# Human palmitoyl protein thioesterase: evidence for lysosomal targeting of the enzyme and disturbed cellular routing in infantile neuronal ceroid lipofuscinosis

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Palmitoyl protein thioesterase (PPT) is an enzyme that removes palmitate residues from various S-acylated proteins in vitro. We recently identified mutations in the human PPT gene in patients suffering from a neurodegenerative disease in childhood, infantile neuronal ceroid lipofuscinosis (INCL), with dramatic manifestations limited to the neurons of neocortical origin. Here we have expressed the human PPT cDNA in COS-1 cells and demonstrate the lysosomal targeting of the enzyme via the mannose 6-phosphate receptormediated pathway. The enzyme was also secreted into the growth medium and could be endocytosed by recipient cells. We further demonstrate the disturbed intracellular routing of PPT carrying the worldwide most common INCL mutation, Arg122Trp, to lysosomes. The results provide evidence that INCL represents a novel lysosomal enzyme deficiency. Further, the defect in the PPT gene causing a neurodegenerative disorder suggests that depalmitoylation of the still uncharacterized substrate(s) for PPT is critical for postnatal development or maintenance of cortical

*Keywords*: INCL/lysosomes/palmitoylation/PPT/thioesterase

# Introduction

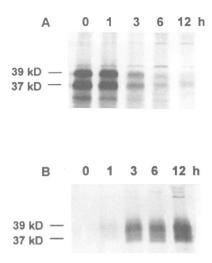
Post-translational processing of a multitude of eukaryotic proteins by fatty acids or prenyl groups appears to play a key role in their anchoring to membranes, a process necessary for their diverse cellular functions including signal transduction, vesicle transport and maintenance of the cytoarchitecture (for review, see Schmidt, 1989). While myristoylation and prenylation represent irreversible protein modifications, addition of palmitate via a labile thioester bond to cysteine residues is a reversible event. Such a dynamic palmitoylation-depalmitoylation cycle suggests a regulatory role for this modification, which is further emphasized by the fast turnover of the proteinbound palmitate (Omary and Trowbridge, 1981; Magee et al., 1987). Intriguingly, many of the palmitoylated proteins have been implicated in signal transduction pathways. For example, H-Ras undergoes prenylation and

palmitoylation both required for its correct membrane attachment (Hancock et al., 1989), and the α-subunits of heterotrimeric G proteins are both myristoylated and palmitoylated (Wedegaertner and Bourne, Wedegaertner et al., 1995). A large number of proteins with diverse cellular functions have been found to be modified by a palmitate group. These include extracellularly anchored acetylcholine esterase (Randall, 1994), the transferrin receptor (Jing and Trowbridge, 1987), the nitric oxide synthase (Robinson et al., 1995), the neuronal growth cone protein GAP-43 (Sudo et al., 1992) and the synaptosomal-associated protein SNAP-25 (Hess et al., 1992).

In spite of the growing number of identified palmitoylated proteins, little is known about the enzymes responsible for palmitoylation and depalmitoylation. Therefore, the biochemical mechanism of palmitoylation and the precise role of the palmitate in individual proteins is still somewhat obscure. Camp and Hofmann (1993) have purified a palmitoyl protein thioesterase (PPT) from bovine brain based on its ability to remove palmitate from palmitoylated H-Ras produced in Sf9 cells. In addition to H-Ras, the purified PPT accepted palmitoylated  $G_{\alpha}$  and long chain fatty acyl-CoA as its substrates in vitro (Camp and Hofmann, 1993). However, the bulk of PPT was suggested to be located extracellularly, and intracellular proteins were not considered as the main in vivo substrates for the enzyme (Camp et al., 1994). The human PPT gene encoding a protein of 306 amino acids has been cloned recently (Vesa et al., 1995). The predicted protein shows 91% identity to the bovine PPT and 85% identity to the rat PPT at the amino acid level. The high degree of conservation suggests an essential role for PPT in the cellular metabolism of vertebrates.

We recently have identified the mutations in the PPT gene causing the autosomal recessive neurodegenerative disorder in childhood, infantile neuronal ceroid lipofuscinosis (INCL) (Vesa et al., 1995). INCL is characterized by early visual loss and mental deterioration, and leads to a vegetative state of the patients by 3 years of age (Santavuori et al., 1974). Characteristic inclusion bodies are found in all tissues of patients (Rapola and Haltia, 1973), and the main protein components accumulating in these inclusions have been shown to be saposins A and D (Tyynelä et al., 1993). The hallmark of the tissue pathology in INCL is the selective loss of cortical neurons; the destructive process leaves, for example, the brain stem and the spinal cord intact. The defect in the PPT gene would thus suggest an essential role for the palmitoylation depalmitoylation cycle of the still uncharacterized substrate(s) for PPT in postnatal development and/or maintenance of cortical neurons.

Here we have analyzed the cellular targeting and processing of the wild-type PPT as well as the polypeptides



**Fig. 1.** Pulse–chase analysis of the wild-type PPT expressed in COS-1 cells. Cells transfected with the wild-type PPT cDNA construct were pulse labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 1 h and chased for 0, 1, 3, 6 or 12 h as indicated on top of the lanes. The PPT polypeptides were immunoprecipitated from (**A**) the cells and (**B**) the medium and subjected to 11% SDS–PAGE followed by fluorography. The molecular weights of the PPT polypeptide chains are indicated.

carrying the most common INCL mutation, Arg122Trp, by expressing the cDNA constructs in COS-1 cells. We demonstrate that PPT is routed to lysosomes. In contrast to the wild-type PPT, the mutant polypeptides were retained in the endoplasmic reticulum (ER), most probably due to the misfolding of the mutant polypeptides. Our findings suggest that INCL belongs to the group of lysosomal enzyme deficiencies.

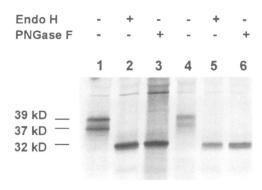
#### Results

# The synthesis and processing of the wild-type PPT

We expressed the wild-type PPT in COS-1 cells to dissect the intracellular processing and trafficking of the enzyme. The polypeptides synthesized in transfected COS-1 cells were labeled for 1 h with [35S]methionine and [35S]cysteine and chased for various periods of time prior to immunoprecipitation with human PPT-specific antibodies.

In SDS-PAGE analysis of immunoprecipitates from the cells harvested directly after a 1 h labeling period or after chase times of 1, 3, 6 or 12 h, the wild-type PPT appeared as a doublet of 39 and 37 kDa bands. The amount of the intracellular PPT decreased with increasing chase times. In the culture medium, the PPT polypeptides became visible after a minimum chase time of 1 h, and the amount of PPT in the medium increased when the chase times were prolonged, evidencing the secretion of PPT. The pattern of the extracellular PPT polypeptides was slightly different compared with the intracellular one. In addition to the 39 and 37 kDa bands, distinct bands of 39.5 and 37.5 kDa were detectable (Figure 1).

The appearance of PPT as a doublet in SDS-PAGE gels may indicate differential glycosylation of the polypeptides, which would be analogous to the bovine PPT (Camp et al., 1994). Based on the deduced amino acid sequence, the human PPT contains three putative *N*-glycosylation sites. We transfected COS-1 cells with the wild-type PPT cDNA construct and labeled the cells for 1 h. The cells



**Fig. 2.** Sensitivity of the PPT polypeptides to Endo H and PNGase F glycosidases. Cells were transfected with the wild-type PPT cDNA construct and labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 1 h and chased for 0 h (lanes 1–3) or 3 h (lanes 4–6). The immunoprecipitates were not treated (lanes 1 and 4) or treated with either Endo H (lanes 2 and 5) or PNGase F (lanes 3 and 6). The molecular weights of the PPT polypeptide chains are indicated.

were harvested for immunoprecipitation after chase times of 0, 3, 6 or 12 h. The immunoprecipitated PPT from the cell lysates was treated with either Endo H or PNGase F glycosidase. Both the Endo H and PNGase F treatment resulted in a single band of 32 kDa in SDS-PAGE analysis. The results obtained using either no chase or a 3 h chase are shown in Figure 2. The data indicate that heterogenous glycosylation is the major cause of the multiple PPT bands observed, and that the oligosaccharides attached to PPT in COS-1 cells are of the high mannose type.

## Evidence for lysosomal targeting of PPT

The localization of the intracellular PPT was studied using immunofluorescence analysis on transfected COS-1 cells. The cells were incubated for 3 h in medium containing 50  $\mu$ g/ml cycloheximide to prevent continuous protein synthesis prior to fixation. The PPT protein perfectly colocalized with the staining of co-transfected lysosomal  $\beta$ -subunit of  $\beta$ -hexosaminidase. The intracellular distribution of the wild-type PPT was distinctly different from the staining obtained with ER- (mAb 1D3) or Golgispecific (anti- $\beta$ -COP mAb maD) antibodies (Figure 3).

Since many mammalian lysosomal enzymes are targeted to lysosomes via a mannose 6-phosphate receptor-mediated pathway, we analyzed the phosphorylation of mannose residues on PPT polypeptides. COS-1 cells transfected with the wild-type PPT construct were labeled with [32P]orthophosphate for 2.5 h, chased for 4 h and the polypeptides were immunoprecipitated. The phosphate label was incorporated into the PPT protein. Further, the observed labeling was sensitive to Endo H glycosidase treatment, indicating the presence of the phosphate group on the oligosaccharides attached to PPT (Figure 4).

Most lysosomal enzymes characteristically are partially secreted after their synthesis, while the bulk of the polypeptides is transported to lysosomes. The secreted enzymes carrying correct targeting signals typically can be endocytosed by the cells. The uptake of recombinant PPT by COS-1 cells was monitored by collecting the extracellular, [35S]methionine and [35S]cysteine-labeled PPT from the medium, and feeding the polypeptides to unlabeled COS-1 cells. As seen in Figure 4, the radio-labeled PPT became cell associated. This cell association

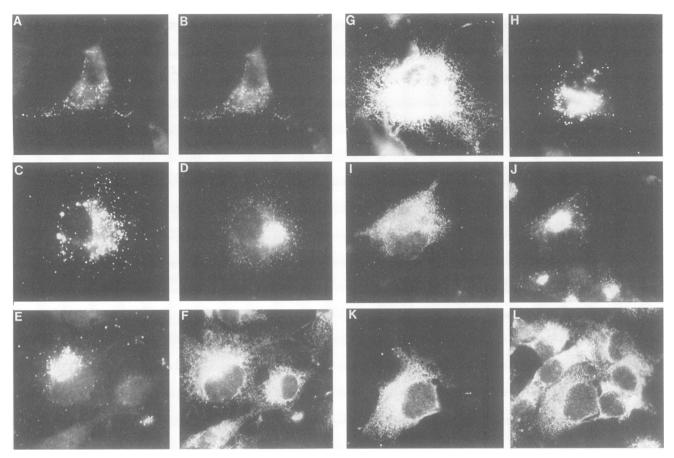


Fig. 3. Intracellular localization of the wild-type and the Arg122Trp mutant PPT in transiently transfected COS-1 cells after blocking the continuous protein synthesis by cycloheximide treatment (50  $\mu$ g/ml) for 3 h. The staining pattern of the cells transfected with the wild-type PPT cDNA construct (**A**, **C** and **E**) and with the Arg122Trp mutant PPT cDNA construct (**G**, **I** and **K**) is shown. Also shown are the lysosomal (**B** and **H**), Golgi (**D** and **J**) and ER (**F** and **L**) staining patterns of the same cells. The lysosomal staining was visualized with antibody against the cotransfected  $\beta$ -subunit of  $\beta$ -hexosaminidase and FITC-conjugated secondary antibody. The Golgi staining was visualized with antibody against  $\beta$ -COP and the ER staining with antibody to the KDEL motif of the ER protein, both detected with TRITC-conjugated secondary antibody. Staining of PPT was visualized with antibody against human PPT and either TRITC-conjugated (A and G) or FITC-conjugated (C, E, I and K) secondary antibody as described in Materials and methods.

was competitively inhibited by the addition of 5 mM mannose 6-phosphate to the culture medium, suggesting that the enzyme was most likely endocytosed by the mannose 6-phosphate receptor-mediated pathway.

We also studied the effect of NH<sub>4</sub>Cl added to the culture medium on the distribution of the cell-associated and extracellular PPT. As expected (Hasilik and Neufeld, 1980), the NH<sub>4</sub>Cl treatment resulted in the enhanced secretion of the PPT polypeptides in the culture medium and decreased amounts of intracellular PPT compared with that of the non-treated cells (data not shown).

#### Abnormal intracellular targeting of the mutant PPT

The cellular consequences of the Arg122Trp mutation on the PPT polypeptide were also analyzed by an *in vitro* expression system. The Arg122Trp mutant polypeptides, like the wild-type PPT, appeared as bands of 39 and 37 kDa in SDS-PAGE gels when immunoprecipitated from cells after their labeling with [35S]methionine and [35S]cysteine for 1 h. The mutant PPT was detectable intracellularly up to a 12 h chase as the 39 kDa/37 kDa doublet. In contrast to the wild-type PPT, the majority of the mutant PPT remained intracellular. Trace amounts of the mutant polypeptides were detectable in the medium after a minimum chase time of 3 h. Secretion of the

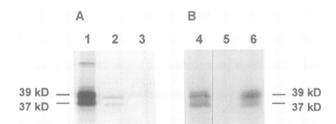
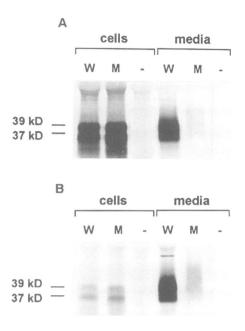


Fig. 4. Phosphorylation and endocytosis of PPT. (A) [35S]methionine-and [35S]cysteine-labeled, secreted PPT collected from the medium (lane 1) was fed back to the unlabeled cells. The endocytosed PPT was detected inside the cells as the 39 kDa/37 kDa doublet after immunoprecipitation with anti-human PPT antibodies (lane 2). Addition of 5 mM mannose 6-phosphate to the culture medium inhibited endocytosis (lane 3). (B) COS-1 cells were transfected with the wild-type PPT cDNA construct, labeled for 2.5 h with [32P]orthophosphate or [35S]methionine and [35S]cysteine and chased for 4 h. The PPT polypeptides were immunoprecipitated from the medium and subjected to 11% SDS-PAGE followed by fluorography. Lane 4: [35S]methionine- and [35S]cysteine-labeled PPT. Lane 5: [32P]orthophosphate-labeled PPT polypeptides treated with Endo H after immunoprecipitation. Lane 6: [32P]orthophosphate-labeled PPT polypeptides.

mutant protein was thus significantly reduced compared with that of the wild-type protein (Figure 5).

In immunofluorescence studies, the Arg122Trp mutant



**Fig. 5.** Pulse–chase analysis of the Arg122Trp mutant PPT expressed in COS-1 cells. Cells transfected with either the wild-type (W) or mutant (M) PPT cDNA constructs or with the pCMV5 vector (–) were pulse labeled with [35S]methionine and [35S]cysteine for 1 h and chased for (**A**) 3 h or (**B**)12 h. The polypeptides were immunoprecipitated from both the cells and media. run on 11% SDS–PAGE gels and visualized by fluorography. The gels were overexposed intentionally for 4 days to detect the intracellular PPT after a chase time of 12 h. The molecular weights of the PPT polypeptide chains are indicated.

protein was localized to the ER. The reticular staining pattern of COS-1 cells transfected with the mutant cDNA construct perfectly co-localized with the staining obtained using the 1D3 monoclonal antibody to the KDEL motif of the ER protein. Staining of the mutant PPT was easily discriminated from that of the co-transfected  $\beta$ -subunit of  $\beta$ -hexosaminidase or that obtained with Golgi-specific (anti- $\beta$ -COP mAb maD) antibodies (Figure 3).

# **Discussion**

Here we have identified the lysosomal targeting of the wild-type PPT via the mannose 6-phosphate receptormediated pathway. This was demonstrated by the phosphorylation of the PPT oligosaccharides, which was sensitive to glycosidase treatment, endocytosis of PPT and its competitive inhibition by mannose 6-phosphate added to the culture medium, NH<sub>4</sub>Cl-induced secretion and co-localization of PPT with the β-subunit of β-hexosaminidase in immunofluorescence. In addition to the lysosomal targeting, PPT was secreted into the medium. Such partial secretion is a feature typical of most lysosomal enzymes (Ludwig et al., 1994). The actual proportion of secreted PPT cannot be determined from the transient expression systems, where overexpression may cause increased secretion, as shown for many lysosomal enzymes (for references, see Ioannou et al., 1992). Establishment of stable cell lines and monitoring the secretion in cells of different tissue origin are required to demonstrate the actual proportions of intra- and extracellular PPT in vivo.

The trafficking of the Arg122Trp mutant polypeptides was strikingly different from that of the wild-type counter-

parts. Most importantly, the mutant polypeptides were retained in the ER and were not targeted to lysosomes. In addition, the mutant PPT expressed in COS-1 cells was inactive, as we have demonstrated previously (Vesa *et al.*, 1995). Arg122 resides immediately adjacent to the consensus lipase sequence (Derewenda and Sharp, 1993), which contains the putative active site serine of thioesterases (Witkowski *et al.*, 1994). The substitution by tryptophan most probably triggers a conformational change in the PPT polypeptide, resulting in incorrect folding of PPT and its inability to pass the quality control in the ER. We also found traces of the inactive mutant PPT in the medium. Whether the mutant polypeptides are partially secreted also in affected human subjects remains to be confirmed.

The defective targeting of the mutant PPT to lysosomes would classify INCL under the lysosomal enzyme deficiencies. INCL could be caused by the neurotoxic effect of the deficiently hydrolyzed membrane-bound palmitoylated proteins which are targeted to lysosomes for degradation. Although the inclusions observed in INCL tissues do not have the vacuolar appearance characteristic of lysosomal accumulations, their lysosomal origin has actually been suggested based on their acid hydrolase activity (Haltia et al., 1973). However, since PPT also seems to be secreted, the existence of an extracellular substrate for the enzyme remains a possibility. For example, many myelin proteins have been shown to be prenylated and palmitoylated (Bizzozero and Good, 1991; Sepp-Lorenzino et al., 1994). Intriguingly, progressive demyelination of cortical neurons has been demonstrated in INCL brains (Haltia, 1982), and this specific destruction is also seen in the retina, the optic nerve being atrophic with complete loss of the myelin sheath (Tarkkanen et al., 1977). However, so far only a single lysosomal enzyme, cathepsin L, is known to have both a lysosomal and an extracellular function (Maciewicz et al., 1989; Yagel et al., 1989). To understand how the defect in the PPT enzyme causes the specific degeneration of cortical neurons requires identification of the *in vivo* substrate(s) for PPT.

PPT clearly removes palmitate from H-Ras and  $G_{\alpha}$ in vitro. However, due to its lysosomal targeting, the PPT enzyme cannot be involved in the cytosolic recycling pathway of the palmitic groups. In addition to PPT, only two other enzymes removing palmitate from distinct proteins have been purified to homogeneity: proteolipid protein fatty acylesterase from rat brain myelin (Bizzozero et al., 1992) and fatty acylesterase depalmitoylating the Semliki forest viral E2 protein in microsomal membranes (Berger and Schmidt, 1986). Palmitoyltransferase activity has been detected in the pre/early Golgi compartment, the plasma membrane and the Golgi complex (Gutierrez and Magee, 1991; Omary and Trowbridge, 1981; Veit and Schmidt, 1993). Recently, a membrane-bound palmitoyl acyltranferase that palmitoylates myristoylated proteins has been purified (Berthiaume and Resh, 1995). This suggests that multiple palmitoyltransferases with different subcellular locations may exist. Accordingly, several thioesterases identifying distinct substrates and occupying the same cellular compartments as the palmitoyltransferases could exist to guarantee the proper functioning of the palmitoylation—depalmitoylation cycles of

proteins. PPT could thus be a lysosomal member of a family of mammalian thioesterases.

## Materials and methods

#### Antibodies

To obtain the PPT protein for immunization, the *PPT* cDNA was subcloned into the pGEX-2T vector (Pharmacia), and the PPT polypeptides were expressed in *Escherichia coli* strain BL21 as a glutathione *S*-transferase (GST) fusion protein and purified from the SDS-PAGE gel. Rabbits were immunized by subcutaneous injection with 300 μg of PPT fusion protein in Freund's complete adjuvant. The blood was collected 1 week after the last immunization and the antibody titers and specificity were determined by immunofluorescence, Western blotting and immunoprecipitation. The antiserum recognized both the native and denatured PPT protein. The mAb 1D3 was a kind gift from Dr Stephen Fuller (Vaux *et al.*, 1990), the mAb maD from Drs Thomas Kreis and Ragna Rönnholm (Pepperkok *et al.*, 1993) and goat anti-β-hexosaminidase A from Dr Richard Proia (Proia *et al.*, 1984).

#### COS cell culture and transfection

COS-1 cells (ATCC CLR 1650) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal calf serum (FCS). One day prior to transfection, the cells were seeded on 3 cm cell culture dishes at  $4\!\times\!10^5$  cells per dish. The cells were transfected either with 5  $\mu g$  of the wild-type or the Arg122Trp mutant PPT cDNA construct in a pCMV5 vector or with the cDNA construct of the  $\beta$ -subunit of  $\beta$ -hexosaminidase (Weitz and Proia, 1992) using the lipofectamine transfection method. Analysis was performed 48 h post-transfection. A pCMV5 expression vector control was used to monitor the background expression of PPT in COS-1 cells, and is designated as (–) in the figures.

#### Pulse labeling and immunoprecipitation

Cells were starved in methionine- and cysteine-free medium for 1 h and thereafter labeled with 100 µCi/ml of both [35S]cysteine and [35S]methionine (Amersham). After a 1 h pulse, the cells and media were either harvested immediately or the cells were subjected to a 1-12 h chase in DMEM without FCS. Labeling with 400 µCi/ml of [32P]orthophosphate was carried out in phosphate-free medium for 2.5 h followed by a 4 h chase. NH<sub>4</sub>Cl (10 mM) was added 2 h before metabolic labeling and was used in the same concentration throughout the experiment. The cells were harvested and lysed by freezing and thawing in phosphate-buffered saline (PBS) containing 1% Triton X-100. Proteins from the collected media were concentrated by Centricon-10 (Amicon) prior to immunoprecipitation. Immune complexes were collected using the rabbit antiserum against human PPT bound to protein A-Sepharose (Sigma), washed twice with 50 mM Tris pH 7.4, 5 mM EDTA, 1% Triton X-100, 500 mM NaCl, 5% sucrose and once with 10 mM Tris pH 6.8, 1 mM EDTA. The polypeptides were separated on 11% SDS-PAGE gels (Laemmli, 1970) and visualized by fluorography (Amplify, Amersham). Endo H and PNGase F digestions were performed as described previously (Tikkanen et al., 1995).

# Endocytosis of recombinant PPT and its inhibition with mannose-6-phosphate

Six 3 cm cell culture dishes each containing  $4\times10^5$  COS-1 cells were transfected with the wild-type PPT cDNA construct and labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 21 h. The media containing the secreted PPT polypeptides were collected and concentrated by Centricon-10 (Amicon). Unlabeled COS-1 cells grown to 80% confluency in a 3 cm dish were incubated in the concentrated media with a total activity of  $2\times10^7$  c.p.m. for 21 h with or without 5 mM mannose 6-phosphate, and the cells were harvested for immunoprecipitation.

#### Immunofluorescence microscopy

COS-1 cells were grown on coverslips and methanol fixed 72 h after transfections using DEAE-dextran (Sussman and Milman, 1984). After washing with PBS, non-specific binding was suppressed by incubating the coverslips in PBS containing 0.5% bovine serum albumin (BSA) for 15 min at room temperature. The coverslips were incubated with rabbit anti-human PPT, goat anti-β-hexosaminidase A, anti-KDEL MAb 1D3 or anti-β-COP MAb maD for 45 min diluted in PBS containing 0.5% BSA at room temperature. This was followed by extensive washes in PBS/0.5% BSA. The primary antibodies were detected by incubation

for 45 min at room temperature with FITC-conjugated goat anti-rabbit antibodies (Immunotech), TRITC-conjugated goat anti-mouse antibodies (Pierce) or, in the double staining experiments, with co-transfected PPT and β-hexosaminidase constructs with TRITC-conjugated goat antirabbit antibodies (Immunotech) and FITC-conjugated mouse anti-goat antibodies (Immunotech) diluted in 0.5% BSA in PBS. The coverslips were mounted in Mowiol and viewed with a Zeiss Axiophot immunofluorescence microscope using 630× magnification.

# **Acknowledgements**

We thank Ms Tuula Airaksinen for expert technical assistance and Dr Stephen Fuller (1D3), Dr Richard Proia ( $\beta$ -hexosaminidase A) and Drs Thomas Kreis and Ragna Rönnholm (maD) for providing the antibodies. This work was financially supported by The Academy of Finland (the Genome program) and the Sigrid Juselius Foundation.

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Received on May 13, 1996; revised on June 17, 1996