Importance of splicing for prosaposin sorting

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The prosaposin gene encodes a 70 kDa protein. This protein might either reach the lysosomes and get processed there to four peptides, which are activators of known lysosomal enzymes, or be secreted by cells as a 70 kDa protein, recently anticipated to have several biological activities. The human prosaposin gene has a 9 bp exon (exon 8) that is alternatively spliced, thus encoding three prosaposin forms: one with an extra three amino acid residues, one with an extra two residues and a third form with no extra residues. With the aim of testing whether there is an association between the alternative splicing and the differential sorting of prosaposins, we cloned two human prosaposin cDNA forms in a T_7/EMC/vaccinia virus-derived vector and expressed them in human cells. The results indicated that the prosaposin containing the three extra residues accumulated faster and in greater amounts in the medium, whereas the prosaposin with no extra residues was mainly destined for lysosomes. Point mutations created by mutagenesis *in vitro* in the 9 bp stretch had a diverse effect on prosaposin secretion. When supplied to cells in the medium, both prosaposins were endocytosed and reached the lysosomes, where they were processed to active saposin B and saposin C. The activities of the saposins were monitored qualitatively and quantitatively. Quantitatively, lipids were extracted from the cells, separated on TLC and measured fluorimetrically. Qualitatively, cells were detected by fluorescence microscopy.

INTRODUCTION

Several lysosomal hydrolases involved in the catabolism of complex sphingolipids require interactions with activator proteins for optimal hydrolytic activity. These activator proteins are heat-stable low-molecular-mass peptides [1,2]. Studies *in vitro* have shown that saposin B (also designated SAP-B or SAP-1, for sphingolipid activator protein-1) participates in the hydrolysis of sulphatide by arylsulphatase A, sphingomyelin by sphingomyelinase and globotriaosylceramide by β-galactosidase A [3]. Saposin C (or SAP-C or SAP-2) enhances the hydrolysis of glucosylceramide by β-glucocerebrosidase [3,4]. Saposin D participates in the hydrolysis of ceramide catalysed by ceramidase [5]; saposin A activates the degradation of galactosylceramide [6]. Another activator protein is required for the degradation of GM2 ganglioside by β-*N*-acetylhexosaminidase A [7]. The physiological significance of these activators was demonstrated by the observation that a deficiency in a specific saposin results in a disease similar to that observed when the activity of the corresponding enzyme is decreased drastically. Thus metachromatic leucodystrophy results from mutations inactivating either the enzyme arylsulphatase A or its activator saposin B (reviewed in [3]). Gaucher disease is due to the inactivation of glucocerebrosidase (reviewed in [8]) or a deficiency of saposin C [9]. Tay–Sachs can result from the inactivation of β-*N*-acetyl hexosaminidase A or an absence of its corresponding activator [3].

Results from several laboratories have demonstrated that one gene, designated prosaposin, encodes for the four saposins [3]. It has already been shown that the prosaposin gene is regulated in a differential manner [10]. Results of *in situ* hybridization experiments on 12.5-day-old mouse embryos indicated high expression in the hindbrain, the dorsal ganglia and the genital ridge (the primordium for both the male and the female gonads). In adults, prosaposin mRNA was detected in the male testis Sertoli cells, Leydig cells and peritubular cells and also in the corpus luteum of the female gonads [11]. Immunohistochemical analyses demonstrated expression in mouse testis Sertoli cells, in small intestine and large intestine enterocytes, in type II pneumocytes and macrophages of the lung, in several neuronal cells of retinal layers and in the epithelial cells lining the proximal convoluted tubules of the kidney [12].

The prosaposin gene is transcribed to two major mRNA species and a minor species in normal individuals (Figure 1). Besides the shortest mRNA, which is co-linear with the amino acid sequence of saposin B, there is a longer species with an additional stretch of nine bases within the saposin B coding domain as well as a minor mRNA species with only six bases of the nine-base stretch [13,14]. These different mRNA species result from alternative splicing. The extra 9 bp represent a separate exon whose sequence is CAGGATCAG and is preceded by the sequence gttcaacag (gttcaacagCAGGATCAG), which serves as an acceptor (3′) splice site. Within the 9 bp exon the AG preceding the six bases GATCAG might also serve as an acceptor site. There is no clue as to the function of the three alternative mRNA species. The shortest mRNA seems to be functional. It is co-linear with the amino acid sequence of saposin B [15].

Prosaposin is not only destined for lysosomes, where it is processed to the four different saposins, which have a known biological function as activators of lysosomal enzymes: its 70 kDa counterpart has been identified as the major product secreted by Sertoli cells [16,17] and in several other body fluids [18,19]. Prosaposin was shown to be active in a variety of neuronal cells including hippocampal neurons [20,21], spinal cord α-motor neurons [22], cerebellar granule cell neurons [23] and neuroblastoma cells [20,22–25]. In each of these cells prosaposin stimulated neurite outgrowth and prevented cell death. In

Abbreviations used: Br-CBE, bromoconduritol-B-epoxide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LR12-CS, lissamine rhodamine-12-cerebroside sulphate (sulphatide); LR12-GC, lissamine rhodamine-12-glucosylceramide (glucocerebroside); MOI, multiplicity of infection.

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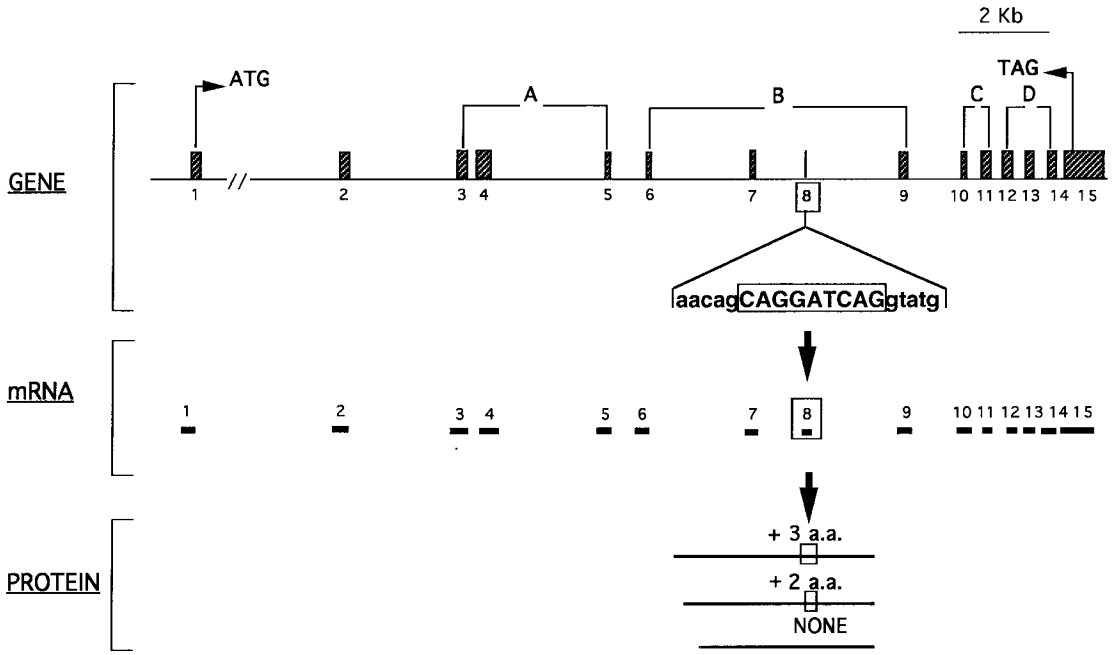

Figure 1 Structure of the human prosaposin gene

The figure depicts the alternatively spliced exon (exon 8) and the three possible proteins that derive from it.

addition, it was shown to stimulate sulphatide synthesis and to increase the sulphatide concentration in Schwann cells and oligodendrocytes, thus indicating its possible role as a trophic factor for myelin formation [26]. Moreover, hypomyelination was demonstrated in the prosaposin knock-out mouse [27]. It was also shown that prosaposin stimulates the phosphorylation of mitogen-activated protein kinase [26].

With the aim of understanding the possible role of alternative splicing in the differential sorting of prosaposins, prosaposin cDNA species with no extra or 9 extra base pairs were cloned into the T_7/EMC/vaccinia virus hybrid expression vector (pTM1). Vaccinia virus, a member of the smallpox virus family, is a cytoplasmic DNA virus, encoding for its own transcription and RNA-modifying enzymes [28]. Vaccinia is widely used as a viral expression vector because of its wide host range (mammalian and avian cells) and because it leads to a high expression level of the target gene. The recombinant vaccinia virus vTF7-3 [29] provides expression of the bacteriophage T_7 RNA polymerase, a prokaryotic enzyme that can transcribe any T_7 promoter-containing DNA introduced into the cytoplasm of the infected cell. The target gene is coupled to the T_7 polymerase promoter, followed by the 5′ encephalomyocarditis virus 5′-untranslated region [30,31]. Recombinant viruses were produced and were used to infect human cells. The results indicated that the prosaposin containing the three extra amino acid residues was mainly secreted to the medium, whereas most of the prosaposin containing no extra residues was sorted to the lysosomes. When expressed as recombinant proteins, both prosaposins reached the lysosomes and were processed there to saposin B and saposin C. When supplied to human prosaposin-deficient cells, they were endocytosed, reached the lysosomes and processed there to saposins B and C, which activated endogenous glucocerebrosidase or arylsulphatase A activity respectively.

MATERIALS AND METHODS

Cells and viruses

HeLa cells and BSC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). Human foreskin fibroblasts (FS11) were grown in DMEM supplemented with 20% (v/v) FCS. Primary Gaucher fibroblasts and human prosaposin-deficient fibroblasts were grown in minimal essential medium containing 20% (v/v) FCS. When cells were grown in FCS-depleted medium they were supplemented with 1 × OPI (100 × OPI is 10 mM Na pyruvate/20 mM oxaloacetic acid/20 i.u./ml insulin). All cells were grown at 37 °C in the presence of air/CO_2 (19:1). Wild-type vaccinia virus (v-WR), vTF7-3 (expressing the T7 RNA polymerase) and recombinant viruses were propagated in HeLa cells. Titres were determined with BSC-1 cells. Stocks were stored at −80 °C.

Primers

The primers used were as follows: 1, TACGCCCTCTTCCTCCTGG (3736); 2, GAGGGTAGAGGAGGAGAG (1642); 3, AGATCTCCTTGGGTTGCATGTGCATCATCATC (LO3); 4, GATGATGATGCACATGCAACCCAAGGAGATCT (LO5); 5, GCCAGAGCAGAGGTGCAG (3655); 6, ATGCACATG**G**AGGATCAG**C**AGCAA (211074); 7, ACATG CAGG**C**TCAG**C**AACC (19823); 8, ATG**C**AGGAT**G**AG**C**AACCCAA (202526).

Plasmid construction

The construction of recombinant vaccinia virus containing the human prosaposin cDNA harbouring the extra 9 bp is depicted in

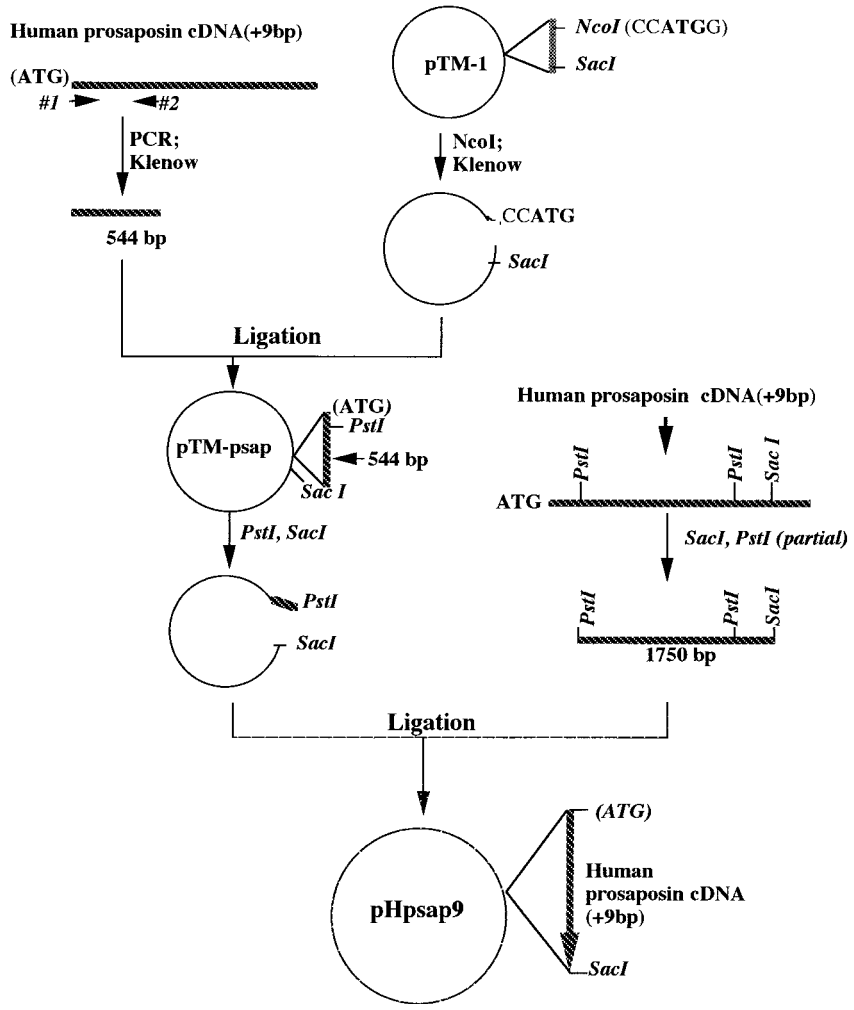

Figure 2 Construction of the pTM1-derived plasmid containing the human prosaposin cDNA with the extra nine base pairs

The 5′ sequence of the prosaposin cDNA was amplified by PCR with primers 1 and 2 (for details see the Materials and methods section). The 544 bp PCR fragment was ligated to the pTM1 vector after its digestion with *Nco*I and blunting of the cohesive ends. The resulting vector (pTM-psap) was digested with *Sac*I and *Pst*I, then ligated with a 1750 bp *Pst*I (partial)– *Sac*I fragment of the 3′ end of the human prosaposin cDNA to yield a plasmid containing the full coding prosaposin cDNA, designated pHpsap9.

Figure 2. The 5′ sequence of the prosaposin cDNA was amplified by the PCR technique by using primer 1, which was designed to amplify sequences immediately downstream of the prosaposin initiating methionine and primer 2, in the anti-sense orientation (see the list of primers). The 544 bp PCR product was ligated to the pTM1 vector after its digestion with *Nco*I and blunting of the cohesive ends with the Klenow fragment of *Escherichia coli* DNA polymerase I. The resulting vector (pTM-psap) was then digested with *Sac*I and *Pst*I and ligated with a 1750 bp *Pst*I–*Sac*I fragment from the 3′ end of the human prosaposin cDNA. This fragment was obtained by cleavage of a plasmid containing the prosaposin cDNA with *Sac*I followed by partial digestion with *Pst*I. The plasmid containing the full coding prosaposin cDNA region was designated pHpsap9.

In vitro mutagenesis

The prosaposin cDNA with no extra base pairs was generated by PCR. As shown in Figure 3, four primers were used to amplify two cDNA fragments (see the list of primers). Primers 1 and 3 amplified a 780 bp fragment and primers 4 and 5 amplified a 410 bp fragment. The two fragments were isolated from a 1% (w/v) agarose gel with the Jetsorb kit (Genomed, GMBH, Bad Oeynhausen, Germany), annealed through their 32 bp identical sequences and the extended fragment was amplified by using primers 1 and 5 to give rise to a 1190 bp fragment. This fragment was digested with the restriction enzymes *Nde*I and *Eco*RI to yield a 238 bp *Nde*I–*Eco*RI fragment (without the extra 9 bp), which was ligated to pHpsap9 after its digestion with *Eco*RI and partial cleavage with *Nde*I. The authenticity of the resulting vector, designated pHpsap0, was confirmed by sequencing.

Point mutations in the extra 9 bp (exon 8) of the human prosaposin cDNA were generated by *in vitro* mutagenesis with the Transformer Site Directed Mutagenesis Kit (Clontech, Palo Alto, CA, U.S.A.) and appropriate primers. Each of the three residues (Gln-Asp-Gln) was mutated independently within the pHpsap9 vector, by using the mutated oligonucleotide primers 6 (for M1), 7 (for M2) and 8 (for M3).

Generation of recombinant vaccinia virus

Generation of recombinant vaccinia viruses was essentially as

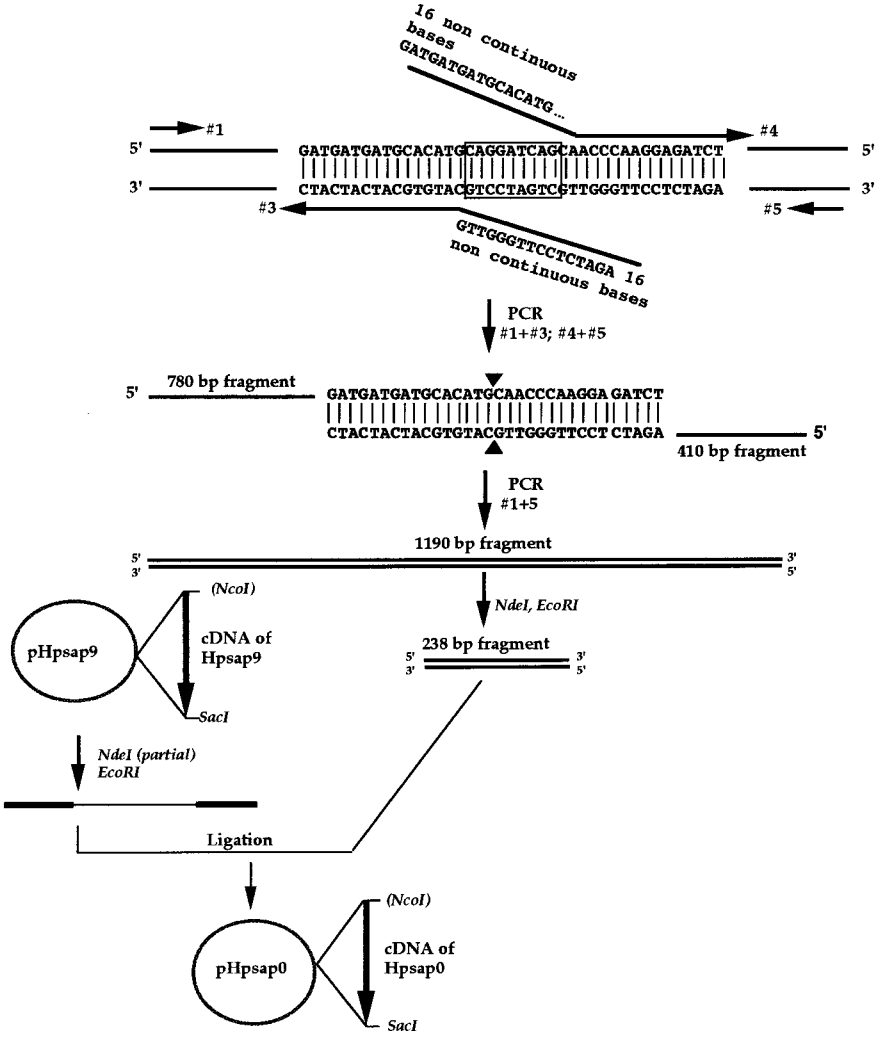

Figure 3 Construction of the pTM1-derived plasmid containing the human prosaposin cDNA without extra base pairs by *in vitro* mutagenesis

Four primers were used to amplify two cDNA fragments. Primers 1 and 3 (see the Materials and methods section) amplified a 780 bp fragment and primers 4 and 5 amplified a 410 bp fragment. The two fragments were isolated from a 1% (w/v) agarose gel and subjected to a second PCR with primers 1 and 5 (see the Materials and methods section). The two fragments were annealed through their 32-bp identical sequence and the extended fragment was amplified to give rise to a 1190 bp fragment. This fragment was digested with *Nde*I and *EcoRI*. The 238 bp NdeI–*Eco*RI fragment was ligated with pTM1-Hpsap9 after its digestion with *Nde*I (partial) and *Eco*RI. Authenticity was determined by sequencing. The resulting vector without the extra 9 bp was designated pHpsap0.

described elsewhere [32]. The virus containing prosaposin with the extra 9 bp was designated Hpsap9 and the one without it Hpsap0. Viruses carrying mutations in the extra 9 bp of the prosaposin cDNA were designated Hpsap9M1 (first residue Glu), HpsapM2 (second residue Ala) and HpsapM3 (third residue Glu).

RNA preparation

Subconfluent HeLa cells (5×10^6 cells per plate) were co-infected with vTF7-3 and one of the recombinant viruses at a multiplicity of infection (MOI) of 10 plaque-forming units per cell. After 48 h the medium was aspirated and RNA was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). The RNA pellet was air-dried and resuspended in 0.1 ml of distilled water.

Northern blot analysis

Loading buffer was added to the RNA samples and incubated for 10 min at 65 °C. Dye marker was added and the samples were loaded on a 6% (v/v) formaldehyde/1% (w/v) agarose gel. After electrophoresis (4 h, 50 V), the gel was soaked for 45 min in $10 \times$ SSC and blotted overnight in $20 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate). The filter (Hybond N) was baked for 2 h at 80 °C [33]. Hybridization and washes were performed as described elsewhere [34].

Metabolic labelling

Cells were infected with the recombinant viruses as described above. After 24 h, medium was removed and methionine-depleted DMEM was added for 30 min; 0.5 ml of methionine-depleted medium containing 190 mM NaCl and 20 μCi of [^{35}S]methionine (3000 Ci/mmol; Amersham) was added to

5×10^5 cells. After a 1 h pulse, medium was removed and fresh DMEM containing either FCS or OPI supplements was added as described. At the end of the labelling or after different durations of chase, medium was collected and the cells were lysed by the addition of 0.5 ml of PBS containing 1 % (v/v) Nonidet P40, 2 mM EGTA, 5 mM $MgCl_2$ and 1 mM PMSF. Cell lysate samples containing the same amount of radioactivity were subjected to SDS/PAGE [10 % (w/v) gel] [35]. Gels were fixed, stained and dried after treatment with Amplify solution (Amersham) for 30 min. Samples of medium containing the same relative amount of cellular radioactivity were subjected to electrophoresis as described above.

Immunoprecipitation

Medium was collected and samples, determined as described above, were immunoprecipitated with anti-(saposin C) polyclonal antibodies [36] by using the method described previously [37]. The precipitates were subjected to SDS/PAGE [15 % (w/v) gel]; the gel was then fixed, dried and exposed to an X-ray film (Rx; Fuji). Quantification was performed with an Agfa PhosphorImager (Fuji bas 1000).

Loading experiments

Loading experiments were performed essentially as described elsewhere [32,38–40]. Cells (5×10^5) were supplemented with 10 nmol/ml lissamine rhodamine-12-glucosylceramide (glucocerebroside) (LR12-GC) or lissamine rhodamine-12-cerebroside sulphate (sulphatide) (LR12-CS) (synthesized by a modification of the method of Marchesini et al. [41]) for 48 h together with 20 μM of bromoconduritol-B-epoxide (Br-CBE) (kindly provided by Dr. Legler, University of Cologne, Cologne, Germany). Cells were infected in the same medium with vTF7-3 and each of the recombinant viruses as described above; 3 h later, medium was removed, cells were washed twice with PBS and DMEM without Phenol Red, supplemented with 20 % (v/v) FCS, was added to cells. After 18 h, cells were washed with PBS and lysed in 0.5 ml of distilled water. After protein determination, cell lysate was extracted with chloroform/methanol (1:1, v/v) and dried. Chloroform/methanol (1:1, v/v) (50 μl) was added and the samples were separated by TLC (Silica gel 60A; Whatman) in chloroform/butanol/ethyl acetate/0.25 % KCl/methanol (25:25:25:9:16, by vol.). Spots on the TLC plates were revealed under a UV lamp. Glucosylceramide or cerebroside sulphate and ceramide were scraped off the plate and eluted with chloroform/methanol (1:1, v/v) and their fluorescence was measured in a spectrofluorimeter (Perkin–Elmer Luminescence Spectrometer LS 50; excitation wavelength 560 nm; emission wavelength 580 nm).

Fluorescent staining of cells

Cells (10^4) were grown on coverslips and loaded with 10 nmol of LR12-GC for 24 h; chase was performed for 24 h. At the end of the chase, the coverslips were fixed in 3.7 % (v/v) formaldehyde for 30 min at room temperature. After washes in PBS, the slides were glued with Galvanol mounting solution.

Preparation of medium containing prosaposin

HeLa cells (5×10^5) were infected with vTF7-3 and each of the recombinant viruses harbouring the prosaposin cDNA species as described above. After 48 h, medium was collected, centrifuged at 15000 ***g*** and filtered through membrane (pore size 0.25 μm) to remove virus residues. Purified medium was frozen in aliquots at −20 °C.

Protein determination

Protein content was determined by the Bradford technique [42].

Restriction enzymes

Restriction enzymes were purchased from several companies and employed in accordance with manufacturers' recommendations.

Random-primed labelling

A kit was purchased from Fermentase and labelling was performed in accordance with the manufacturer's recommendations.

RESULTS

Construction of recombinant vaccinia virus containing the human prosaposin cDNA

To establish an efficient expression system for the human prosaposin cDNA species that would enable us to study their sorting, we used the hybrid T_7/EMC/vaccinia virus-derived expression system. The prosaposin cDNA species containing no extra or 9 extra base pairs were introduced into the pTM1 vector as shown in Figures 2 and 3 respectively and as explained in the Materials and methods section. Recombinant viruses were produced and designated Hpsap9 and Hpsap0 respectively.

RNA expression

To follow protein processing it was important to demonstrate that the steady-state RNA levels directed by the different recombinant viruses are comparable. To that end, HeLa cells were co-infected with vTF7-3 and either Hpsap9 or Hpsap0 at an

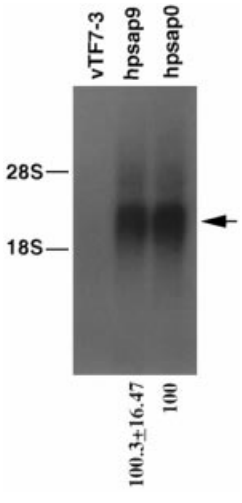

Figure 4 Northern blot analysis of human prosaposin RNA

HeLa cells were co-infected with vTF7-3 and the viruses harbouring the different prosaposin cDNA species. At 18 h after infection, RNA was extracted and subjected to formaldehyde/agarose gel electrophoresis, then blotted and hybridized with ^{32}P-labelled human prosaposin cDNA. The blot was washed and rehybridized with ^{32}P-labelled human rRNA cDNA (results not shown). The numbers underneath the blot summarize PhosphorImager measurements of four independent experiments, with the results normalized to represent the amount of prosaposin mRNA relative to the rRNA present in each sample. RNA derived from pHpsap0 was taken as 100 %.

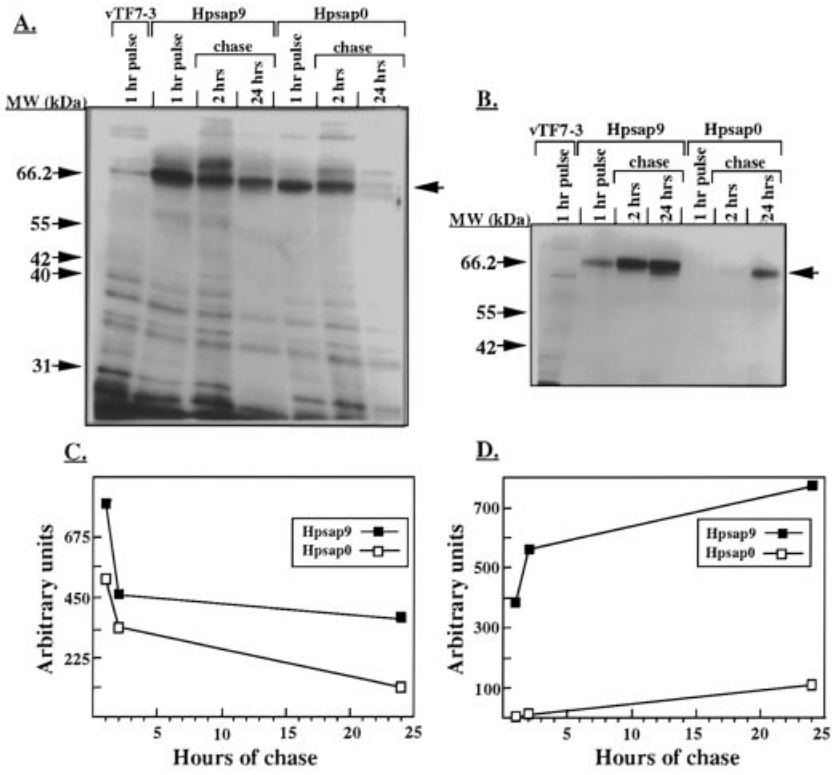

Figure 5 Metabolic labelling of cells

Subconfluent monolayers of normal human fibroblasts were co-infected (MOI = 10 for each virus) with vTF7-3 and each of the different recombinant viruses in serum-free medium supplemented with OPI. After 20 h, cells were metabolically labelled for 1 h with [^{35}S]methionine followed by 2 or 24 h of chase. Cell lysates (**A**) and medium (**B**) were collected. Samples were subjected to SDS/PAGE [10% (w/v) gel]; the gel was then fixed, dried, exposed to an X-ray film, and a PhosphorImager analysis was performed. [Panels (**C**) and (**D**) represent quantification of results presented in (**A**) and (**B**) respectively]. The arrows show the position of prosaposin.

MOI of 10 plaque-forming units per cell of each virus. After 48 h, RNA was extracted and subjected to Northern analysis with prosaposin cDNA as a probe. As shown in Figure 4, a major RNA species, 3 kb in size, was detected. This RNA was highly overexpressed as exemplified by the difference between HeLa cells infected only with vTF7-3 in comparison with cells co-infected with vTF7-3 and each of the recombinant viruses. Under the exposure conditions used in this experiment the endogenous prosaposin RNA levels in HeLa cells infected with vTF7-3 were below the detection level. To quantify prosaposin RNA in the different clones, the filters were rehybridized with a human rRNA cDNA probe and the amount of the prosaposin RNA species was calculated relative to that of the rRNA species. The results indicated that the amounts of steady-state prosaposin RNA originating from the two clones were comparable. Therefore any difference between the two prosaposins could not have been due to different mRNA levels.

Protein expression and processing

To follow the processing pattern and the fate of the two prosaposins, pulse–chase experiments were performed. FS11 subconfluent monolayers were co-infected with vTF7-3 and each of the recombinant viruses. After a 1 h pulse, a chase was performed in serum-free medium supplemented with OPI. At the end of the pulse and after different durations of chase, medium was collected and cell lysates were prepared and subjected to electrophoresis as described in the Materials and methods section. As shown in Figure 5(A), both prosaposin cDNA species directed the synthesis of a predominant 67–70 kDa prosaposin protein that was visible after 1 h of labelling. The amount of this protein decreased in the cells during the chase period. The mature low-molecular-mass saposins could not be detected. However, immunoprecipitation of cell lysates with anti-(saposin C) antibodies revealed a 9–12 kDa protein (see Figure 8A). In the medium (Figure 5B), the prosaposin containing the three extra residues accumulated more rapidly and to higher levels. No immunoprecipitations were needed to detect the prosaposin in the medium when we grew the cells in serum-free medium. To prove that the accumulated 67–70 kDa protein was prosaposin we performed immunoprecipitation. To that end, HeLa cells were co-infected with vTF7-3 and each of the recombinant viruses, and pulse–chase analysis was performed. Medium was collected and cell lysates were prepared. Samples of medium as well as samples from cell lysates were immunoprecipitated with anti-(saposin C) antibodies and subjected to SDS/PAGE. The results again indicated that the prosaposin containing the three extra residues was preferentially secreted from the cells and accumulated in the medium (Figure 6). It is clear from the results presented that the use of the T_7/EMC/vaccinia virus hybrid expression system obviates the need for antibodies for the detection of prosaposin.

In vitro mutagenesis

To investigate the importance of the extra three residues, encoded

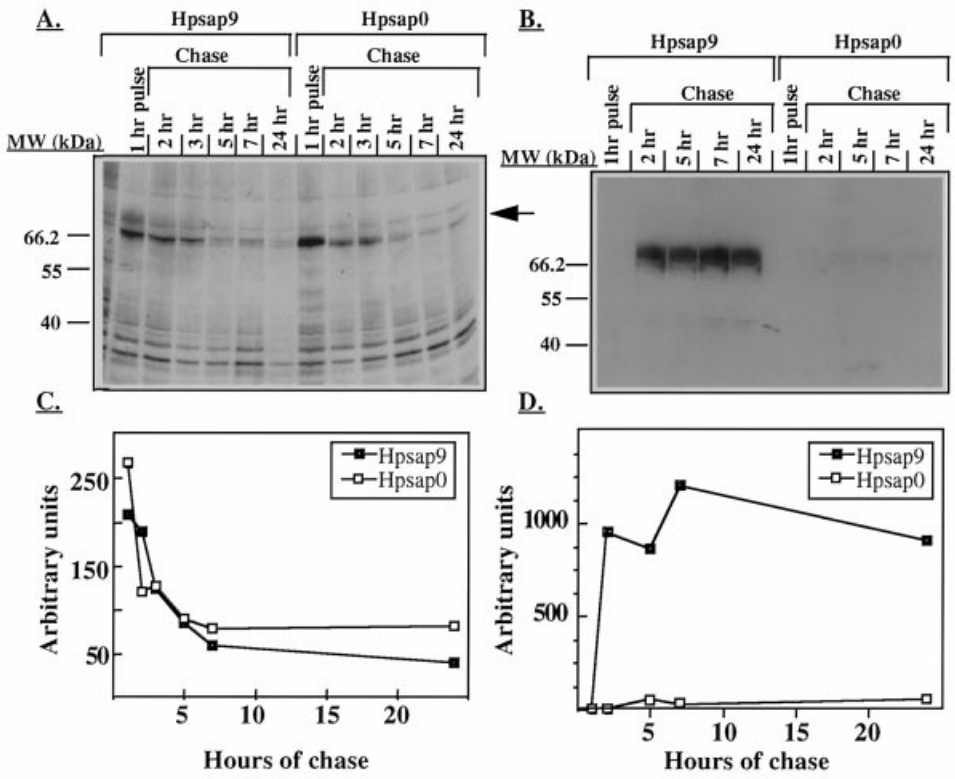

Figure 6 Immunoprecipitation analysis with anti-(saposin C) antibodies

Subconfluent monolayers of normal human fibroblasts were co-infected (MOI = 10 for each virus) with vTF7-3 and each of the different recombinant viruses. After 20 h, cells were metabolically labelled for 1 h with [^{35}S]methionine followed by 2, 3, 5, 7 or 24 h of chase. Cell lysates and medium were collected. Medium samples were immunoprecipitated with anti-(saposin C) antibodies. Samples from cell lysates (**A**) and from immunoprecipitated medium (**B**) (only four chase points were tested) were subjected to SDS/PAGE [10% (w/v) gel]; the gel was then fixed, dried and exposed to an X-ray film, or a PhosphorImager analysis was performed. [Panels (**C**) and (**D**) represent quantification of results presented in (**A**) and (**B**) respectively]. The arrow shows the position of prosaposin.

by exon 8, in prosaposin secretion, mutations were introduced into this exon within the pHpsap9 vector and recombinant viruses were produced. The mutations are depicted in Figure 7. To follow the level of secretion of the mutated and the normal prosaposins, FS11 cells were co-infected with vTF7-3 and each of the mutated recombinant viruses as well as their normal counterparts. After a 1 h pulse and a 3 or 6 h chase, cell lysates and medium were collected, immunoprecipitated with anti-(saposin C) antibodies and subjected to electrophoresis as described in the Materials and methods section. As shown in Figure 8(A), all prosaposin forms (Hpsap0, Hpsap9, HpsapM1-M3) directed the synthesis of a 67–70 kDa protein, which decreased in amount within the cells during the chase period. There was a notable accumulation of one major lysosomal intermediate approx. 15 kDa in size [43]. After 6 h of chase there was already a visible accumulation of saposin C deriving from Hpsap0. The immunoprecipitates of medium samples were subjected to electrophoresis as mentioned above and the gels were subjected to PhosphorImager analysis. The results obtained for prosaposin secretion at 3 h of chase in three independent experiments were analysed statistically by using a Kruskal–Wallis one-way variance test. As shown in Figure 8(B), the level of secretion of the mutated prosaposins differed significantly from that of the normal prosaposin. However, none of the mutations created in exon 8 of the prosaposin cDNA caused a decrease in its secretion to the level observed for the prosaposin containing no extra residues. There was 7.5–15-fold more secretion directed by the different prosaposins containing the three extra residues (normal or mutated) in comparison with the prosaposin without the three extra residues. Replacement of the first glutamine residue by glutamic acid (Hpsap9M1) resulted in a significant decrease ($P = 0.0139$) in prosaposin secretion to the medium. Replacement of the aspartic residue by alanine and the third glutamine residue by glutamic acid resulted in a significant elevation ($P = 0.0193$ for each of them) of the prosaposin levels in the medium after 3 h of chase.

Biological activity

The results presented indicate that the prosaposin with the three extra residues is preferentially secreted from the cells, whereas the other form of prosaposin is mainly sorted to the lysosomes. We wished to test whether both prosaposins, when reaching the lysosomes, could be processed there to active saposins. To that end, prosaposin-deficient cells [44,45] were loaded with either fluorescent glucosylceramide or fluorescent sulphatide, and glucocerebrosidase or arylsulphatase A activities were tested after infection with the different recombinant viruses. As shown in Figure 9(A), the infection of cells, loaded with LR12-GC, with either one of the prosaposins resulted in a more than 2-fold elevation in the amount of LR-ceramide in the cells. As shown in Figure 9(B), infection of cells, preloaded with LR12-sulphatide,

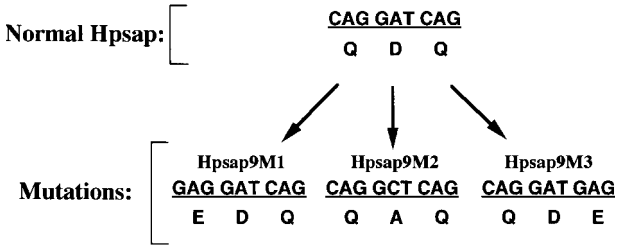

Figure 7 Mutations created in exon 8 of the prosaposin cDNA

Mutations created within the extra three residues are shown.

with the viruses containing the prosaposin cDNA species with an extra 0 bp or 9 bp resulted in 2.9-fold and 1.7-fold elevations respectively in the amount of LR-ceramide in the cells. Thus both prosaposins, when reaching the lysosomes, were processed there to active saposin B and saposin C, which enhanced the hydrolysis of the fluorescent cerebroside sulphate or the glucosylceramide respectively to fluorescent ceramide. Because we have shown that most of the prosaposin containing the three extra residues is secreted (Figures 5 and 6) and can be processed intracellularly to saposins B and C (Figure 9), we assumed that extracellular prosaposin can be endocytosed by cells, reach the lysosomes and be processed there to saposins B and C. To demonstrate directly that, when supplied to cells in the medium, prosaposin can reach lysosomes and be processed there to active saposins, fluorescence microscopy was performed. Prosaposin-deficient human cells (5×10^5) were loaded with 10 nmol/ml LR12-GC for 24 h together with 20 μM bromoconduritol-β-epoxide (Br-CBE). Then 0.5 ml of medium containing prosaposin, collected as explained in the Materials and methods section, was added to the cells for 24 h. The cells were then fixed and visualized in a fluorescence microscope. We have already shown that the fluorescent substrate is endocytosed by the cells and accumulates in the lysosomes [32]. If active glucocerebrosidase and saposin C are present there, the substrate is hydrolysed to fluorescent ceramide, which leaves the lysosomes to a different cellular compartment (most probably the Golgi apparatus), resulting in a different staining pattern. Lysosomal compared with non-lysosomal staining can be revealed by fluorescence microscopy. As demonstrated in Figure 10, supplying either one of the prosaposins resulted in the accumulation of the fluorescent product in a non-lysosomal cellular compartment, in contrast with the lysosomal localization in the non-treated prosaposin-deficient cells. The numbers of cells exhibiting lysosomal and non-lysosomal staining were counted and the percentage of cells presenting non-lysosomal staining was calculated. Non-lysosomal fluorescence was observed in 38% and 33% of cells supplemented with medium containing Hpsap9 and Hpsap0 respectively, compared with 8.7% of cells supplemented with medium obtained from cells infected with vTF7-3 only. We therefore conclude that both prosaposins reached the lysosomes and were processed there to active saposin C, which enhanced the hydrolysis of the fluorescent glucocerebroside to fluorescent ceramide.

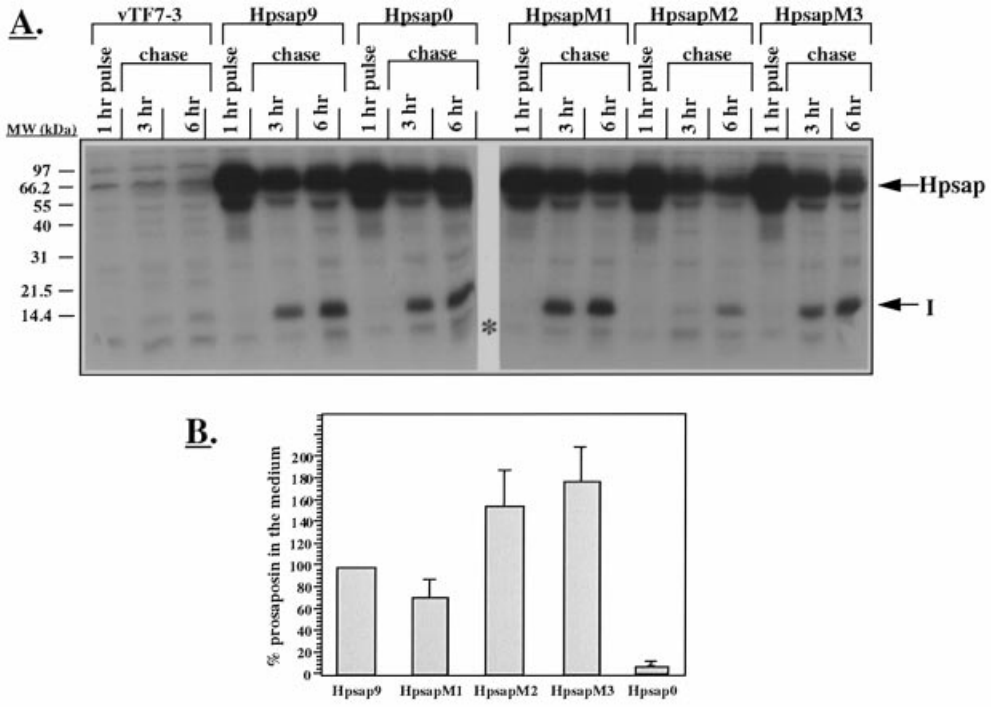

Figure 8 Secretion of normal and mutated recombinant prosaposins

(**A**) Subconfluent monolayers of normal human fibroblasts were co-infected (MOI = 10 for each virus) with vTF7-3 and each of the different recombinant viruses. After 20 h, cells were metabolically labelled for 1 h with [^{35}S]methionine followed by 3 or 6 h of chase. Cell lysates and medium were collected. Samples from cell lysates were immunoprecipitated and subjected to SDS/PAGE [15% (w/v) gel]; the gel was then fixed, dried and exposed to an X-ray film. Labels: I, prosaposin intermediate; *, saposins; Hpsap, human prosaposin. (**B**) Samples from medium collected after 3 h of chase were immunoprecipitated and subjected to SDS/PAGE [10% (w/v) gel]; the gel was then fixed, dried and a PhosphorImager analysis was performed. The results shown were obtained for prosaposin secretion at 3 h of chase in three independent experiments after statistical analysis with the Kruskal–Wallis one-way variance test.

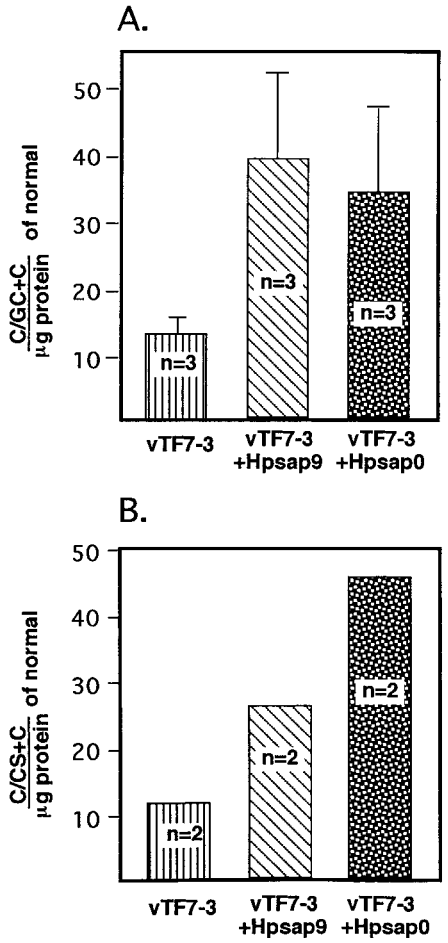

Figure 9 Intracellular fate of the recombinant prosaposins

Human prosaposin-deficient cells (5×10^5) were supplemented with 10 nmol/ml LR12-GC for 48 h together with 20 μM Br-CBE. Then cells were infected in the same medium with vTF7-3 and each of the recombinant viruses. After 3 h, the medium was removed, the cells were washed twice with PBS, then minimal essential medium without Phenol Red, supplemented with 20% (v/v) FCS, was added to the cells. A further 18 h later the medium was collected, then the cells were washed with PBS and lysed in 0.5 ml of distilled water. After protein determination, cell lysates and medium were extracted with chloroform/methanol (1:1, v/v) and dried. chloroform/methanol (1:1, v/v) (50 μl) was added and the samples were separated by TLC. The spots were scraped, extracted with chloroform/methanol and quantified in a spectrofluorimeter. Data are presented as percentages (ceramide/glucosylceramide per μg of protein) of normal [where normal, 100%, is the result obtained for 5×10^5 FS11 cells loaded with 10 nmol of LR12-GC or LR12-CS for 48 h]. The data are means $\pm$ S.E.M. for three different experiments, each performed in duplicate.

DISCUSSION

Because alternative splicing of a 9 bp exon was demonstrated for the prosaposin gene, it was interesting to test whether this alternative splicing is the mechanism participating in the differential sorting of prosaposins. We decided to express two prosaposin cDNA species, one containing the extra exon and the other without it, in the T_7/EMC/vaccinia virus-derived expression system. This system has several advantages: (1) a wide range of hosts can be very efficiently infected with the chimaeric viruses; (2) it provides efficient transcription and translation of the target gene; and (3) there is a shut-off of host protein synthesis, obviating the need for antibodies to follow expression of the target gene. Because transcription is initiated at the T_7 RNA polymerase promoter by the binding of the enzyme to its recognition site, high RNA levels are obtained from any introduced cDNA. It is therefore convenient to test the stability of different RNA species by following their steady-state levels in infected cells. In doing so, we were able to demonstrate that the two recombinant viruses directed the synthesis of prosaposin RNA with comparable stability (Figure 4). Translation in the T_7/EMC/vaccinia virus hybrid expression system is directed by the internal ribosomal entry site conferred by the encephalomyocarditis virus 5′-untranslated region. Therefore the ATG that is located at the 3′-most end of the encephalomyocarditis virus segment and is part of the *Nco*I site (CCATGG) is employed as the initiator ATG for translation. Because the same viral signals and cellular machinery participate in translating both prosaposins, any difference in their intracellular or extracellular levels can be attributed to a difference in post-translational processing. Both prosaposin DNA species directed the synthesis of a 67–70 kDa prosaposin protein. The 9–12 kDa mature saposins could be detected only by immunoprecipitation. This was most probably because only a small portion of the expressed recombinant protein reaches the lysosomes [32].

Pulse–chase experiments indicated that the prosaposin containing the three extra residues accumulated to much higher levels in the medium, in comparison with the prosaposin with no extra residues (Figures 5, 6 and 8). Because there was no concomitant increase in the intracellular amount of the prosaposin containing the three extra residues, this reflects preferential secretion of the prosaposin containing the three extra residues to the medium. The intracellular amounts of Hpsap0 and Hpsap9 were comparable. We cannot exclude the possibility that a certain fraction of Hpsap9 within the cells resulted from its endocytosis from the medium. The results indicated (Figure 8A) that Hpsap0 directed a faster accumulation of at least saposin C in the lysosomes, already visible at 6 h of chase (denoted by an asterisk). This might indicate the more efficient intralysosomal processing of Hpsap0 to saposins.

Both prosaposins directed the activities of saposin B and saposin C (Figure 9). Because, as discussed, most of the prosaposin containing the three extra residues was secreted, it seems that this form of prosaposin was efficiently endocytosed by cells, reached the lysosomes and was processed there to saposins B and C. However, the prosaposin containing no extra residues directed a higher saposin B activity than the prosaposin containing the three extra residues. This might indicate that the former is either more efficiently processed to saposin B or that the saposin B form without the three extra residues is more active than saposin B containing them.

Both prosaposins, when supplied to the cells in the medium, reached the lysosomes and were processed there to at least mature saposin C. The results indicated that the two prosaposins directed comparable saposin C activities (Figure 10B). Taking into consideration the fact that there was approx. 10-fold more of the prosaposin containing the three extra residues in the medium that was added to the cells than the form containing no extra residues (Figure 8B), the latter directed at least 10-fold more saposin C activity. Again, this high activity might reflect the more efficient lysosomal processing of the prosaposin containing no extra residues. It is evident from the results that there was some activator non-dependent hydrolysis of the fluorescent substrate within lysosomes [10–15% of the hydrolysis compared with normal; see Figures 9(A) and 10(B)]. It has recently been shown [46] that when supplied to the medium of prosaposin-deficient cells, both prosaposins could revert the mutant phenotype.

It has already been shown that alternative splicing, causing polymorphism at the prosaposin protein level, might alter the

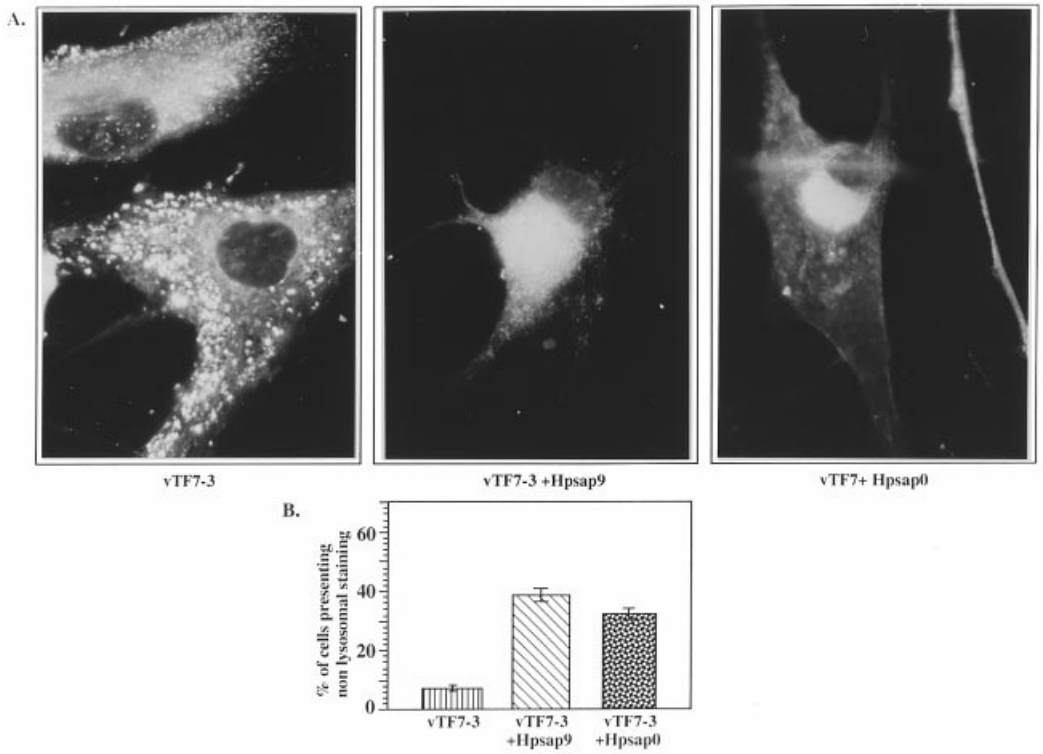

Figure 10 Cellular localization of fluorescent substrate and product in prosaposin-deficient cells supplemented with recombinant prosaposins

(**A**) Human normal and prosaposin-deficient cells (5×10^5) were grown on coverslips in the presence of 10 nmol of LR12-GC. After 48 h, prosaposin-deficient cells were supplemented with the different recombinant prosaposins, as described in the Materials and methods section. A further 24 h later, the cells were fixed and revealed by fluorescence microscopy. (**B**) Cells (100) from three different fields were counted and the number of cells presenting non-lysosomal staining was noted. Data are presented as the percentages of cells showing non-lysosomal staining. The data are means ± S.E.M.

binding specificity and/or function of the expressed proteins. Lamontagne and Potier [14] have shown that synthetic peptides derived from the saposin B domain of prosaposin (from Ser^{246} to Glu^{266}), with or without the three-residue insertion, had different binding affinities for GM_1 ganglioside, sulphatide and sphingomyelin. It has also been demonstrated that a five-residue stretch, EGHRG, can serve as an endoplasmic reticulum retention signal for the asialoglycoprotein receptor H2A [47]. The fact that the addition of the prosaposin containing the three extra residues, namely QDQ (Gln-Asp-Gln) within the saposin B domain, leads to the secretion of prosaposin would indicate that they can function either as a signal for active secretion or as an inhibiting signal for lysosomal sorting. A mutation in the first residue of the QDQ domain decreased the level of secretion, whereas substitutions of the other two residues resulted in an elevation of secretion. From these results we cannot conclude which of the alternative mechanisms is responsible for prosaposin secretion.

Henseler et al. [48] recently expressed the three alternative forms of prosaposin in baby hamster kidney (BHK) cells. The authors concluded that all three prosaposins encoded by the different cDNA species were transported and processed in the same manner. Their expression studies in BHK cells revealed that most of the expressed prosaposins were secreted into the culture medium, whereas a minor fraction was targeted to the acidic organelles of the BHK cells, where proteolytic cleavage to the mature saposins took place. The authors noted that owing to the different expression efficiencies, the yield of purified proteins varied between the different transfected BHK cells. The yields were 15 and 150 mg/l of medium for the prosaposins containing no and the three extra residues respectively. Because there were no results (for example RNA quantification) to support this hypothesis, we assume that these results indicate that the prosaposin containing the three extra residues was preferentially secreted from the cells. Moreover, because protein sequencing revealed [49] that only one form of saposin B, without the three extra residues, exists in cells, it is plausible that the prosaposin form without the three residues is sorted to the lysosomes. Recently, Igdoura et al. [17] have found two forms of sgp-1 (prosaposin) in rat Sertoli cells: a 65 kDa form that was shown to be targeted to lysosomes, and a 70 kDa form that was secreted extracellularly. It is plausible that the secreted form contains the three extra residues, whereas the form targeted to the lysosomes contains no extra residues.

The function of the secreted form of prosaposin is still unclear. One can envisage that this prosaposin form might serve as a ligand or a neurotrophic factor, as speculated [23,26], in several neuronal cells or it might be secreted by cells that serve as the reservoir of prosaposin for the benefit of cells that need functional saposins but are unable to produce them in the desired quantities. Immunohistochemical analysis of mouse tissues and embryos with anti-(mouse prosaposin) antibodies revealed a high expression of prosaposin in secreting cells such as the endometrial and tubular epithelium of the uterus, in Sertoli cells of the seminiferous epithelium, in Leydig cells in the testes and in the proximal convoluted tubules of the kidney. High expression was

also observed in the cerebellum Purkinje cells, in several types of neuron of the cerebrum, in neurons of dorsal ganglia and in neuroepithelial cells of the eye and the nose [12] (N. Altman and M. Horowitz, unpublished work). It is possible to envisage that Sertoli cells secrete prosaposin [16] that might be taken up by sperm cells, targeted to their lysosomes and there be used as a precursor for active saposins.

In summary, by using the T_7/EMC/vaccinia-derived hybrid system to express two human prosaposin cDNA species, we were able to show that alternative splicing is most probably the mechanism responsible for differential sorting of the two forms of prosaposin. The secreted form can be endocytosed by cells and can be processed in their lysosomes to active saposins B and C.

We thank Dr. Klaus Harzer (Tübingen, Germany) and Barbara Paton (Adelaide, Australia) for the gift of human prosaposin-deficient cells, and Dr. Gunther Legler (Cologne, Germany) for a gift of Br-CBE. This work was supported by grants from the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities (to M.H.) and from the 'Eshkol' fund administered by the Ministry of Science and the Arts (to L.M.S.).

REFERENCES

1 Mehl, E. and Jatzkewitz, H. (1964) Hoppe-Seyler's Z. Physiol. Chem. **339**, 260–276
2 Li, S. C. and Li, Y. T. (1976) J. Biol. Chem. **251**, 1159–1163
3 Sandhoff, K., Harzer, K. and Furst, W. (1995) in The Metabolic and Molecular Basis of Inherited Disease (Scriber, S., Beaudet, A., Sly, W. S. and Valle, D., eds.), pp. 2427–2441, McGraw Hill, New York
4 Ho, M. W. and O'Brien, J. S. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 2810–2813
5 Azuma, N., O'Brien, J. S., Moser, H. M. and Kishimoto, Y. (1994) Arch. Biochem. Biophys. **311**, 354–357
6 Harzer, K., Paton, B. C., Christomanou, H., Chatelut, M., Levade, T., Hiraiwa, M. and O'Brien, J. S. (1997) FEBS Lett. **417**, 270–274
7 Conzelmann, E. and Sandhoff, K. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3979–3983
8 Beutler, E. and Grabowski, G. A. (1995) in The Metabolic and Molecular Basis of Inherited Disease (Scriber, S., Beaudet, A., Sly, W. S. and Valle, D., eds.), pp. 2641–2663, McGraw Hill, New York
9 Christomanou, H., Chabas, A., Pampols, T. and Guardiola, A. (1989) Klin. Wochenschr. **67**, 999–1003
10 Reiner, O., Dagan, O. and Horowitz, M. (1989) J. Mol. Neurosci. **1**, 225–233
11 Sprecher, L. H., Orr, U. A., Lonai, P. and Horowitz, M. (1993) Cell. Mol. Biol. (Noisy le Grand) **39**, 287–299
12 Morales, C. R., El, A. M., Zhao, Q. and Igdoura, S. A. (1996) J. Histochem. Cytochem. **44**, 327–337
13 Nakano, T., Sandhoff, K., Stumper, J., Christomanou, H. and Suzuki, K. (1989) J. Biochem. (Tokyo) **105**, 152–154
14 Lamontagne, S. and Potier, M. (1994) J. Biol. Chem. **269**, 20528–20532
15 Furst, W. and Sandhoff, K. (1992) Biochim. Biophys. Acta **1126**, 1–16
16 Collard, M. W., Sylvester, S. R., Tsuruta, J. K. and Griswold, M. D. (1988) Biochemistry **27**, 4557–4564
17 Igdoura, S. A., Rasky, A. and Morales, C. R. (1996) Cell Tiss. Res. **283**, 385–394
18 Hineno, T., Sano, A., Kondoh, K., Ueno, S., Kakimoto, Y. and Yoshida, K. (1991) Biochem. Biophys. Res. Commun. **176**, 668–674
19 Kondoh, K., Hineno, T., Sano, A. and Kakimoto, Y. (1991) Biochem. Biophys. Res. Commun. **181**, 286–292
20 Sano, A., Matsuda, S., Wen, T. C., Kotani, Y., Kondoh, K., Ueno, S., Kakimoto, Y., Yoshimura, H. and Sakanaka, M. (1994) Biochem. Biophys. Res. Commun. **204**, 994–1000
21 Kotani, Y., Matsuda, S., Wen, T. C., Sakanaka, M., Tanaka, J., Maeda, N., Kondoh, K., Ueno, S. and Sano, A. (1996) J. Neurochem. **66**, 2197–2200
22 Kotani, Y., Matsuda, S., Sakanaka, M., Kondoh, K., Ueno, S. and Sano, A. (1996) J. Neurochem. **66**, 2019–2025
23 O'Brien, J. S., Carson, G. S., Seo, H. C., Hiraiwa, M., Weiler, S., Tomich, J. M., Barranger, J. A., Kahn, M., Azuma, N. and Kishimoto, Y. (1995) FASEB J. **9**, 681–685
24 O'Brien, J. S., Carson, G. S., Seo, H. C., Hiraiwa, M. and Kishimoto, Y. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 9593–9596
25 Campana, W. M., Hiraiwa, M., Addison, K. C. and O'Brien, J. S. (1996) Biochem. Biophys. Res. Commun. **229**, 706–712
26 Hiraiwa, M., Taylor, E. M., Campana, W. M., Darin, S. J. and O'Brien, J. S. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 4778–4781
27 Fujita, N., Suzuki, K., Vanier, M. T., Popko, B., Maeda, N., Klein, A., Henseler, M., Sandhoff, K., Nakayasu, H. and Suzuki, K. (1996) Hum. Mol. Genet. **5**, 711–725
28 Moss, B., Elroy, S. O., Mizukami, T., Alexander, W. A. and Fuerst, T. R. (1990) Nature (London) **348**, 91–92
29 Fuerst, T. R., Niles, E. G., Studier, F. W. and Moss, B. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8122–8126
30 Elroy-Stein, O., Fuerst, T. R. and Moss, B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6126–6130
31 Elroy, S. O. and Moss, B. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6743–6747
32 Pasmanik-Chor, M., Madar-Shapiro, L., Stein, E. O., Aerts, H., Gatt, S. and Horowitz, M. (1997) Hum. Mol. Genet. **6**, 887–895
33 Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
34 Reiner, O., Wilder, S., Givol, D. and Horowitz, M. (1987) DNA **6**, 101–108
35 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
36 Vaccaro, A. M., Salvioli, R., Barca, A., Tatti, M., Ciaffoni, F., Maras, B., Siciliano, R., Zappacosta, F., Amoresano, A. and Pucci, P. (1995) J. Biol. Chem. **270**, 9953–9960
37 Pinhasi, O. and Oren, M. (1984) Mol. Cell. Biol. **4**, 2180–2186
38 Agmon, V., Monti, E., Dagan, A., Augusto, P., Marchesini, S. and Gatt, S. (1993) Clin. Chim. Acta **218**, 139–147
39 Pasmanik-Chor, M., Elroy-Stein, O., Aerts, H., Agmon, V., Gatt, S. and Horowitz, M. (1996) Biochem. J. **317**, 81–88
40 Pasmanik-Chor, M., Laadan, S., Elroy-Stein, O., Zimran, A., Abrahamov, A., Gatt, S. and Horowitz, M. (1996) Biochem. Mol. Med. **59**, 125–133
41 Marchesini, S., Preti, A., Aleo, M. F., Casella, A., Dagan, A. and Gatt, S. (1990) Chem. Phys. Lipids **53**, 165–175
42 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
43 Hiraiwa, M., Martin, B. M., Kishimoto, Y., Conner, G. E., Tsuji, S. and O'Brien, J. S. (1997) Arch. Biochem. Biophys. **341**, 17–24
44 Schnabel, D., Schröder, M., Fürst, W., Klein, A., Hurwitz, R., Zenk, T., Weber, J., Hauzer, K., Paton, B. C., Poulos, A. et al. (1992) J. Biol. Chem. **267**, 3312–3315
45 Paton, B. C., Schmid, B., Kustermann, K. B., Poulos, A. and Harzer, K. (1992) Biochem. J. **285**, 481–488
46 Burkhardt, J. K., Huttler, S., Klein, A., Mobius, W., Habermann, A., Griffiths, G. and Sandhoff, K. (1997) Eur. J. Cell. Biol. **73**, 10–18
47 Lederkremer, G. Z. and Lodish, H. F. (1991) J. Biol. Chem. **266**, 1237–1244
48 Henseler, M., Klein, A., Reber, M., Vanier, M. T., Landrieu, P. and Sandhoff, K. (1996) Am. J. Hum. Genet. **58**, 65–74
49 Furst, W., Schubert, J., Machleidt, W., Meyer, H. E. and Sandhoff, K. (1990) Eur. J. Biochem. **192**, 709–714

Received 20 March 1998/17 September 1998; accepted 16 October 1998