible at 2 months of age, and these cells remained viable, but markedly affected, for the life of the animals.

Ovary and uterus

The zona pellucida and the granulosa cells, that surround the developing follicle and serve as support and/or feeder cells for the follicle, expressed high levels of PPCA transcripts (data not shown). The corpus luteum, which originates from the differentiated granulosa cells after follicle maturation and secretes estrogen and progesterone, displayed even higher levels of mRNA and protein, whereas expression was very low in the interstitial cells (not shown). High expression was also apparent in the columnar epithelial cells and branched tubular glands of the uterus and, during pregnancy, within the rapidly dividing endometrium/trophoblasts. In the affected mouse, foamy macrophages infiltrated the stroma of the ovary and uterus, but the secretory epithelial cells appeared normal (data not shown).

DISCUSSION

It is becoming increasingly clear that the notion of lysosomal genes as simple housekeeping genes (i.e. requiring little or no regulation) may be inaccurate. These enzymes are expressed at basal levels in virtually all cells, but are expressed differentially in specific cell types. For instance, murine prosaposin mRNA is seen in Leydig, Sertoli and peritubular cells but not in the

spermatogenic cells of the testis (29,30). In contrast, acid phosphatase is detected only in the spermatocytes of this organ (31). Acid lipase is expressed basally throughout the testis (32), while acid α-glucosidase is seen in the seminiferous tubules but not in the spermatogonia (33). In this study, we have identified another pattern of expression in the testis, that of PPCA being evident in Leydig and Sertoli cells but not in spermatogenic cells. Similar differential expression for PPCA mRNA and protein exists for virtually all other tissues of the body, being highest in metabolically active or phagocytic cells. This is particularly evident in specific subpopulations of neurons.

The distribution pattern of PPCA and other lysosomal enzymes necessitates some kind of regulation that cannot be anticipated by the housekeeping characteristics of their promoters. Regulation of each of these genes could occur at the transcriptional and/or post-transcriptional levels. It has been reported that human aspartylglucosaminidase is regulated predominantly at the translational level (34). Moreover, the activity of lysosomal enzymes that rely on other proteins or cofactors/activators for function may be modulated in a more complex fashion. For example, some lysosomal enzymes require other lysosomal proteins for intracellular transport and stability (neuraminidase/PPCA), activity (sphingolipid degradative enzymes/saposins, β-hexosaminidase/G_{M2}-activator) or substrate specificity (homo- and heterodimers of β-hexosaminidase) (8,29,30,35–37). Despite differences in the expression patterns of many lysosomal proteins, it is noteworthy that their interacting and cooperating proteins are usually co-expressed in specific cells. Little is known about the physiological and pathological stimuli or molecular mechanisms that affect the activity of lysosomal enzymes; however, it is clear that steroid hormones, such as androgens and estrogens, have a general inductive effect on the activity levels of many lysosomal enzymes (38–40).

Comparing PPCA levels in normal mouse tissues with the distribution of storage in PPCA knockout mice revealed that, in most cell types, a higher level of expression correlated with more extensive storage. This was particularly evident in the brain where specific neurons, such as the Purkinje and mitral cells, and the cells of the amygdala and entorhinal cortex displayed the most extensive storage. In some neuronal cells such as those of the hippocampus and dentate gyrus, however, this pattern was not maintained. Also, the glomerulus of the kidney expressed virtually no PPCA and yet that of the PPCA(–/–) mouse stored extensively. This apparent discrepancy between endogenous expression levels and the degree of storage in diseased mice could be due to a difference in the spectrum and amounts of substrates, derived during normal cellular processes. Alternatively, PPCA may be expressed in selected cells primarily for its enzymatic function on bioactive peptides, such as oxytocin, substance P or endothelin I (19–21), rather than for its ability to protect neuraminidase and β-galactosidase. In this latter situation, undegraded products would not be expected to accumulate in the knockout model. It is worth noting that the substance P receptor (NK-1 receptor) is well expressed in the hippocampus (41,42); however, it remains to be seen whether the primary requirement for PPCA in these cells is for its role in neuropeptide inactivation.

In PPCA(–/–) mice, we observed differences in the storage within different cell types. Purkinje cells accumulated only a few large PAS-positive storage granules prior to cell death, whereas cerebellar basket cells, and the cells of the amygdala and anterior olfactory nucleus, were almost completely full of fine storage granules before they deteriorated. Epithelial cells of the choroid plexus, renal proximal convoluted tubule and caput epididymis accumulated PAS-negative material and appeared to survive for the entire lifespan of the animal. From these results, it seems clear that some stored intermediates have more toxic effects on cell viability than do others, or that certain cells may be more vulnerable to toxicity. In this regard, both G_{M2}-ganglioside and one of its downstream degradation products, ceramide, have been implicated in the induction of apoptosis (43–47).

The lifespan of cells may also play a role in the correlation between PPCA expression and storage. In the small intestine, we found PPCA mRNA transcripts in the crypts of Lieberkuhn and expression of the protein only in the mucosal epithelium of the microvilli. This is consistent with the newly dividing mucosal cells, differentiating in the crypts to express PPCA transcripts and then translating these transcripts into protein as they move toward the tips of the microvilli. As might be expected, in PPCA(–/–) mice, storage products were never apparent in these cells, probably because their lifespan is too short to allow for accumulation at detectable levels.

The reproductive organs also differentially express PPCA, which is not surprising as lysosomal enzymes have been implicated in reproduction for many years (38,48–51). Both male and female mice express PPCA differentially at specific sites. In the knockout mice, the lack of PPCA considerably disturbs the reproductive capacity of the mice. Although fertile, they mate poorly, and the frequency of pregnancies is less than that for wild-type mice. Litter sizes and delivery, however, appear normal. We do not know at the moment whether this reduced number of pregnancies, which worsens with age, is a result of decreased fertility or whether it is secondary to pathological effects on other organs, including the brain, which may affect performance. It is noteworthy that one of the peptide hormones known to be inactivated by PPCA is oxytocin (19). The hormone is found in the corpus luteum, which also expresses PPCA at high levels, and stimulates growth during early blastocyst development (52). Oxytocin and its receptor are also important for parturition and lactation in mammals (53–60). It is unclear whether the absence of PPCA in the knockout mouse model hampers processing or clearance of oxytocin in the uterus, which could have detrimental effects on the continuing pregnancy. Curiously, it has been reported that mothers of human GS patients, who have 50% PPCA activity, often suffer from spontaneous abortions (61).

This detailed study highlights some important factors regarding the expression of lysosomal enzymes and the consequences of their functional loss. It is clear that there are marked differences in the expression levels of lysosomal enzymes in different cell types. Questions remain as to the specific cellular distribution of enzyme substrates and the differential toxicity of accumulated substrates in different cell types throughout the body. A detailed map of expression and storage will be extremely valuable in the accurate assessment of correction following therapeutic regimes.

MATERIALS AND METHODS

Materials

Chemicals were obtained from standard commercial suppliers. [α-^{35}S]UTP (>1000 Ci/mmol; Amersham), was used to prepare

riboprobes for *in vitro* transcription assays, which included either T7 or T3 RNA polymerase (Promega Biotec).

In situ RNA hybridization

FVB mice (8–12 weeks old) were anesthesized sublethally with avertin and subsequently perfused with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Tissues were isolated and post-fixed for 2–4 h, before being processed for cryostat sectioning. Sections (10–14 μm) were placed on Superfrost/Plus (Fisher Scientific) glass slides and hybridized for 16–20 h at 50°C with a 272 bp murine PPCA ^{35}S-labeled riboprobe (position 904–1176 bp of the mouse PPCA cDNA), as previously described (23,62). This probe detects both the predominant ubiquitous 1.8 kb and the minor 2.0 kb PPCA mRNA. The washed slides were dipped in photographic emulsion (Kodak NTB-2) and developed after 5–8 days with Kodak D-19 developer and fixer. The slides were then counterstained with 0.1% toluidine blue and mounted.

Immunocytochemical and histological staining

Mice were perfused via the left cardiac ventricle at a rate of 3.5 ml/min for 2 min with phosophate-buffered saline (PBS) to remove circulating blood cells. They were perfused further for 15 min with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. Tissues were removed and fixed for a further 4 h in the same fixative before being paraffin embedded. Tissue sections (10 μm for brain; 4 μm for all other tissues) were deparaffinized and rehydrated and the antigen was retrieved by microwave boiling in 0.1 M citrate, pH 6.0, and cooling for 30 min. The sections were then blocked in PBS (containing 0.05% Tween-20 and 0.1% bovine serum albumin) and 10% normal goat serum for 30 min before being incubated overnight at room temperature with anti-mouse PPCA antibodies. The latter were raised in rabbit against the native mouse PPCA precursor, overproduced in insect cells infected with a PPCA recombinant baculovirus construct. The polyclonal antibody preparation was shown previously to be monospecific for the mouse 54 kDa precursor and mature subunits (63). The Vector ABC–HRP system was used for detection. After a secondary antibody incubation of 2 h, endogenous peroxidase activity was removed by adding PBS containing 0.3% hydrogen peroxide and 0.02% sodium azide for 30 min. The ABC–HRP complex was then added for 1 h and visualized by adding a VIP substrate (Vector) for 3–8 min. Nuclei were counterstained with methyl green. For histological staining with hematoxylin/eosin, tissues were paraffin embedded (see above), and, for PAS staining, tissues were processed for frozen sectioning (17).

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Chapter 6

A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse

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A point mutation in the *neu-1* locus causes the neuraminidase defect in the SM/J mouse

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Lysosomal neuraminidase (sialidase) occurs in a high molecular weight complex with the glycosidase β-galactosidase and the serine carboxypeptidase protective protein/cathepsin A (PPCA). Association of the enzyme with PPCA is crucial for its correct targeting and lysosomal activation. In man two genetically distinct storage disorders are associated with either a primary or a secondary deficiency of lysosomal neuraminidase: sialidosis and galactosialidosis. In the mouse the naturally occurring inbred strain SM/J presents with a number of phenotypic abnormalities that have been attributed to reduced neuraminidase activity. SM/J mice were originally characterized by their altered sialylation of several lysosomal glycoproteins. This defect was linked to a single gene, *neu-1*, on chromosome 17, which was mapped by linkage analysis to the H-2 locus. In addition, these mice have an altered immune response that has also been coupled to a deficiency of the Neu-1 neuraminidase. Here we report the identification in SM/J mice of a single amino acid substitution (L209I) in the Neu-1 protein which is responsible for the partial deficiency of lysosomal neuraminidase. We propose that the reduced activity is caused by the enzyme's altered affinity for its substrate, rather than a change in substrate specificity or turnover rate. The mutant enzyme is correctly compartmentalized in lysosomes and maintains the ability to associate with its activating protein, PPCA. We propose that it is this mutation that is responsible for the SM/J phenotype.

INTRODUCTION

Neuraminidases (sialidases) constitute a large and important family of hydrolytic enzymes that cleave the terminal sialic acid residues from a variety of sialoglycoconjugates [for a review see (10)]. This event influences many cellular processes, including cell–cell interaction/adhesion, protection from pathogens and antigen recognition (10–14). Some family members share certain characteristic features, including the F(Y)RIP domain in the N-terminal region of the protein, where the arginine is part of the active site, and two to five evenly spaced Asp boxes (S/T-X-D-X-G-X-T-W/F), which are located C-terminal of the F(Y)RIP sequence (15,16). The three-dimensional structure of bacterial and viral sialidases has shown that these enzymes have a common catalytic core of ~40 kDa with a characteristic six-bladed β-propeller fold (17,18). Human lysosomal *N*-acetyl-α-neuraminidase is deficient or defective in two distinct metabolic storage disorders: sialidosis, which is caused by structural lesions in the neuraminidase gene; and galactosialidosis, in which neuraminidase deficiency is secondary to a primary defect in the serine carboxypeptidase protective protein/cathepsin A (PPCA) (1,4). Recently we and others cloned the human neuraminidase cDNA and identified a number of independent mutations in the neuraminidase gene that we associated with different clinical variants of sialidosis (3,19,20). The neuraminidase locus maps to the HLA region on chromosome 6p21 (3,21,22).

In the mouse a partial deficiency of a neuraminidase was first identified in the naturally occurring strain SM/J (23). These inbred mice had already been selected in the early 1940s for their relative small body size following cross matings with seven different inbred strains. Later, biochemical analysis demonstrated abnormal sialylation of at least four lysosomal glycoproteins that showed an altered migration pattern on starch gel electrophoresis. This defect was corrected by treatment with bacterial sialidase [reviewed in (5)]. This hypersialylation was attributed to a reduction in activity of a liver-specific sialidase (5,24,25), although some reports suggested that other organs were also affected (23,26). The responsible gene was designated *neu-1* and mapped, by linkage analysis, to the histocompatibility locus on chromosome 17, in the region between H-2D and H-2E_α (6,7), which is syntenic to the human HLA locus on chromosome 6p21. Besides the abnormal sialylation of lysosomal glycoproteins, SM/J mice also have an impaired immune response, which is thought to result from the altered processing of sialic acids present on cell surface molecules of subpopulations of T cells (8,9,27–31). An important step in the development of an immune response is differentiation of activated naive T cells into either IFN-γ-producing (T_{h1}) or IL-4-producing (T_{h2}) cells [for a review see (32)]. Although SM/J mice can stimulate a T_{h1}-mediated immune response, they cannot stimulate the conversion of naive T cells into IL-4-producing T_{h2} lymphocytes. This altered response has been attributed to reduced activity of Neu-1 neuraminidase, which is thought to result in: (i) improper desialylation of surface antigens on T_{h2}-committed cells; (ii) reduction in early IL-4 production; and (iii) absence of IgG1 and IgE production by B cells after *in vivo* immunization of SM/J mice with pertussis toxin (31). T cell Neu-1 neuraminidase has

also been implicated in conversion of vitamin D_3 binding protein into macrophage activating factor (27). Together these data suggest an important role for Neu-1 neuraminidase in processing of selected sialoglycoconjugates at either the plasma membrane or within intracellular compartments.

In this paper we describe identification of a single amino acid substitution, L209I, in the *neu 1*-encoded lysosomal neuraminidase of SM/J mice. Analysis of the biochemical properties of this mutant enzyme demonstrates that its reduced neuraminidase activity is indeed caused by the presence of this mutation and not by improper compartmentalization of the protein, altered turnover or a lack of association with PPCA.

RESULTS

Isolation of the murine neuraminidase cDNA and expression pattern of *neu-1* in mouse tissues

Two murine neuraminidase cDNAs (1.8 and 2.4 kb) were isolated using the human cDNA as probe. Both contained the same open reading frame, but the 2.4 kb clone lacked the first two codons and had an extended 3′-untranslated region (UTR). The deduced amino acid sequence of the mouse protein is 91% similar to its human counterpart: the N-terminus begins with a conventional 39 amino acid signal sequence (33) and includes a FRIP domain as well as three conserved and two degenerated Asp boxes. The protein has four potential N-linked glycosylation sites; the first three are found at identical positions in the human neuraminidase, whereas the fourth, which is close to the C-terminus, is only present in the mouse sequence (Fig. 1). Northern blot analysis of multiple tissues, using probes spanning the cDNA (Fig. 1) demonstrated two major and two minor transcripts, which vary in length from 1.8 to 4.0 kb (Fig. 1). The 3′-UTR probe, unique for the 2.4 kb cDNA, recognized only the 2.4 and 4.0 kb transcripts, indicating that the four mRNAs use alternative 3′-UTRs. The hybridization results suggest that all four transcripts contain the same protein encoding sequence. The 1.8 and 2.4 kb mRNAs were the most abundant and displayed a differential pattern of expression which closely correlated with expression of PPCA, which forms a complex with the neuraminidase protein (34,35). The murine gene coding for the isolated cDNAs contains six coding exons (Table 1). The gene spanned a small region of 4 kb and was mapped, using the 1.8 kb cDNA insert as probe, to the H-2 region of chromosome 17 (data not shown).

Phenotypic characterization of SM/J mice and identification of the mutation in the *neu-1* gene

All four neuraminidase transcripts displayed similar patterns of expression in kidney, brain, liver and spleen RNA preparations from SM/J mice (Fig. 1). In addition, a single polypeptide was immunoprecipitated with anti-human neuraminidase antibodies (anti-neur) from radiolabeled lysates of SM/J fibroblasts; this immunoprecipitated protein was comparable in size with the normal murine protein (data not shown). Although we found no overt changes at the RNA or protein level, we did find that neuraminidase activity of SM/J Neu-1 differed from that of wild-type Neu-1. Using sodium 4-methyl-umbelliferyl-α-D-*N*-acetylneuraminate (4-MU-NANA) as substrate, SM/J Neu-1 activity was significantly reduced in lysosomal/mitochondrial extracts derived from several SM/J tissues and this partial deficiency was clearly not restricted to any one tissue (Fig. 2). SM/J neuraminidase activity in kidney and liver extracts was also lower than that of control values when assayed with either α-2,3- and α-2,6-sialyllactose (α-2,3- and α-2,6-NANA-lactose) as substrate, thus demonstrating that the defective enzyme did not show altered specificity for either of the two linkages (Fig. 2). However, using fibroblast extracts we could demonstrate that SM/J neuraminidase assayed with substrate concentrations ranging from 0.1 to 1.5 mM 4-MU-NANA had an ~3-fold lower V_{max} than the wild-type enzyme (Fig. 2). This suggests that the L209I substitution influences the kinetic properties of the mutant enzyme. Furthermore, the mutant mice displayed an abnormal pattern of urinary oligosaccharides (Fig. 2), indicative of oligosaccharidiuria, a condition commonly observed in galactosialidosis mice (2). Histological analysis of the SM/J mice showed evidence of storage products in specific cells, such as the Purkinje cells of the cerebellum and the glomerular epithelium, which appeared to accumulate over time (data not shown). Because these parameters are commonly used in biochemical diagnosis of sialidosis and galactosialidosis patients (1,4), it is clear that SM/J mice share similar phenotypic abnormalities with these two human diseases.

To identify the underlying genetic lesion responsible for these abnormalities we searched for a mutation(s) in the neuraminidase gene. Using RT-PCR on brain and liver RNA derived from SM/J mice of different ages and from different litters we amplified four overlapping fragments that span the entire neuraminidase cDNA (Fig. 3). Three mouse strains, BALB/c, 129/Sv and FVB, were used as controls. Sequence comparison identified seven nucleotide changes in the SM/J cDNA; four involved the wobble base of amino acid codons Lys93, Arg202, Thr295 and Ala316, two were present in the 3′-UTR and one was a C→A transversion at nt 625 within exon IV of the gene. This transversion resulted in the amino acid change Leu209 to Ile (L209I). Because exon IV is present in all four neuraminidase transcripts (Fig. 1), we inferred that this point mutation must be present in all of the mRNAs and in the corresponding protein.

Table 1. Sizes and locations of exons and introns and sequences at the exon/intron boundaries of the *neu-1* gene

Exons		cDNA position	Introns			Intron size	
Number	Size		5′ splice site		3′ splice site		
I	171	−30 to 141	AGCCTGgtgagc	.....	gcgcagGTGCAG	365	(1)
II	190	142 to 331	ACCAGGgtaaca	.....	ttctagGTAGCA	453	(2)
III	266	332 to 597	ATTCAGgtttca	.....	taacagAAACAG	1200	(3)
IV	183	598 to 780	TGCCAGgtcagg	.....	acgcagCCCTAC	97	(4)
V	221	781 to 1001	AGTTCCgtgagt	.....	tcctagGAGTGA	99	(5)
VI	1365	1002 to 2366					

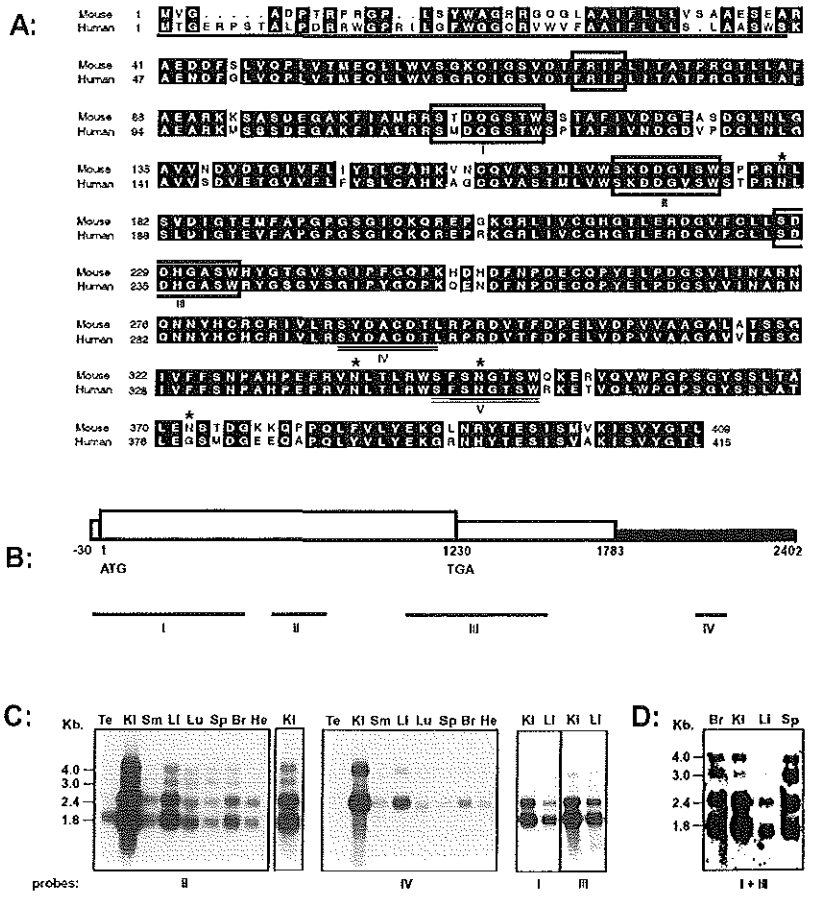

Figure 1. (A) Comparison of the amino acid sequences of mouse and human neuraminidases. Identical residues are shown in black and similar residues in gray. The signal sequence is underlined; the FRIP sequence and the conserved Asp boxes (I–III) are boxed; the degenerate Asp boxes (IV and V) are double underlined. The glycosylation sites are indicated by an asterisk above the sequence. (B) Linear representation of the two neuraminidase cDNAs: the coding region is indicated as an open box; the 5′-UTR, unique for the 1.8 kb clone, and the part of the 3′-UTR shared by both cDNA clones are indicated by gray boxes. The part of the 3′-UTR unique for the 2.4 kb clone is shown as a smaller black box. Numbers represent nucleotide positions. The different probes used to hybridize the northern blot are: I, nt −23 to 491; II, nt 601 to 780; III, nt 1049 to 1539; IV, nt 2072 to 2168. (C) Northern blot analysis using the probes outlined in (B) and indicated under each panel. Exposure times were 3 days for blots probed with II and IV, 16 h for blots probed with I and III. The blot hybridized with probe II was also exposed for 16 h to resolve the different transcripts in the kidney sample (shown as separate lane). Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Sm, smooth muscle; Sp, spleen; Te, testis. (D) Northern blot prepared with total RNA from SM/J-derived tissues and hybridized with a combination of probes I and III. Exposure time was 5 days.

Expression of L209I mutant neuraminidase in deficient fibroblasts

To assess the impact of the L209I change on biochemical properties of the normal enzyme we engineered this mutation into the normal 1.8 kb murine cDNA. The resulting mutant clone (Mo-smj) was completely sequenced to confirm correct introduction of the mutation. This mutant cDNA was transiently expressed in two human sialidosis type II fibroblasts. These cells were chosen because, unlike SM/J fibroblasts, they totally lack neuraminidase activity (3). Mo-smj cDNA only partially corrected the deficient fibroblasts, generating neuraminidase activity of between 40 and 65% of that of the wild-type murine (Mo-neur) enzyme. Given the strict dependence of neuraminidase on PPCA for full enzymatic activity (1,2), we also tested the effect of both mouse and human PPCA on SM/J neuraminidase. Co-transfection of the Mo-smj and Mo-neur cDNAs with either the mouse or human PPCA cDNA (Mo-ppca and Hu-ppca) resulted in a clear

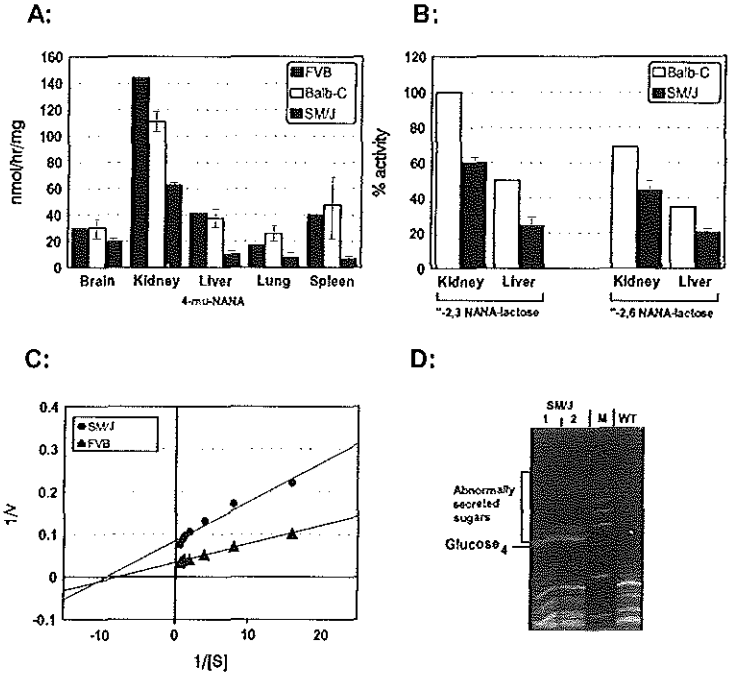

Figure 2. (A) Lysosomal/mitochondrial fractions of different mouse tissues assayed with the 4-MU-NANA substrate. Values shown represent the average of three independent experiments. (B) Lysosomal/mitochondrial fractions of different mouse tissues assayed with either α-2,3-NANA-lactose or α-2,6-NANA-lactose. Values given are the average of three independent experiments. Activities are expressed as a percentage of normal kidney neuraminidase activity assayed with α-2,3-NANA-lactose. Both control and SM/J mice were between 3 and 4 months old. (C) Lineweaver–Burke analysis showing dependence of the 4-MU-NANA substrate concentration on initial rate of neuraminidase activity. V_{max} for the SM/J neuraminidase is ~12 nmol/h/mg, while the wild-type enzyme has a V_{max} of ~30 nmol/h/mg. Activities were assayed as described in Materials and Methods; v is the velocity rate of the reaction in nmol/h/mg and S is the substrate concentration in mM. (D) Urine analysis of SM/J and control mice, displaying an abnormal pattern of oligosaccharides. SM/J 1 and 2 represent two independent urine samples from two different SM/J mice, M is the OLIGO ladder standard from Glyko Inc. and WT refers to the urine sample collected from a wild-type mouse.

increase in neuraminidase activity for both the normal and mutant protein (Fig. 3). The induced SM/J activity, however, remained lower than that of the wild-type. The L209I mutation was also introduced into the human neuraminidase cDNA (Hu-smj). Expression of this mutant clone alone or in combination with human or mouse PPCA again resulted in reduced neuraminidase activity (Fig. 3), unequivocally demonstrating that the L209I substitution is responsible for the enzyme defect. Immunofluorescence analysis of singly and doubly transfected cells showed that the presence of the mutation in either the mouse or the human neuraminidase molecule does not alter the subcellular distribution of the enzyme, which maintained a typical punctate lysosomal staining (Fig. 3). The lysosomal localization of the mutant enzyme was confirmed using Percoll density gradients with transfected COS-1 cells (data not shown). Co-expression of either mouse or human PPCA clearly enhanced the lysosomal signal, further indicating that PPCA has a stabilizing effect on the mutant protein.

Biosynthesis of the L209I mutant in COS-1 cells and its association with the PPCA precursor

The increase in SM/J neuraminidase activity in cells co-expressing mutant Neu-1 neuraminidase and PPCA suggested that interaction between the two proteins was not affected by the L209I mutation. We tested this assumption by overexpressing the Smj-neu1 and the PPCA cDNAs in COS-1 cells and then immunoprecipitating radiolabeled proteins with anti-neur or anti-PPCA antibodies (Fig. 4). Although the anti-neur antibodies recognized the murine protein with lower affinity than they did the human protein, more SM/J protein than wild-type mouse protein was immunoprecipitated from equally transfected cells (Fig. 4, lanes 2 and 3). Nevertheless, neuraminidase activity in singly transfected cells was again 50% of that of control values (data not shown). In co-transfected cells both the mouse and human PPCA precursors were co-precipitated with anti-neur antibodies, together with the SM/J polypeptide (Fig. 4, lanes 8

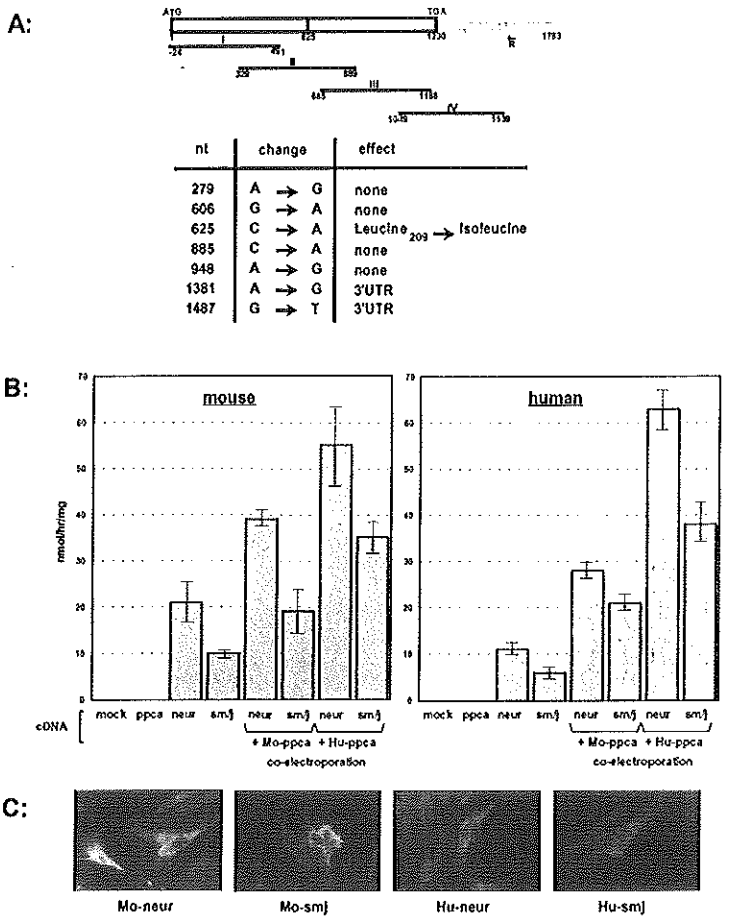

Figure 3. (A) Strategy used to screen for mutations in the *neu-1* cDNA. R indicates the primer used to reverse transcribe the mRNA; individual fragments were amplified using the gene-specific primers listed in Materials and Methods. The table represents the results obtained with this screening procedure. (B) Neuraminidase activity in cell lysates of electroporated GM01718 sialidosis type II fibroblasts using 4-MU-NANA as substrate. Values represent the average of four independent electroporations. The diagram to the left shows results obtained with the murine neuraminidase cDNAs, whereas the diagram to the right shows results with the human cDNA samples. The last four samples on the right of each panel represent the neuraminidase cDNAs co-electroporated with either mouse (Mo-ppca) or human (Hu-ppca) protective protein/cathepsin A cDNA. ppca, protective protein/cathepsin A cDNA; neur, wild-type 1.8 kb neuraminidase cDNA; smj, 1.8 kb neuraminidase cDNA containing the SM/J mutation. (C) Immunofluorescence with anti-human neuraminidase antibodies of fibroblasts electroporated with the mouse neuraminidase cDNA (Mo-neur), the SM/J cDNA (Mo-smj), human neuraminidase cDNA (Hu-neur) and human mutant cDNA (Hu-smj). Lysosome-like punctated staining was evidenced in the different electroporated fibroblasts.

and 11). The mutant protein, the wild-type mouse protein and human neuraminidase all co-precipitated the PPCA precursor equally efficiently (lanes 7, 9, 10 and 12). Therefore, the L209I substitution did not interfere with association between the mutant protein and either mouse or human PPCA, excluding the possibility that the SM/J mutation affects complex formation. Sequential immunoprecipitation of all samples with anti-PPCA antibodies explained the difference in the ability of the mouse and human PPCA to enhance neuraminidase activity (Fig. 3). The murine PPCA precursor in overexpressing cells was not as well processed to the mature two chain form as the human PPCA precursor (Fig. 4, lanes 7–9 and 10–12). This reduced level of processing could have led to a smaller pool of mature PPCA available for 'protection' of the lysosomal neuraminidase.

Turnover of the SM/J neuraminidase was apparently not influenced by the L209I substitution, as determined by pulse-

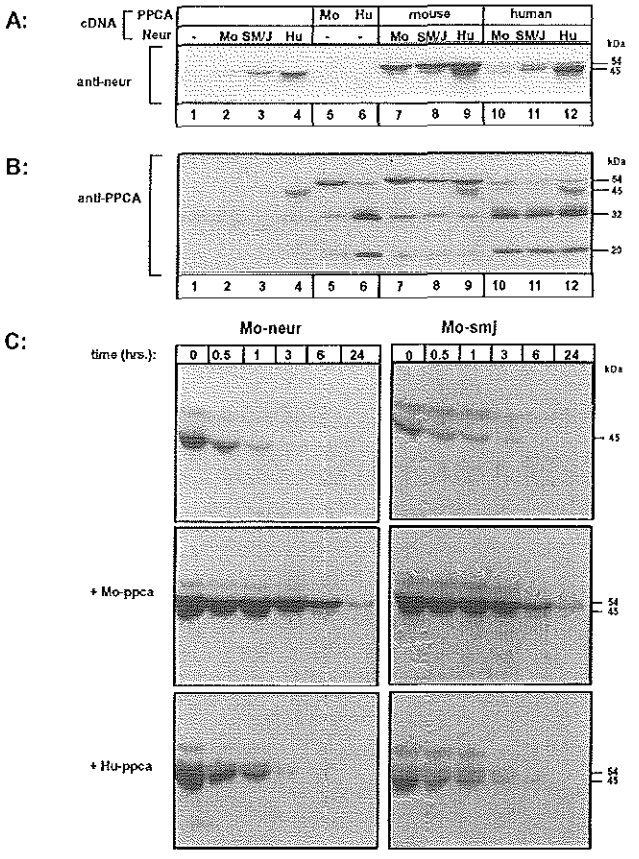

Figure 4. (A) Immunoprecipitation of radiolabeled cell lysates from transiently transfected COS-1 cells using anti-human neuraminidase antibodies. Cells were either singly or doubly transfected with the indicated cDNA clones and then labeled for 16 h with 50 µCi [^{3}H]-4,5-leucine before harvesting. Lane 1, mock-transfected cells. Mo, mouse; SM, SM/J; Hu, human. (B) Sequential immunoprecipitation of the same lysates as used in (A) using either anti-mouse (lanes 1–5 and 7–9) or anti-human (lanes 6 and 10–12) PPCA antibodies. In lanes 4, 9 and 12 small quantities of the 45 kDa neuraminidase protein are still visible, because the samples were not precleared prior to performing the second immunoprecipitation. (C) Pulse-chase analysis of transiently transfected COS-1 cells. Cells were labeled for 1 h with 50 µCi [^{3}H]-4,5-leucine and then chased for the indicated times with non-radioactive medium. Samples were then immunoprecipitated with the anti-human neuraminidase antibodies. (Top panel) Pulse-labeled COS-1 cells transfected with the mouse neuraminidase cDNA (Mo-neur) or the mutant cDNA (Mo-smj); (middle panel) COS-1 cells co-transfected with mouse neuraminidase and PPCA (Mo-ppca) cDNAs; (lower panel) COS-1 cells co-transfected with mouse neuraminidase and human PPCA (Hu-ppca) cDNAs. Molecular weights were calculated on the basis of protein standards.

chase labeling of transfected COS-1 cells (Fig. 4). Both the mutant and wild-type neuraminidase appeared to be stabilized upon co-expression of mouse PPCA, since immunoprecipitable material could still be detected at the 3–6 h time points (Fig. 4, middle panels). The stabilizing effect was less apparent, but still recognizable, when human PPCA was co-expressed with mutant or wild-type neuraminidase (Fig. 4, lower panels). These results clearly correlate with the observed increase in enzyme activity in cells co-expressing mutant or wild-type neuraminidase with PPCA.

DISCUSSION

Overall the results we present here provide strong evidence that the subtle L209I substitution is responsible for the altered neuraminidase activity in SM/J mice. Leu209 in the murine enzyme falls in an amino acid stretch that is highly conserved among the different sialidases (10,36). This residue coincides with Leu221 and Leu199 of the *Micromonospora viridifaciens* and *Salmonella typhimurium* sialidases respectively, which are located in the three-dimensional structure of these enzymes in the

vicinity of the active site (17,18). It is therefore conceivable that this amino acid substitution in the SM/J neuraminidase could affect substrate recognition, rate of substrate cleavage or release of the product, as evidenced by the altered V_{max} of the mutant protein. Although SM/J mice present with some of the biochemical abnormalities that are associated with the human lysosomal disorder sialidosis, the relatively high residual neuraminidase activity prevents the occurrence in young mice of excessive storage in their tissues. Older mice, on the other hand, eventually develop visible cellular changes, especially in the CNS. Therefore this animal model may be regarded as a mild form of sialidosis.

The residual SM/J activity varied slightly in different tissues. This could be attributed to the occurrence in some tissues, like brain, of neuraminidase 'isoenzymes' thought to be localized in the lysosomal (37,38) or plasma membrane (38–42) and the cytosol (16,38,43–45). However, the existence of various lysosomal neuraminidases is questionable, since in PPCA-deficient mice no residual neuraminidase activity is detected at acidic pH (2). The same holds true for human sialidosis patients with structural mutations in the neuraminidase gene that result in complete loss of neuraminidase activity (3).

Modification of sialic acid residues, which are present as terminal sugars on various types of sialoglycoconjugates, is essential for regulation of many cellular activities. The Neu-1 neuraminidase plays a key role in such modifications, for example in processing of cell surface molecules that are involved in modulating an immune response (9,27–31). T lymphocyte activation is normally accompanied by an increase in endogenous Neu-1 neuraminidase (30,31), which, in turn, results in hyposialylation of glycoproteins on the surface of activated T cells (9,46–49). These surface glycoproteins are required for T cell differentiation [for a review see (32)] and several of them are known to be internalized from the plasma membrane and subsequently re-exposed by a 'recycling' process. MHC class I and class II molecules and the T cell receptor are examples of such molecules (50–53). Therefore, it may be that processing of the sialic acid residues on these and other glycoproteins present on the surface of specific T cells is mediated intracellularly by lysosomal neuraminidase. If this enzyme is part of the main mechanism for sialic acid processing in T cells then the altered V_{max} value of SM/J neuraminidase would quite logically account for the abnormal sialylation of these molecules. Our data suggest that the mutant enzyme retains the capacity to recognize its substrate but that its rate of catalysis and/or release of product is impaired. The type of substrates that are cleaved by the enzyme may determine whether or not a certain cell type can compensate for a reduction in activity of mutant neuraminidase. Again, this is best exemplified in the T cell system, where the immune response in SM/J mice involves differentiation of naive T cells to T_{h1} but not to T_{h2} cells.

Interestingly, reduced neuraminidase activity has also been detected in rat strain KGH (54). The responsible gene, *neu-1*, was mapped to the RT1 locus (55), which is syntenic to the mouse H-2 and human HLA loci. It is unclear whether this defect results in the same phenotypic alterations identified in SM/J mice. It will be instructive to identify the molecular basis of the defective neuraminidase activity in this rat strain and to compare it with that found in SM/J mice. A second gene, *neu-2*, has also been described in both mouse and rat (54,56). However, the encoded enzyme is localized in the cytoplasm and does not cleave the fluorimetric substrate (57). Furthermore, linkage analysis demonstrated that the *neu-2* is not linked to the *neu-1* locus (54).

Once the three-dimensional structure of the lysosomal mammalian neuraminidase becomes available we could gain a better understanding of the impact of the L209I mutation on structure and function of the enzyme. Our findings on SM/J mice will hopefully facilitate further genetic and immunological studies on this animal model.

MATERIALS AND METHODS

Isolation of the mouse cDNA

A mouse BALB/c cDNA library was screened according to the manufacturer's instructions (Clontech). cDNA clones were sequenced with the Amersham thermocycler kit and subcloned into the mammalian expression vector pSCTOP (58).

RNA isolation and Northern blot analysis

RNA was prepared from SM/J mouse tissues by the LiCl/urea method as previously described (59). Total RNA was separated on 1% agarose gels that contained 0.66 M formaldehyde in MOPS buffer, was then blotted onto Zetaprobe membranes (BioRad) and was finally hybridized under standard conditions (60). The multiple tissue northern blot was purchased from Clontech and handled according to the manufacturer's instructions.

Mutation analysis and mutagenesis

Total RNA preparations from different SM/J mice and FVB controls were subjected to RT-PCR (3,61). The following primers were used in the reactions: 5′-CCCTAGGACACCGGGCCTTC-3′ (antisense, primer R); 5′-CCTGGACAGGGATCGCCG-3′ and 5′-GTAGAGGCCACCTGGCAG-3′ (fragment I); 5′-CGGACCAGGGTAGCACGTGG-3′ and 5′-GGGTGTCACAGGCGTCATAG-3′ (fragment II); 5′-GATGACCACGGTGCCTCC-3′ and 5′-GGTGTACCGGTTCAGGCC-3′ (fragment III); 5′-CCTGGCAGAAGGAGAGGG-3′ and 5′-CTGTTCATCTCTCCAGGG-3′ (fragment IV).

Amplified products were purified by phenol/chloroform extraction, on Centricon-100 columns (Amicon) and by ethanol precipitation. The purified products were directly sequenced with the fmol sequencing kit (Promega). The mutation was inserted into the wild-type cDNA by combining fragments II and III (Fig. 3) and using the *Stu*I restriction sites at positions 377 and 1168 to substitute the *Stu*I fragment for the wild-type fragment.

Cell culture, electroporation of fibroblasts and transfection of COS-1 cells

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum (FBS). COS-1 cells were maintained in DMEM supplemented with 5% FBS. Fibroblasts were electroporated according to the manufacturer's instructions (BioRad) with the following modifications. Cells were harvested by trypsinization and washed once in Iscove's medium. They were then counted and 25 µg plasmid DNA were electroporated into 1×10^6 cells suspended in 500 µl Iscove's medium using a BioRad Gene Pulser set at 0.32 kV and 500 µF. Electroporated cells were seeded in 6-well plates for 14–18 h before the medium was changed. They were harvested 72 h later. Immunofluores-

cence of electroporated fibroblasts was performed as described previously (3). COS-1 cells were transfected with Qiagen's Superfect according to the manufacturer's instructions and harvested 72 h post-transfection. Transfection efficiency was checked by immunofluorescence and the total amount of synthesized neuraminidase protein was estimated by western blot analysis of total cell lysates. Comparable transfection efficiencies were obtained among samples within each experiment and similar levels of neuraminidase protein were synthesized.

Lysosomal/mitochondrial extract

Mice were sacrificed by cervical dislocation and their tissues immediately isolated and placed in ice-cold 10 mM HEPES, pH 7.4, 250 mM sucrose. After the tissues were washed several times in this buffer they were weighed and homogenized in a tight-fitting dounce (Kontes) in 4 vols HEPES-buffered sucrose. A lysosomal/mitochondrial extract was prepared according to the procedure described by Gieselmann (62). The resulting lysosomal/mitochondrial pellet was dissolved in HEPES-buffered sucrose and analyzed for enzyme activity.

Enzyme activities and urine analysis

Lysosomal/mitochondrial extracts and cell lysate from either transfected COS-1 cells or electroporated fibroblasts were assayed for neuraminidase activity using the artificial substrate 4-MU-NANA according to Galjaard (63). Protein concentrations were determined using the BCA kit from Pierce Chemical Co. Neuraminidase activity also was assayed with α-2,3- and α-2,6-NANA-lactose as substrates, according to the procedure described previously (64,65). Urine samples were collected and analyzed using a FACE® Urinary Carbohydrate Analysis kit purchased from Glyko Inc. following the manufacturer's instructions.

Immunoprecipitation

Transfected COS-1 cells were seeded in 6-well plates and labeled for 16 h with 50 μCi [^{3}H]-4,5-leucine. Radiolabeled proteins were immunoprecipitated with anti-neur antibodies, as described previously (66). For the pulse-chase experiment transfected cells were labeled with 50 μCi [^{3}H]-4,5-leucine for 1 h and then chased in fresh DMEM over different time periods (67).

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Chapter 7

Discussion and future prospects

A: Human and murine genomic organization

The results presented in chapters 2 to 5 have emphasized the concept that lysosomal proteins, until now viewed as structural enzymes merely fulfilling a digestive function, have characteristics that demand a level of regulation not previously anticipated. I have studied two mammalian PPCA genes, the human and mouse, and found that they are very homologous, both in organization of the exon/intron boundaries as well as in exon sequences (chapter 2, 51). Comparison of their genomic organization has enabled the identification of conserved domains that could be important in the regulation of PPCA expression, either by influencing the rate of transcription or the splicing efficiency. It was unexpectedly discovered that even sequences within introns were very conserved, as we found for introns 3,4 and 5 (unpublished result). The homology extents into the promoter regions, which share some features of housekeeping genes, like G/C-rich sequences and SP1 boxes (17). In addition, the murine gene has a unique, TATA-box containing additional promoter that could control expression of the gene under the influence of tissue-specific stimuli. It is noteworthy, for instance, that PPCA mRNA expression gradually increases in the uterus during pregnancy with a clear peak right before parturition after which the level drops drastically. This pattern closely coincides with that of oxytocin and its receptor, a peptide hormone known to be inactivated by PPCA (27). This, together with the observation that this promoter also contains a GRE half-site, is suggestive for hormone dependent or inducible expression, which would ensure the generation of sufficient protein to inactivate the hormone at the proper time and target site. Another lysosomal gene, the aspartyl proteinase cathepsin D, has been shown to be activated by estrogens, which direct transcription from a TATA-box present in a mixed promoter context (3, 12, 32, 33).

It was already shown that the last 58 nucleotides of the last PPCA exon are reverse complementary to the 3'UTR of the *Phospholipid transfer protein* gene (Pltp, 51). Through our analysis of the promoter region, we have discovered that both the human and mouse PPCA loci are flanked by and partially overlapped by yet another gene of unknown function so far (chapter 3). This novel gene is transcribed from the opposite strand and results in a mRNA of 1 kb. Similar, but not identical, situations have been observed in other genes, like *tenascin-X*, *erb-A* and *cytochrome P450c27/25* (2, 9, 30, 35, 50, 54, 63). Aside from the PPCA, two other loci encoding lysosomal enzymes were shown to have overlapping transcriptional units, β-glucuronidase (*Gus-s*) and glucocerebrosidase (*Gba*). *Gus-s* contains a complex transcriptional unit on the same strand, called *Gig*, which has its promoter within intron 1 of *Gus-s*. Although the two genes intermingle and partially overlap, their transcripts share little, if any, sequence similarity (62). The *Gba* gene and its adjacent pseudogene are flanked both upstream

and downstream by several other loci, that do not, however, overlap with the *Gba* transcript (64). Interestingly, the generation of the Gaucher mouse led to the identification of one of these genes, called *metaxin* (*Mtx*). The *Mtx* gene, located immediately downstream of the GBA pseudogene, is transcribed convergently to the *Gba* gene and shares a bidirectional promoter with another gene, encoding for thrombospondin 3 (Thbs 3, 7). Upstream of the *Gba* gene, and transcribed from the same strand, are at least three other genes, *Clk2*, *Propin 1*, *Cote 1* and a non-transcribed pseudogene of *Mtx* is in between the *Gba* gene and its pseudogene (64).

Nonetheless, the PPCA locus is unique since it overlaps both at the 5' and 3' end with two other genes, both of which are transcribed from the complementary strand with respect to PPCA. This adds complexity to the PPCA locus and the mode of regulation of the three genes. It is remarkable that the human and mouse PPCA TATA-less promoters contain three conserved E-boxes to which transcription factors like MyoD, kE2-binding protein and c-myc bind (5, 6, 36). It is unclear at the moment, whether these elements are implicated in the regulation of the PPCA gene, or of the 5' overlapping gene, which is primarily expressed in heart and skeletal muscle. It would be interesting to examine severe early infantile galactosialidosis patients, who completely lack PPCA mRNA, for the influence of the two overlapping genes on the phenotypic expression of the disease. Mutations or deletions in the PPCA promoters may result in the silencing of the PPCA gene, but could simultaneously abolish or influence expression of the 5' overlapping gene, or cause mutations in the encoded protein. The heterogenous clinical manifestations of galactosialidosis can include the involvement of heart problems, consisting of cardiomegaly, thickening of the septum and cardiac failure. The interesting possibility arises that mutations in this 5' overlapping gene, which is highly expressed in heart, are responsible for these symptoms. Recently, it was demonstrated that in cases of severe Hunter syndrome mutations in the FMR2 locus, neighboring the α-iduronate-2-sulphatase gene, could contribute to the clinical manifestations (59). These authors showed that patients suffering from seizures combined with atypical symptoms had a genomic deletion that also involved the FMR2 locus.

The extensive homology between the human and mouse PPCA genes could aid in the identification of mutations within important regulatory elements in the genes of patients who completely lack PPCA mRNA (chapters 2 and 3). Analysis of the complete PPCA locus may facilitate the understanding of the phenotypic variation observed in galactosialidosis patients and improve our ability to relate genotype with phenotype in order to provide appropriate therapies. Clinical manifestations, unusual for a lysosomal storage disorder, could eventually be explained by the prolonged presence or abnormally high levels of active peptide-hormones, which are known to be a natural substrate of PPCA. The expression of the two flanking genes and their influence on the

features of the disease may also be worth investigating, because it could reveal new clinical features in patients unknown until now.

B: Mouse model for human galactosialidosis

Knowledge of the genomic organization of the mouse PPCA gene has helped in the generation of a mouse model for galactosialidosis by targeted gene disruption (chapter 4). The PPCA knockout mouse, or GS mouse, displayed progressive nephropathy, ataxia and premature death, a phenotype that closely resembled the most severe form of the human disease. The early infantile type patients suffer from fetal hydrops, kidney and heart involvement, visceromegaly, psychomotor delay, skeletal abnormalities, mental retardation and death occurs within the first year of life (15). The mouse model provides an excellent *in vivo* system and has led to a better understanding of the development of the disease pathology. Analysis of the GS mouse unexpectedly revealed a remarkable regional distribution of cells with lysosomal storage in different tissues, in spite of the presence of lysosomes in virtually every cell type. For example, the epithelial cells of the proximal tubuli in the kidney clearly show storage, whereas the epithelium of the distal tubuli in the kidney is devoid of detectable storage. In brain, the entorhinal cortex contains more PAS positive neurons than the somatosensory cortex, the epithelial cells of the choroid plexus are extensively vacuolated, while the Purkinje cells in the cerebellum only have a few defined vacuoles before they die. The neurons of the hippocampus and dentate gyrus do not show signs of storage at all throughout the lifespan of the animals. For a clearer interpretation of the GS phenotype, we have examined the site and level of *in vivo* PPCA expression in normal mice (chapter 5). We found a regional distribution of both PPCA mRNA and protein, which in general colocalized with the detected lysosomal storage pattern. However, also some interesting discrepancies were noted as cells with high levels of PPCA expression showed no detectable storage in the GS mouse (hippocampus, dentate gyrus), whereas low expressing sites displayed high levels of storage (glomerulus). At this moment it is not clear why such differences occur, but it may reflect the importance of the catalytic activity of PPCA, known to inactivate specific bioactive peptides, rather than its protective function in specific cell types, as discussed in chapter 5. Alternatively, since the glomerular epithelium filters the blood, it may collect blood-derived metabolites that cannot be cleared once internalized. Other lysosomal genes, like murine prosaposin (55, 56), acid phosphatase (20), acid lipase (16) and acid α-glucosidase (42), show a regional distribution of mRNA and/or protein. However, a comparison between storage and normal gene expression has not been performed in these cases.

A close examination of the galactosialidosis phenotype may also help in the identification of novel metabolic pathways in which PPCA is involved. This in view of

what has been discovered through the analysis of mouse models for the lysosomal storage disorders G_{M1} gangliosidosis (β-galactosidase), Tay-Sachs (β-hexosaminidase A), Sandhoff (β-hexosaminidase B) and G_{M2} activator deficiency (14, 22, 31, 40, 49, 57, 58, 65). These models elucidated the importance of a parallel metabolic pathway that converts G_{M1} - and G_{M2} gangliosides into the asialo types G_{A1} and G_{A2} through a sialidase active in mice, but absent or less active in humans (see also general introduction, part 1.5). The asialo glycolipids could be further cleared by the remaining hexosaminidase isoenzyme in Tay-Sachs and G_{M2} activator deficient mouse models. The slow metabolic turnover results in modest storage and a mild phenotype in comparison with the Sandhoff mouse, which misses sufficient residual hexosaminidase activity to process the asiolo derivatives and thus accumulates considerable amounts of G_{M2} and G_{A2}. It is not clear yet what the potential contribution of G_{A1} and G_{A2} is in relation to the emerging disease patterns, but it may be responsible for significant differences between the human and mouse neuropathology.

Finally, the availability of the GS mouse model may be very useful for the understanding of the physiological role of PPCA in the conversion of its biological substrates. The knowledge that the PPCA carboxypeptidase activity can be separated from its protective function towards β-galactosidase and N-acetyl-α-neuraminidase could be applied to the generation of catalytic mutants that maintain the protective activity (19). Analysis of these mouse mutants may lead to the identification of individuals with mutations that only hamper the cathepsin A activity. Those mutations are not noticed yet since they lack overt clinical signs typical for galactosialidosis.

C: Experimental approaches to treatment

The importance of a detailed expression map of the gene in relation with the disease pattern in the knockout mouse is crucial for the precise assessment of correction of the phenotypic abnormalities after treatment. In fact, one of the reasons for creating mouse models is the development of an *in vivo* system to examine the feasibility of different therapeutic approaches to treat these diseases (21, 37, 57). Therapeutic strategies have relied on the unique capacity of soluble, lysosomal enzyme precursors to be secreted by one cell type and internalized via receptor-mediated endocytosis by other cells at distant sites (reviewed in 8). Methods such as enzyme replacement therapy (ERT), bone marrow transplantation (BMT), organoid implantation and gene therapy have been attempted in patients with moderate succes (reviewed in 23, 25, 26, 43). Each approach presents inherent problems mainly related to the difficulty of correcting bone abnormalities and the CNS pathology. ERT, which to date has been succesful for the treatment of humans with the non-neuropathic form of Gaucher disease, could be the treatment of choice, but only for non-neuropathic patients. However, it is costly and

requires repeated administrations of normal enzyme. BMT implies a "once-only" practice, and relies on delivery of enzyme from donor-derived normal hematopoietic cells to affected host cells after treatment. This approach has been widely used to treat lysosomal diseases in both human and animal studies (1, 15, 23), but the outcome has been inconsistent, primarily with regard to CNS correction. Other methods, including *ex vivo* gene therapy, have suffered from poor transduction efficiencies, short-term or silenced gene expression *in vivo* and the difficulty of delivering therapeutic protein to target cells. To overcome these obstacles, we have used a novel approach based on the generation of transgenic mice, expressing a human PPCA minigene under the control of the β-globin promoter and the LCR. Contrary to what is achieved with *ex vivo* systems using viral vectors, these transgenic mice would mimic 100 % transduction efficiency of bone marrow cells, maintain high level of expression of the therapeutic gene and express it in a cell specific manner. It was not known, however, how the heterologous lysosomal protein would behave during differentiation and maturation of the erythrocytes. The results of the analysis of this transgenic line were very encouraging, since the human PPCA minigene was expressed in erythroid precursors at high levels and depended on the copy number of the transgene. Furthermore, the overexpressed precursor protein was secreted by erythroid cells in large amounts and appeared stable in the circulation. The synthesized protein was correctly glycosylated, since *in vitro* uptake studies showed correction of galactosialidosis fibroblasts and thus their therapeutic value. So, to test whether sustained expression of corrective protein in specific bone marrow derived cells can revert, diminish or prevent lysosomal storage in all tissues affected in the GS mouse, including the brain, we transplanted bone marrow derived from wild type and transgenic mice into deficient mice (chapter 4). The treated animals showed a reduction of excreted sialyloligosaccharides, correction of all the affected systemic organs and a prolonged lifespan (chapter 4). The brain was only partially corrected, showing persistent storage in neurons, but a normal microscopic appearance of the choroid plexus, the trigeminal ganglia and the pituitary. The partial reversal of the galactosialidosis phenotype was more pronounced with the overexpressing transgenic bone marrow, indicating that the level of correction is dependent on the amount of normal enzyme. These studies demonstrate the importance of cell-specific expression of the therapeutic enzyme, since it facilitates the interpretation of the *in vivo* effects of overexpression.

The correction obtained after bone marrow transplantation of GS mice using our strategy based on this novel approach are very promising. A number of reports have been published describing the results of different treatments for lysosomal storage disorders in animals, including correction of the visceral organs and a prolonged lifespan, but minimal improvement of the CNS involvement and bone abnormalities (4, 11, 38, 44, 45, 47, 48, 52, 53). It was shown that administered β-glucuronidase through intravenous injection into newborn mice was taken up within one hour by many tissues,

including the meninges, choroid plexus and vessels (61). However, no detectable enzyme levels were found in CNS neurons and small amounts were identified in neurons in the peripheral nervous system. Bone marrow transduced with a retrovirus expressing glucocerebrosidase was transplanted into normal mice to assess the possibility of hematopoietic cells to contribute to the CNS. All macrophage populations, including CNS microglia, appeared to be partially replaced with donor cells, which continued to express glucocerebrosidase as long as 8 months after transplantation (28). These and other studies demonstrated the possibility that donor-derived cells, most likely monocytes and macrophages, have the capacity to infiltrate the CNS and differentiate into the microglial population (24, 29, 39). In addition, it was noted that treatment initiated early after birth was more effective than those started later in life, most likely because the blood-brain barrier is not yet fully developed in very young mice (34, 45, 46).

Considering the encouraging results obtained with the β-globin/LCR mediated, erythrocyte-specific expression of PPCA and in order to target more specifically the CNS, we have developed another transgenic mouse, which expressed the human PPCA minigene under the control of the CSF-1-receptor promoter (Hahn *et al.*, submitted for publication). These mice expressed the therapeutic protein exclusively in the monocyte/macrophage lineage, as well as in brain microglia. Transplantation of transgenic bone marrow into deficient mice resulted in a direct reversal of the oligosaccharidurie, and an impressive correction of the visceral organs, even if GS mice were transplanted late in life. This could be relevant for future applications in human patients. Infiltration of expressing macrophages in the brain clearly improved perivascular sites, although scattered neurons still accumulated undegraded products, while the cerebellar Purkinje cells continued to store and die. So, either the therapeutic macrophages and monocytes were not introduced at an early enough stage, or the level of expression was not high enough to clear the storage, as cultured bone marrow macrophages secreted only low amounts of PPCA. It remains to be seen whether enough macrophages will ever repopulate the CNS, even following BMT at an early age, to allow for sufficient neural correction prior to the onset of irreversible damage.

D: The role of lysosomal neuraminidase

The loss of neuraminidase activity in galactosialidosis patients and in the PPCA knockout mouse, together with the biochemical evidence that, in addition to β-galactosidase, neuraminidase is in complex with PPCA, encouraged the isolation of the murine lysosomal neuraminidase gene. In chapter 6 the characterization of the mouse neuraminidase cDNA and gene are described, including the determination of the molecular basis for reduced neuraminidase activity in the naturally occurring mouse

inbred strain SM/J. The importance of lysosomal neuraminidase in the de-sialylation of cell-surface molecules is demonstrated by the inability of these mice to produce T_{h2}-cells (13). It would be interesting to investigate if the galactosialidosis mouse behaves in a similar fashion with respect to T-cell differentiation. If this phenotypic abnormality is not present in the GS mouse, we could speculate on a novel role for lysosomal neuraminidase outside the complex with PPCA. In line with this, it is known that in the absence of PPCA, endogenous neuraminidase resides in a prelysosomal/endocytic compartment and is not transported to mature lysosomes (60). However, it is well documented that exogenous addition of PPCA to galactosialidosis fibroblasts triggers the transport of neuraminidase to the lysosomes and its activation (18, 19, 60). So, it can be postulated that a cell-surface, sialylated receptor molecule could be internalized in a prelysosomal/endocytic compartment, associate with neuraminidase and serve as "protective factor". This complex could then be transported to the lysosome where the activated neuraminidase would process the receptor molecule, which is then rerouted to the cell surface. Once dissociated from the "receptor", neuraminidase remaining in the mature lysosome would be quickly degraded due to the absence of PPCA.

The identification of the mutation in the neuraminidase gene of the SM/J mouse enabled us to link the observed phenotypic abnormalities with a partial neuraminidase deficiency. However, these mice have only a partial neuraminidase deficiency, so the generation of a model completely lacking neuraminidase activity will be important for the study of the pathogenesis of the severe type of sialidosis, as well as the development of therapeutic strategies. The generated mouse model may resemble the galactosialidosis mouse, since the storage pattern observed in the GS mouse is primarily determined by the absence of neuraminidase activity. Comparison between these two knockout mice may answer questions about the catalytic activity of PPCA, which is separable from its protective function. Moreover, if lysosomal neuraminidase is responsible for the turnover of G_{M1} and G_{M2} gangliosides into the asialo G_{A1} and G_{A2}, as previously discussed, then the neuraminidase knockout mouse may also accumulate these gangliosides, aside from sialylated compounds. It would be interesting to analyze crosses between the sialidosis mouse and G_{M1} gangliosidosis, Tay-Sachs and Sandhoff mice to check whether the sialidase activity that processes the G_{M1} - and G_{M2} gangliosides into the asialo derivatives is identical to the lysosomal protein.

E: Concluding remarks

The work described in this thesis has contributed to the further understanding of galactosialidosis and sialidosis and will help to elucidate the molecular pathology of patients with these diseases. Furthermore, the obtained knowledge can be used to generate mouse models of these diseases carrying sophisticated mutations, which in turn

will help in the understanding of the pathogenesis of the disorders and the exact biological functions of the proteins. The extrapolation from animal models to humans is very difficult and requires careful examination of the variable factors involved. For instance, murine models are genetically identical and live in a controlled environment, whereas human patients are genetically different and often their environmental factors vary as well. On the other hand, murine models for human disorders have been very instructive, as has been shown in this thesis. For instance, the discovery of alternative biochemical pathways in animal models may be helpful in the identification of new therapeutic strategies for human patients.

The results of therapeutic applications in patients with lysosomal storage disorders have so far been modest. One of the problems is the targeting of the normal, therapeutic protein to the affected cells and tissues and also the occurrence of antibodies against the corrective factor may cause unwanted clinical manifestations (10, 41). Nevertheless, both the studies of animal models and the results of several clinical trials are encouraging to continue our efforts to solve these problems.

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SUMMARY

Lysosomes are a heterogeneous population of cellular organelles involved in the degradation of a wide variety of intra- and extra-cellular substrates. These acidic compartments contain proteins that orchestrate the stepwise degradation of macromolecules and the emerging products are either recycled or further degraded. Lysosomal storage disorders are characterized by the deficiency of proteins involved in one of these catabolic pathways, leading to the accumulation of undegraded substrates in lysosomes. The continuous accumulation of storage products results in cells containing numerous enlarged lysosomes and ultimately to cellular dysfunctioning. Since lysosomes are present in virtually every cell type, the partial or complete loss of their function causes multi-systemic clinical manifestations in humans, which may include CNS involvement, skeletal abnormalities, cardiovascular problems and organomegaly. These diseases can be subdivided into glycoproteinoses, glycolipidoses and mucopolysaccharidoses, depending on the nature of the main storage product. In addition, the biosynthetic pathway of lysosomal hydrolases may be affected, as has been demonstrated for I-cell disease and pseudo-Hurler polydystrophy, or membrane transport could be affected, as in Salla disease.

The lysosomal storage disorder galactosialidosis is one of the glycoproteinoses and is biochemically characterized by the loss of activity of two hydrolases, acid β-D-galactosidase and N-acetyl-α-neuraminidase, although the primary defect is a mutation in the protective protein/cathepsin A (PPCA) gene. The PPCA protein associates with the two hydrolases and forms an active complex in lysosomes. In the absence of PPCA, sialylated oligosaccharides, the substrates for N-acetyl-α-neuraminidase, are the primary storage products in the lysosomes. In addition and fully separable of its protective function towards these two hydrolases, PPCA has catalytic activity towards a selective set of bioactive peptides.

Chapter 2 and 3 describe the isolation and characterization of the organization of the murine PPCA gene, as well as the analysis of the promoter region. The organization appears to be very homologous to the human, both in the position of the exon/intron boundaries as well as their exonic sequences. Comparison of the human and mouse genes may reveal important conserved domains that could facilitate the molecular analysis of galactosialidosis patients. The promoter regions are also highly conserved and contain several elements that are shared by the family of housekeeping genes. Family members are known to be expressed ubiquitously and encode proteins that fulfill

metabolic functions. Surprisingly, two promoters rather than one control the murine gene. This additional promoter contains a TATA-box and two imperfect CAAT boxes, suggesting a special regulatory mechanism for its activation. Northern blot analyses and reverse transcriptase coupled polymerase chain reactions revealed that both promoters drive transcription in every tissue tested, but the level of transcript derived from the "housekeeping" promoter is generally higher. These results were paralelled by *in vitro* activity studies carried out using the CAT reporter gene, which allowed us to map the minimal promoter region. The *in vivo* activity of both promoters was demonstrated using *in situ* hybridization. In addition, it showed that the TATA-box containing promoter was not active in all cells that express PPCA from the housekeeping promoter.

Chapter 4 describes the generation of a mouse model for galactosialidosis using targeted disruption of the PPCA gene by inserting a selectable marker in exon II. The mice appeared to be phenotypically and histologically similar to the human disease, displaying a high urinary excretion of sialylated oligosaccharides, nephropathy, ataxia, hepatosplenomegaly and a shortened lifespan. Transplantation of these -/- mice with transgenic bone marrow overexpressing the human PPCA in the erythroid lineage resulted in a complete reversal of the diseased phenotype in the visceral organs, and a partial correction of the CNS. It was clear that higher levels of therapeutic protein from overexpressing bone marrow cells afford a more timely and complete correction of the disease phenotype than normal bone marrow. These encouraging data demonstrated the effectiveness of cell therapy in the treatment of galactosialidosis and form the basis for the development of new therapeutic strategies in the future. Furthermore, the usage of transgenics that specifically express the PPCA in the erythroid lineage enabled us to examine the effect of a single cell population after bone marrow transplantation.

In order to understand the pathophysiology of galactosialidosis, a fine mapping of the storage in the PPCA (-/-) mice was performed, as described in chapter 5. The distribution was remarkably regional within different tissues, as some cell types displayed clear signs of storage, while others did not. This pattern did not consistently correlate with the expression of PPCA mRNA and protein, since some high expressing cells did not store at all in the affected mouse, and certain low expressing cells accumulated high amounts of storage products. This discrepancy suggests that at these sites the catalytic functions of PPCA could be more important than its protective function. Furthermore, a fine mapping of the storage distribution in different tissues allows for a more precise assessment of the results of specific therapeutic approaches.

Chapter 6 describes the isolation of the murine N-acetyl-α-neuraminidase, one of the hydrolases in complex with PPCA. The protein has many of the typical sialidase superfamily features and is highly homologous to the human counterpart. In addition, molecular analysis of the murine inbred strain SM/J identified a point mutation resulting in a aminoacid substitution (L209I), which was responsible for the reduced activity observed in these mice. The mutation hampers the efficiency of neuraminidase to cleave its substrates, while its specificity remains unchanged. The isolation of the murine genomic region will be used to generate a mouse model for the severe form a sialidosis by inactivating the neuraminidase gene by targeted gene disruption.

The studies presented in this thesis will eventually contribute to a better understanding of both galactosialidosis and sialidosis. They will facilitate studies on the PPCA and neuraminidase interactions and elucidate important regulatory factors involved in the expression of both genes. A broader and deeper knowledge on the way these proteins function *in vivo* will be beneficial for the testing and evaluation of therapeutic approaches and to set up *in vivo* systems for the analysis of the two separate functions of PPCA.

SAMENVATTING

De kleinste bouwsteen van het leven, de cel, is opgebouwd uit verscheidene compartimenten, die elk afzonderlijk een specifieke functie vervullen. In de kern van de cel bevindt zich het erfelijke materiaal, het DNA, wat onder meer verdeeld is in stukken welke informatie bevatten voor het aanmaken van eiwitten (codering), alsmede de stukken met instructies wanneer deze eiwitten gemaakt moeten worden (regulatie). Het stuk DNA wat de combinatie van codering en regulatie van een eiwit bevat, heet een gen. Het coderende gedeelte van een gen, de exonen, zijn onderbroken door niet coderende stukken, intronen. Onder invloed van het regulerende deel van het gen, wordt een kopie gemaakt waarbij de intronen verwijderd worden, en het kopie, het boodschapper RNA (mRNA), fungeert als mal voor het aanmaken van het eiwit. Veranderingen in DNA waardoor er wijzigingen optreden in de regulatie of de codering van eiwitten worden mutaties genoemd. Mutaties kunnen leiden tot het volledig stil leggen van het gen of kunnen een verandering in het eiwit veroorzaken waardoor het niet goed meer functioneert.

Lysosomen zijn een heterogene populatie van compartimenten, afgesloten van de rest van de cel door een membraan, waarin zich vele eiwitten bevinden die betrokken zijn bij de afbraak van macro-moleculen van uiteenlopende oorsprong. Deze afbraak vindt plaats via enzymen die nauw op elkaar afgestemde processen begeleiden en de ontstane producten kunnen gebruikt worden als bouwstenen van nieuwe moleculen. Lysosomale stapelingsziekten zijn erfelijke aandoeningen, die worden gekenmerkt door een mutatie in één van de vele enzymen betrokken bij deze afbraakprocessen. Hierdoor vindt er een voortdurende opslag van substraten plaats die niet langer kunnen worden verwerkt. Uiteindelijk leidt dit tot de aantasting van de lysosomale functie en de zieke cel kan geïdentificeerd worden door de aanwezigheid van vele, gezwollen lysosomen. Aangezien lysosomen in bijna elk cel type voorkomen, hebben patiënten uiteenlopende klinische klachten, zoals beschadigingen aan het centrale zenuwstelsel en zwakzinnigheid, botafwijkingen, hart en vaat problemen en opgezette organen, zoals lever en milt. Lysosomale stapelingsziekten worden ingedeeld op grond van, en vernoemd naar, de moleculen die de voornaamste bron van de stapeling vormt.

Het werk dat beschreven is in dit proefschrift is gebaseerd op de lysosomale stapelingsziekte galactosialidosis. Mutaties in het protective protein/cathepsin A gen (PPCA) vormen de genetische basis voor deze ziekte. Het PPCA eiwit heeft de capaciteit om een complex te vormen met twee andere lysosomale eiwitten, β-

galactosidase en neuraminidase, waardoor deze twee eiwitten beschermt worden tegen afbraak in de lysosomen. Een erfelijke aandoening die het functioneren van het PPCA eiwit verstoort, leidt tot destabilisatie van het complex, waardoor de substraten die normaal door β-galactosidase en neuraminidase worden afgebroken, nu opgeslagen worden in de lysosomen. Het PPCA eiwit heeft naast de beschermende functie in het complex, ook een enzymatische activiteit, welke in staat is om bepaalde hormonen te inactiveren. Deze enzymatische activiteit vindt plaats buiten de lysosomen en is volledig onafhankelijk van de beschermende rol van het PPCA eiwit.

In hoofdstuk 2 en 3 wordt de isolatie en karakterisatie van het muize PPCA gen beschreven, alsmede de analyse van het gebied wat de basale functies voor de gen-regulatie bevat, de promoter. Uit vergelijkend onderzoek is gebleken dat het muize gen vele overeenkomsten vertoont met het humane gen. Gebieden die erg aan elkaar verwant zijn kunnen duiden op belangrijke domeinen in het gen, bijvoorbeeld voor de regulatie. Deze informatie kan van belang zijn bij de analyse van patiënten. De promoters van het humane en muize gen vertonen veel overeenkomsten met een familie van genen die omschreven worden als "huishoud" genen. Genen die tot deze familie behoren worden in vrijwel elk cel type tot expressie gebracht en zijn vaak betrokken bij algemene processen die nodig zijn voor het onderhoud van de cel. Naast de promoter met de beschreven karakteristieken, heeft het muize gen nog een extra promoter, welke kenmerken heeft die de suggestie wekken dat het muize PPCA gen op een meer specifieke manier kan worden geactiveerd. Het bestaan en de activiteit van de beide promoters van het muize gen zijn aangetoond met verschillende experimenten. Een belangrijke observatie is dat de meer specifieke muize promoter niet in elk cel type, waar de "huishoud" promoter actief is, geactiveerd wordt.

Met de verkregen informatie uit de gen analyse, is vervolgens een muis model van galactosialidosis gemaakt door het PPCA gen specifiek te inactiveren, zoals beschreven is in hoofdstuk 4. De ontstane muis vertoont grote gelijkenis met het humane ziektebeeld: verhoogde excretie van suikers met sialyl groepen, nier afwijking, ataxia, vergrootte lever en milt en een verkorte levensduur. Transplantatie van beenmerg afkomstig van normale muizen, of van gemanipuleerde muizen die het humane PPCA eiwit hoog tot expressie brengen in de rode bloedcellen, is in staat de aangetaste cellen van de galactosialidosis muizen volledig te herstellen, met uitzondering van het centrale zenuwstelsel wat slechts gedeeltelijk geneest. Dit experiment heeft tevens duidelijk gemaakt dat de hoge expressie van het humane PPCA eiwit, afkomstig van de gemanipuleerde muis, een beter herstel van de galactosialidosis

muis gaf dan normale hoeveelheden PPCA, wat uit het beenmerg van wild type muizen komt. Deze bemoedigende resultaten hebben aangetoond dat cel therapie effectief is in de behandeling van galactosialidosis, en zij vormen een basis voor het ontwikkelen van nieuwe behandelingen in de toekomst. De transplantatie van beenmerg van muizen die het PPCA eiwit alleen in de rode bloedcellen tot expressie brengen, heeft het mogelijk gemaakt om een specifieke populatie cellen te onderzoeken en het effect van hun bijdrage aan het genezingsproces van de galactosialidosis muis te meten.

Het in kaart brengen van de verschillende cellen die het typische beeld van een stapelingsziekte vertonen in de galactosialidosis muis, zoals beschreven in hoofdstuk 5, kan bijdragen tot het begrijpen van het ziektebeeld. Het verspreidingspatroon van deze cellen vertoont een opvallende regionaliteit: sommige cellen vertonen duidelijke tekenen van stapeling en andere cellen weer absoluut niet. Het vergelijken van het expressie niveau van het PPCA mRNA en eiwit in normale muizen met de stapeling in galactosialidosis muizen vertoont een duidelijke correlatie in de meeste gevallen: cellen die veel PPCA eiwit hebben, vertonen ook een hoge mate van stapeling. Het patroon komt echter niet altijd overeen met de expressie van het PPCA eiwit, aangezien sommige cellen die een hoge expressie hebben, totaal geen tekenen van stapeling vertonen, en vice versa. Deze discrepantie suggereert dat op die plaatsen, waar een hoge gen/eiwit expressie is, maar geen aantoonbare stapeling aanwezig is in de galactosialidosis muis, de enzymatische activiteit van het PPCA eiwit een belangrijkere functie vervult dan zijn beschermende functie ten aanzien van β-galactosidase en neuraminidase. Tevens is het precies in kaart brengen van het stapelingspatroon in de muis belangrijk voor een correcte interpretatie van de resultaten die verkregen worden met therapeutische behandelingen van de galactosialidosis muizen.

In hoofdstuk 6 wordt de isolatie en karakterisatie van het muize neuraminidase gen beschreven, één van de enzymen die in complex is met het PPCA eiwit in lysosomen. Het neuraminidase eiwit vertoont grote gelijkenis met het humane eiwit en heeft de typische kenmerken van de neuraminidase familie, bestaande uit neuraminidases afkomstig van verscheidene organismen. Naast de isolatie van het gen, wordt een mutatie beschreven die voorkomt in een muize stam dat een verlaagde neuraminidase activiteit vertoont. De mutatie verlaagt de efficiëntie waarmee het neuraminidase zijn substraten kan verwerken, terwijl de specificiteit van het eiwit voor zijn substraten onveranderd is. De karakterisatie van het gen zal het creëren van een muis model voor sialidosis, de ziekte waarbij het neuraminidase gen defect is, vergemakkelijken.

De studies beschreven in dit proefschrift dragen bij tot een beter inzicht in het ziektebeeld van galactosialidosis en sialidosis. Met name de interactie tussen het PPCA en het neuraminidase eiwit kunnen nu beter en in meer detail bestudeerd worden. Ook kunnen belangrijke factoren die een rol spelen bij de expressie van beide genen opgehelderd worden. Een betere en uitgebreidere kennis van het gedrag van beide eiwitten komen het testen en de evaluatie van toekomstige therapieën ten goede. Tevens kan de informatie dienen om de twee functies van het PPCA eiwit, de beschermende rol ten opzichte van β-galactosidase en neuraminidase en de enzymatische activiteit, beter te analyseren.

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