Expression of the Three Alternative Forms of the Sphingolipid Activator Protein Precursor in Baby Hamster Kidney Cells and Functional Assays in a Cell Culture System*

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Sphingolipid activator proteins (SAPs) are non-enzymatic glycoproteins required for lysosomal degradation of various sphingolipids with short oligosaccharide chains by their respective exohydrolases. Four of these (SAP-A to SAP-D or saposins A to D) are derived from a common precursor by proteolytic processing. Alternative splicing of the SAP-precursor gene results in insertion of additional 6 or 9 bases of exon 8, respectively, into the SAP-B coding region of the transcribed mRNAs.

To examine the features of the three different SAP-precursor proteins (prosaposins), the respective cDNAs were stably expressed in baby hamster kidney cells. Pulse-chase experiments with transfected cells and endocytosis studies on human fibroblasts showed that synthesis, transport, and maturation of all SAP-precursor led to formation of the four mature SAPs (SAP-A to SAP-D).

In order to determine the biological function of the three different SAP-B isoforms, SAP-precursor-deficient human fibroblasts were loaded with recombinant SAP-precursor proteins with or without 2- and 3-amino acid insertions, respectively, purified from the medium of the baby hamster kidney cells. They were found to stimulate at nanomolar concentrations the turnover of bio-
synthetically labeled ceramide, glucosylceramide, and lactosylceramide. Since the physiological function of SAP-B is to stimulate the degradation of sulfatide by arylsulfatase A (EC 3.1.6.1) and globotriaosylceramide by β-galactosidase (EC 3.2.1.23) loading studies with the respective exogenously labeled lipids on SAP-precursor-deficient fibroblasts were performed. Addition of different purified SAP-precursors to the medium of the lipid-loaded fibroblasts showed positive stimulation of the lipid degradation by all three SAP-B isoforms derived from the SAP-precursors. These findings establish that all three forms of the SAP-B can function as sulfatide/globotriaosylceramide activator.

The physiological degradation of glycosphingolipids with short oligosaccharide chains is catalyzed by lysosomal exohydrolases in the presence of small, heat-stable glycoproteins, so called sphingolipid activator proteins (SAPs)* or saposins (1). Four of the five known activator proteins (SAP-A to SAP-D or saposins A–D) are derived from a common precursor polypeptide by proteolytic processing (2, 3). This precursor also called prosaposin was identified as a protein of 68 kDa in human skin fibroblasts by studying the synthesis and processing of SAP-C (4). After modification of its N-linked oligosaccharides in the Golgi apparatus, a 73-kDa form is secreted into the culture medium. Processing to the mature polypeptides of 8–13 kDa takes place after the transport of the precursor to the acidic organelles of the cells.

Although different activating properties for the SAPs (SAP-A to SAP-D) have been described, their physiological relevance has been established only for SAP-B and SAP-C (1). The absence of SAP-C in human patients with Gaucher disease shows the relevance of this activator protein for the degradation of glucosylceramide by glucosylceramidase (EC 3.2.1.45) (1, 5). While SAP-A is known to stimulate the breakdown of several glycosphingolipids in vitro (6, 7), SAP-D has recently been shown to be involved in ceramide degradation in vivo (8). This was established in an in vivo test system, in which the break-
down of [3H]serine-labeled sphingolipids accumulating in hu-
mans fibroblasts derived from patients with SAP-precursor deficienc (9) was stimulated by addition of different SAPs (SAP-A to SAP-D) to the culture medium. SAP-B itself has capacity to bind several sphingolipids, but its genetic defect leads to an accumulation of sulfatides, glo-

* The abbreviations used are: SAP, sphingolipid activator protein; pHBE0, pHBE6, and pHBE9, expression plasmid pHBE containing the human prosaposin cDNA without additional base pairs, with 6 base pairs, or 9 base pairs, respectively; BH cells, baby hamster kidney cells; SAP-precursor0, SAP-precursor6, and SAP-precursor9; proteins (SAP-precursor, 46- and 30 kDa-protein forms) purified from the medium of transfected BHK cells; MEM, minimal essential medium; FCS, fetal calf serum; Endo H, endo-β-N-acetylgalactosaminidase H; Cer, cer-
amide, N-acetylsphingosine; GlcCer, glucosylceramide, Glcβ1–1Cer; galactosylceramide, Galβ1–1Cer; LacCer, lactosylceramide, Galβ1–4Glcβ1–1Cer; digalactosylceramide, Galα1–4Galβ1–1Cer; sulfatide, Gal(β-sulfate)β1–1Cer; ELISA, enzyme-linked immunosorbent assay. The abbreviated designations of the gangliosides are according to Sven-
nerholm’s nomenclature for ganglio series gangliosides (Svennerholm, L. (1963) J. Neurochem. 10, 613–623). To date there are different nomenclatures used throughout the literature for the sphingolipid activa-
tor proteins and their precursor protein: sphingolipid activator pro-
tein precursor = prosaposin or SAP-precursor; sphingolipid activator proteins A–D: saposin A = SAP-A, saposin B = SAP-B, saposin C = SAP-C, and saposin D = SAP-D. In this article we use the designation SAP-precursor, SAP-A, SAP-B, SAP-C, and SAP-D for the correspond-
ing proteins.
botriaosylceramide, digalactosylceramide, and ganglioside G\textsubscript{M13} in a disorder classified as metachromatic leukodystrophy (1, 10).

The SAP-precursor gene localized on the long arm of human chromosome 10 (10q21–22) (11) consists of at least 15 exons, four of which (exon 6–9) code for SAP-B. Exon 8 consists of only 9 base pairs (12) and can be spliced differentially, generating three different cDNAs (13, 14): one containing all 9 bases of exon 8, one containing the downstream 6 bases of exon 8 (also called exon 8') and one completely lacking this exon (Fig. 1). At the protein level, only SAP-B without the amino acids derived from exon 8 has been detected so far (10, 15). A recent report shows a differential tissue distribution of the mRNAs with or without exon 8 in different cell lines or tissues (16). The multiple mRNA species were also detected in the mouse (17). These findings prompt the question whether the different sequences within the SAP-B domain in the SAP-precursor have any biological significance. In the present report we describe the expression and processing of the three SAP-precursor forms in BHK cells stably transfected with the respective cDNAs. In addition, the stimulation of sulfatide and globotriaosylceramide degradation by the three SAP-B isoforms was tested in vitro in a cell culture system by adding purified recombinant precursor proteins (designated as SAP-precursor 0, 6, or 9) to the medium of lipid loaded human SAP-precursor-deficient fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction and modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Federal Republic of Germany), Life Technologies, Inc. (Eggenstein, Federal Republic of Germany), and New England Biolabs (Schwalbach, Federal Republic of Germany). Peptide-N\textsubscript{glycanase P and Endo H were from New England Biolabs (Schwalbach, Federal Republic of Germany). Radiolabeled chemicals were obtained from Amersham Corp. (Braunschweig, Federal Republic of Germany). Cultura media for bacterial and eukaryotic cells were obtained from Life Technologies, Inc. Biochrom (Berlin, Federal Republic of Germany), Sigma (Deisenhofen, Federal Republic of Germany), and ICN (Meckenheim, Federal Republic of Germany). Fetal calf serum was purchased from Cytogen (Berlin, Federal Republic of Germany). Puro- mycin was from Sigma (Deisenhofen, Federal Republic of Germany) and G418 sulfate from Life Technologies, Inc. Protein A-Sepharose was obtained from Sigma, and Protein G-agarose was from Calbiochem-Novabiochem (Bad Soden, Federal Republic of Germany).

All other commercially available materials were obtained from the following suppliers: Sigma, Merck (Darmstadt, Federal Republic of Germany), Pharmacia LKB Biotechnologies (Freiburg, Federal Republic of Germany), and New England Biolabs.

![Fig. 1. Relationship of SAP-B to the human SAP-precursor gene (according to Ref. 14).](image)

**Antisera—**Human SAP-A and SAP-C were purified from spleen of a Gaucher patient essentially as described earlier (8, 20). Polyclonal anti-SAP-A and anti-SAP-C antisera were raised in rabbits by injection of 100 \(\mu\)g of pure protein in combination with Freund's complete adjuvant followed by three booster injections of 100 \(\mu\)g protein each in Freund's incomplete adjuvant given in 4-week intervals. The animals were bled 10 days after the final injection. The antisera had ELISA titer of 1.950 and 1.900 (half-maximal \(A_{405}\) of 3-fold serum dilutions on a 50-ng antigen coat) against SAP-A and SAP-C, respectively. Specificity of the antisera was assessed by ELISA, immunoblot and immunoprecipitation, and in the case of the anti-SAP-C antisera by comparison with the rabbit anti-SAP-C antiserum described earlier (21). The preparation of the goat anti-SAP-B and anti-SAP-D antiser has been described earlier (8).

**Cells and Cell Culture—**Human skin fibroblasts and baby hamster kidney cells (BHK 21 ATCC CCC 10) were maintained at 37 °C in 5% CO\textsubscript{2} in Dulbecco's modified Eagle's medium supplemented with antibiotics, 4.5 mM NaHCO\textsubscript{3} and 10% fetal calf serum (FCS) as described previously (22). Transfected BHK cells were cultured in medium supplemented with 5 \(\mu\)g/ml puromycin. The fibroblasts from a SAP-precursor deficient human fetus have been described earlier (9, 23, 24).

**Expression of the SAP-precursor Proteins in BHK Cells—**The expression plasmid pBHE9 was constructed by placing the entire SAP-precursor cDNA (from nucleotide -9 to 1597, numbered from A of the ATG initiation codon) which contained the 9 base pairs of exon 8 into the XhoI and SmaI site upstream of the SV40 early promoter in the expression vector pBHE (25). The expression plasmid pBHE0 containing the SAP-precursor cDNA without exon 8 and the expression plasmid pBHE6 containing the cDNA with the 6 bases of exon 8' were constructed by replacing nucleotides 543 to 797 (using the BsmBI and BglI restriction sites on the precursor cDNAs) in the expression plasmid pBHE9 by the respective cDNA segments of the other SAP-precursor cDNAs. These cDNA segments were derived from partial cDNAs obtained by reverse transcriptase-polymerase chain reaction of total RNA isolated from human fibroblasts (9). The correct cDNA sequence of each expression plasmids pBHE0, pBHE6, and pBHE9 was confirmed by DNA sequencing (28).

Stably transfected BHK cells were obtained by cotransfection of the expression plasmids and of the plasmids pSV2 pac and pSV2 neo conferring resistance to puromycin and G418 sulfate to the cells (27), as described previously (28). BHK cells transfected only with the expression plasmid pBHE were used as control.

**Processing and Endocytosis Studies—**Confluent cultures of transfected BHK cells in 25-cm\textsuperscript{2} tissue culture flasks were incubated for 1 h in 1 ml of methionine-free modified Eagle's medium (MEM) containing 4% dialyzed and heat-inactivated FCS. The cells were labeled by incubation in 0.7 ml of this medium supplemented with 1.85 MBq of [3H]Sulfatide, which was labeled in the sphingoid moiety, and [3H]globotriaosylceramide labeled in the terminal galactosyl residue were synthesized in our laboratory according to published procedures (1, 10).

**Materials—**Restriction and modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Federal Republic of Germany), Life Technologies, Inc. (Eggenstein, Federal Republic of Germany), and New England Biolabs (Schwalbach, Federal Republic of Germany). Peptide-N\textsubscript{glycanase P and Endo H were from New England Biolabs (Schwalbach, Federal Republic of Germany). Radiolabeled chemicals were obtained from Amersham Corp. (Braunschweig, Federal Republic of Germany). Culture media for bacterial and eukaryotic cells were obtained from Life Technologies, Inc. Biochrom (Berlin, Federal Republic of Germany), Sigma (Deisenhofen, Federal Republic of Germany), and ICN (Meckenheim, Federal Republic of Germany). Fetal calf serum was purchased from Cytogen (Berlin, Federal Republic of Germany). Puro- mycin was from Sigma (Deisenhofen, Federal Republic of Germany) and G418 sulfate from Life Technologies, Inc. Protein A-Sepharose was obtained from Sigma, and Protein G-agarose was from Calbiochem-Novabiochem (Bad Soden, Federal Republic of Germany).

All other commercially available materials were obtained from the following suppliers: Sigma, Merck (Darmstadt, Federal Republic of Germany), Pharmacia LKB Biotechnologies (Freiburg, Federal Republic of Germany), and New England Biolabs.
RESULTS

Isolation and Purification of Recombinant SAP-precursor Proteins—The three different SAP-precursor differing in the additional amino acids encoded by exon 8 or 8 as described previously (9). Deglycosylation of the precipitated proteins with peptide-N-glycanase F and Endo H was performed in 1% Nonidet P-40, 10 mM EDTA, 100 mM NaCl, 0.1% FCS, and the same concentration of the SAP-precursor 0, 6, or 9 as indicated. Subsequently the cells were permeabilized with 0.22% aqueous CaCl₂ (60:35:8 by volume). All glycosphingolipids were identified from their RF and disappearance forms described earlier in expression of a SAP-precursor with an apparent molecular mass of 73 kDa, which was already detectable after 1 h of pulse. In addition, additionally processed forms of about 46 and 30 kDa were revealed that after 1 h of pulse labeling a SAP-precursor of t-[³⁵S]methionine (specific radioactivity > 37 TBq/nmol) for 1 h. The chase periods were started by addition of unlabeled methionine (final concentration 10 mM). For endocytosis studies, 0.7 ml of medium from transfected BHK cells labeled for 5 h with 2.5 MBq of t-[³⁵S]methionine (37 TBq/mmol) for 1 h. The major immunoprecipitable protein of the cell extracts and denoted by an asterisk, is caused by unspecific contaminations of immunoprecipitates.

Presentation of Data—Radioactivity of individual sphingolipids is expressed as percentage of total radioactivity of the sphingolipid fraction. Total radioactivity is normalized to the same amount of cellular protein. All data are given as means of at least two separate experiments.

Expression and Maturation of the Three Different SAP-precursor Forms in BHK Cells—In order to follow synthesis and transport of the different SAP-precursor isoforms and their maturation in cell culture, stably transfected BHK cells were prepared. Metabolic labeling of transfected BHK cells with t-[³⁵S]methionine and immunoprecipitation of the expressed proteins from the cell extracts with an anti-SAP-C antiserum revealed that after 1 h of pulse labeling a SAP-precursor of apparent 68 kDa was generated by all three cDNA forms (Fig. 2). Additionally processed forms of about 46 and 30 kDa were detectable. Within 2 h of chase there was significant biosynthesis of radiolabeled mature SAP-C with an apparent mass of 13 kDa. This protein was further processed to 11- to 8-kDa forms during an additional chase of 18 h. The major immunoprecipitable protein, secreted into the culture media, was the SAP-precursor with an apparent molecular mass of 73 kDa, which was already detectable after 1 h of pulse. In addition, processed proteins of molecular masses of 46 and 30 kDa were precipitable from the media, presumably identical with the tri- and disaposin forms described earlier in expression of a SAP-
precursor in insects cells (27). During the pulse and chase periods more than 60% of the immunoprecipitable proteins were secreted into the medium of the transfected cells. Secretion of the 73-kDa forms started 30 min after a short pulse labeling of 5 h (data not shown). No cross-reacting material was precipitated from extracts of control BHK cells (Fig. 2, Co).

This labeling experiment indicated formation of similar polypeptides in BHK cells transfected with the different SAP-precursor cDNAs as described for in human fibroblasts (4). The different amounts of immunoprecipitable proteins in the transfected BHK cells were due to the fact that the totality of BHK cells resistant to the antibiotics were used for pulse-chase experiments and that the selection leaves varying numbers of non-resistant cells.

In order to assess correct processing of all three different recombinant SAP-precursor to the four mature SAPs (SAP-A to SAP-D) and to analyze their carbohydrate structures, the BHK transfecants were metabolically labeled for 5 h, and the cell extracts were subjected to immunoprecipitation with polyclonal antisera against the corresponding mature SAP-A to SAP-D (Fig. 3). All three SAP-precursors resulted in formation of mature SAP-A, SAP-B, SAP-C, and SAP-D, differing with respect to their carbohydrate types as analyzed previously (34, 35). In our hands, mature SAP-A never yielded a clear band in its glycosylated form, but only a broad patch ranging from 14.5 to 29 kDa. Upon deglycosylation, however, a clear band comperible in size with deglycosylated mature SAP-B, SAP-C, and SAP-D was obtained. The carbohydrate chains of SAP-A were Endo H-resistant, presumably being of the complex type, whereas the carbohydrate moieties of SAP-C and SAP-D were predominantly of the Endo H-sensitive high mannose or hybrid type. It is noteworthy that each SAP-precursor generated a mature SAP-B with an Endo H-resistant carbohydrate chain.

Endocytosis of the SAP-precursor Isoforms—To confirm that human cells process the three SAP-precursors in the same manner as the BHK transfecants, BHK cells were labeled with $[^35]$S)methionine, and the medium containing the secreted proteins was added to human fibroblasts derived from a SAP-precursor-deficient fetus (9). After a 24-h incubation the media and cell extracts were analyzed for immunoprecipitable SAP-B, SAP-C, and SAP-D with the corresponding antisera (Fig. 4). Some SAP-precursor was still detectable in the medium. About 50% of each of the different radiolabeled SAP-precursor forms had been internalized by the SAP-precursor deficient fibroblasts and processed to the mature SAP-A to SAP-D. Repeating the endocytosis experiment with normal human fibroblasts showed an uptake of about 30% of each prosaposin form and the same correct processing to the mature proteins (data not shown). Thus, no difference with regard to maturation was observed for any of the three SAP-precursors in human fibroblasts in comparison with BHK cells.

Purification of the SAP-precursor Isoforms—The three different isoforms of the recombinant SAP-precursors were purified from the medium of transfected BHK cells by a two-step procedure as described for the purification of SAP-precursor expressed in insect cells (29). Affinity chromatography on a concanavalin A-Sepharose was performed, followed by reversed-phase chromatography. Since not only the SAP-precursor forms of 73 kDa, but also processed forms of 46 and 30 kDa, were detectable in the medium of the transfected BHK cells (Fig. 2), it was not surprising that all these proteins were copurified from the media. A separation of these protein forms was not achieved under the chosen high performance liquid chromatography conditions. The three precursor isoforms showed mutually identical elution profiles with no significant difference in retention time. The purification progress was monitored by ELISA and immunoblot analysis using an anti-SAP-D antiserum, which confirmed that the 46- and 30-kDa forms were also SAP-precursor forms (data not shown). Due to the different expression efficiencies of the BHK cells the yield of purified proteins varied among the different transfected BHK.
cells. The yields were 15 μg/liter medium for SAP-precursor0, 55 μg/liter for SAP-precursor6, and 150 μg/liter for SAP-precursor9, respectively. When the medium of BHK control cells was subjected to the same purification procedure, no immunologically cross-reactive material and no significant amount of other proteins were detected in the fractions, which should have contained the expression products. Nevertheless, these fractions were pooled to serve as control (Co) in the further experiments to eliminate the influence of the endogenous BHK proteins.

Effect of the SAP-precursor 0, 6, or 9 on the Lipid Turnover in Human Fibroblasts—Metabolic labeling of sphingolipids with [14C]serine in cultured human fibroblasts followed by a long chase period resulted in labeling of the most prominent sphingolipids of normal fibroblasts, sphingomyelin, and globosides, while in fibroblasts derived from a patient affected with SAP-precursor deficiency an increased labeling of the storage compounds characteristic for this disease, e.g., ceramide, glucosylceramide, lactosylceramide, and ganglioside G_{M3}, occurred (Fig. 5). Since it had become clear from the endocytosis experiments that the SAP-precursor isoforms were taken up and processed effectively, we attempted to restore the lipid metabolism in the mutant fibroblasts by administration of recombinant SAP-precursors. The cells were preincubated and chased for 120 h in the presence of different concentrations (from 0.025 to 0.9 μg/ml medium) of purified SAP-precursor9 added to the culture medium (Fig. 5). The addition of different concentrations of SAP-precursor9 resulted in reduction of the storage of ceramide, glucosylceramide, and lactosylceramide. The fraction of labeled ceramide, for example, which accounted for 12.8 ± 2.0% of total sphingolipid-associated radioactivity in untreated SAP-precursor-deficient fibroblasts, decreased to 10.34 ± 0.8% or even 1.82 ± 0.3% (out of 58,758 dpm/mg of the total sphingolipid fraction) by administration of 0.025 or 0.9 μg/ml medium of SAP-precursor9, respectively. In normal cells, ceramide made up 1.3 ± 0.6% (out of 39,967 dpm/mg) of the total detectable radioactivity.

In order to demonstrate the same effect on the lipid turnover for all three purified prosaposins, the [14C]serine pulse-chase labeling experiment was repeated, adding each SAP-precursor (0, 6, or 9, 0.5 μg/ml medium of each) to the culture medium of the deficient fibroblasts during the preincubation and chase periods (Fig. 6). To evaluate the influence of the material co-

Alternative Spliced SAP-precursor cDNAs and Mature SAP-B

| + SAP-precursor | NC | SAP-precursor deficient |
|-----------------|----|------------------------|
| 9               |    | 0.025 0.05 0.1 0.25 0.5 0.7 0.9 |
| Cer             |    |                        |
| GlcCer          |    |                        |
| LacCer          |    |                        |
| Globosides      |    |                        |
| SM              |    |                        |
| GM3             |    |                        |
| GM2             |    |                        |
| GM1             |    |                        |
| GD3/GD1a        |    |                        |

FIG. 5. Concentration dependence of the turnover of labeled sphingolipids in cultured fibroblasts on the complementation of the culture medium with SAP-precursor9. Fibroblasts from a patient with SAP-precursor deficiency and a normal control (NC) were incubated for 24 h with medium supplemented with recombinant SAP-precursor9 as indicated. Subsequently the cells were incubated with [14C]serine (1 μCi/ml) for 24 h. The medium was changed and the cells were chased for 120 h with a medium containing unlabeled serine and the same concentration of recombinant SAP-precursor9 used during the preincubation period. After harvesting of the cells, the sphingolipid fraction was isolated, equal amounts of radioactivity were separated by TLC, and the radioactive spots identified by autoradiography as described under "Experimental Procedures." SM, sphingomyelin.

FIG. 6. Effect of the different SAP-precursor isoforms on the turnover of labeled sphingolipids in cultured fibroblasts. Fibroblasts from a patient with SAP-precursor deficiency and a normal control (NC) were incubated for 24 h with recombinant SAP-precursor isoforms purified from the medium of transfected BHK cells (0.5 μg/ml medium SAP-precursor 0, 6, or 9) and with an equal volume of control protein fraction isolated from BHK cells transfected with the expression plasmid pBHE (Co). Subsequently the fibroblasts were incubated for 24 h with [14C]serine (1 μCi/ml). Then the medium was changed, and the cells chased for 120 h with medium supplemented with unlabeled serine and with recombinant SAP-precursor 0, 6, or 9 as used in the preincubation period. After the cell harvest the sphingolipid fraction was isolated, equal amounts of radioactivities were separated on TLC and identified as described under "Experimental Procedures." SM, sphingomyelin.

purified with recombinant SAP-precursors, the control fractions from the media of untransfected BHK cells, as described above, was also offered to labeled mutant fibroblasts. Addition of all three SAP-precursors decreased the amount of accumulated ceramide, lactosylceramide, and glucosylceramide in quantitatively similar manner, while no significant influence on the turnover of these lipids was detectable by the BHK control fraction (Co).

One known physiological function of SAP-B is to stimulate the degradation of sulfatide by arylsulfatase A. Since the met-

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abolic labeling of sphingolipids with $[^{14}C]$serine in cultured fibroblasts resulted only in the pronounced labeling of those sphingolipids which are endogenously synthesized in fibroblasts, the effect of SAP-B isoforms on the turnover of sulfatide was determined by loading experiments. $[^{3}H]$Sulfatide was added to the medium of the cultured fibroblasts and during the preincubation and chase periods the culture medium was supplemented with 0.5 $\mu$g/ml of each purified SAP-precursor isoform. The metabolic fate of the $[^{3}H]$sulfatide taken up by the cells was examined by TLC (Fig. 7). While in untreated SAP-precursor-deficient fibroblasts about 97.6 $\pm$ 6.0% of the total radioactivity remained in the accumulated sulfatide, its proportion decreased to 89.2 $\pm$ 4.1% in the cells treated with each of the recombinant SAP-precursor isoforms. In contrast, treatment with the control protein fractions (Co) had no effect on sulfatide turnover. In normal fibroblasts up to 45.2 $\pm$ 15.7% of the radioactivity was detectable in the sulfatide fraction.

The presence of the radiolabeled ceramide and sphingomyelin, arising from degradation of sulfatide and reutilization of ceramide in all SAP-deficient fibroblasts cultured in presence of the purified proteins as well as in the normal control clearly pointed out that all three SAP-B isoforms have been able to stimulate the sulfatide degradation in vivo (Fig. 7).

An analogous feeding experiment was performed with $[^{3}H]$globotriaosylceramide in order to examine the function of endocytosed $[^{3}H]$globotriaosylceramide (Fig. 8). Due to the fact that the label was located in the terminal galactose residue, no metabolic products were detectable. In the normal control cells, the radioactivity of the isolated globotriaosylceramide constituted 32.2 $\pm$ 17.5% of total radioactivity. In the mutant fibroblasts, untreated and treated with the control protein fractions (Co), globotriaosylceramide bound radioactivity remained 78.9 $\pm$ 21.8% and 81.9 $\pm$ 18.1% of the total radioactivity, respectively. In SAP-precursor-deficient fibroblasts cultured in the presence of purified SAP-precursor 0, 6, or 9, a decrease of the accumulated globotriaosylceramide was generally detectable (62.9 $\pm$ 11.1% of the total radioactivity), indicating that all three SAP-B isoforms stimulate degradation of globotriaosylceramide to a similar degree.

**DISCUSSION**

That the SAP-precursor gene is alternatively spliced producing three different cDNAs, with or without inclusion of 9 or 6 base pairs of exon 8 has been known for several years (14), but no protein product encoded by the longer cDNAs has been detected in tissues so far. This can be due to several reasons: the translation products of the longer cDNAs may be processed differently and generate different sets of final products, the longer SAP-B forms may be unstable, or expression pattern of the sources from which SAP-B was usually purified favors the protein without the insertion (15). The results presented in this report indicate that all three polypeptides encoded by the different SAP-precursor cDNAs are transported and processed in the same manner as described for SAP-precursor in human fibroblasts and that all three SAP-B isoforms are stable and have the same sphingolipid activator function (4).

Recently, the relative abundance of the alternatively spliced mRNA with or without 9 bases was determined by reverse transcriptase-polymerase chain reaction in various human tissues and cell lines (16), but it was difficult to correlate the presence of the mRNA forms with the occurrence of mature SAP-B at the protein level, especially as there are no antibodies available to differentiate between the three protein forms. Since we have shown that the maturation of SAP-B proceeds in a comparable way in BHK cells and in human fibroblasts, the question arises if there is a significance for the alternative spliced mRNAs and their protein products. The detection of immunoprecipitable, stable mature SAP-B derived from the different SAP-precursors allowed the conclusion that the stability of mature SAP-B is not affected dramatically by these
insertions. Examination of several patients suffering from genetic SAP-B deficiency demonstrated that disruption of the correct protein structure of SAP-B led to lack of cross-reacting insertions. Cross-reacting insertions. A deletion at the N terminus of the SAP-B domain due to a splice site mutation in the prosaposin gene had the same effect (37). In a third case, the insertion of additional bases by a point mutation introducing a new splice site at the 5’ end of exon 8 resulted in the insertion of 11 amino acids into the SAP-B region of the SAP-precursor. No stable SAP-B was released from this precursor either (38, 39).

The expression studies in BHK cells revealed that the major part (more than 60%) of the expressed SAP-precursors was secreted into the culture medium and that a minor amount was targeted to the acidic organelles of the BHK cells where proteolytic cleavage to the mature SAPs (SAP-A to SAP-D) took place. An analogous phenomenon was reported for the expression of cathepsin D precursor in BHK cells and the authors concluded that if synthesized at a higher rate a particular group of lysosomal proteins was secreted into the medium without concomitantly compromising the targeting of other lysosomal enzymes (40).

Observations that the SAP-precursor occurs in different human fluids such as milk, cerebrospinal fluid, and seminal plasma (41, 42) and the fact that all three isoforms were secreted into the medium of the transfected BHK cells call for a detection system for the individual SAP-precursor isoforms. Such a method would allow determination of the existence and amounts of the different protein forms in different body fluids. In addition to findings that demonstrated binding and transport of gangliosides by the SAP-precursor (43), this protein has been suggested to act as a neuroprotective or regenerative agent in vivo (44). More recently, the SAP-precursor has been suggested to be a neurotrophic factor (45), because it elicited differentiation of neuroblastoma cells when applied in the nanomolar range. The neurotrophic function is said to be localized on the SAP-C domain (46), indicating that alternative splicing does not modulate this function.

For our studies on the stimulatory effect of SAP-B on the lysosomal degradation of sphingolipids we purified the prosaposin isoforms from the media of transfected BHK cells. In all preparations obtained by the purification procedure, partially processed intermediates copurified with 73-kDa precursor form. A similar phenomenon was observed during the first purification steps of SAP-precursor from the medium of infected C127 cells, seminal plasma, and milk (29). The intermediates were identified to be tri- and disaposins, and a possible pathway of proteolysis of SAP-precursor was discussed (29). However, our endocytosis studies demonstrate clearly that all endocytosed higher protein forms were processed within 24 h to the mature SAPs (SAP-A to SAP-D). Therefore all effects shown on lipid degradation (in the time period between 24 and 168 h after feeding of SAP-precursor 0, 6, or 9, see Figs. 4 and 5) were caused by mature SAP forms rather than by the SAP-precursor or partially processed intermediates.

It has been shown previously that alternative splicing causing a polymorphism at the protein level may alter the binding specificity and/or function of the expressed proteins, including hormone precursors, DNA-binding proteins and structural proteins (47, 48). A recent report indicated that synthetic peptides derived from the SAP-B amino acid sequence (from Ser246 to Glu266 with or without the Gln-Asp-Gln insertion) have different binding affinities for GM1 ganglioside, sulfatide, and sphingomyelin (16). From these results, the authors suggested that alternative splicing of the SAP-precursor gene may change binding specificity in the encoded SAP-B presumably to adapt to the variable sphingolipid composition of tissues. However, our observations indicate that all SAP-B isoforms are able to stimulate the degradation of sulfatide and globotriaosylceramide in cultured fibroblasts without significant differences among them.

Two models have been proposed for the structure of SAP-B. Potier based his model on sequence homology of SAP-B with influenza virus neuraminidase (49). The model predicts a high proportion of β-sheet and one α-helix at the C-terminal end of SAP-B, which should contain the additional two or three amino acids inserted by alternative splicing (16). Based on the disulfide bonding pattern the second model suggests a four-helix bundle structure (11, 50). In this model the additional amino acids would also be placed into a α-helical structure with the majority of the hydrophobic residues forming a potential lipid binding core in the interior of the molecule. A mechanism has been proposed for the interaction of SAP-B with membrane surfaces and for the binding of single lipid molecules (11). In this model the helices are oriented parallel to the lipid head group. After extraction of the lipid from the membrane the SAP-B helices should create a hydrophobic environment for the lipid hydrocarbon tail.

Recently, a structural model for a family of proteins called saposin-like proteins (SAPLIP) was discussed basing on the four α-helical bundle first proposed by Stevens et al. (50) and using crystal structural data derived from hemerythrin as a template for modeling SAP-B (51). In this model the inserted 3 amino acids are located between helices III and IV and might affect lipid binding directly or indirectly.

All these different models point to the fact that only structural work on SAP-B and its different isoforms can test the validity of the above models and may indicate the possible functional significance of the amino acids insertions.

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