Biosynthesis, Processing, and Targeting of Sphingolipid Activator Protein (SAP) Precursor in Cultured Human Fibroblasts

MANNOSE 6-PHOSPHATE RECEPTOR-INDEPENDENT ENDOCYTOSIS OF SAP PRECURSOR*

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The lysosomal degradation of most glycosphingolipids with short oligosaccharide chains by acidic hydrolases is dependent on the action of small, nonenzymic glycoproteins, so-called short oligosaccharide chains by acidic hydrolases. SAP-A, -B, -C, and -D derive from proteolysis of a 73-kDa glycoprotein, the SAP precursor. In the present publication, we studied the intracellular transport and the endocytosis of SAP precursor in human skin fibroblasts. Our data indicate that SAP precursor bears phosphate residues on noncomplex carbohydrate chains linked to the SAP-C and the SAP-D domain and sulfate residues on complex carbohydrate chains located within the SAP-A, -C, and possibly the SAP-D domain. Treatment of fibroblasts with either bafilomycin A1 or 3-methyladenine indicates that proteolytic cleavage of SAP precursor begins as early as in the late endosomes. To determine whether targeting of SAP precursor depends on mannos 6-phosphate residues, we analyzed the processing of SAP precursor in I-cell disease fibroblasts. In these cells nearly normal amounts of newly synthesized SAP-C were found, although secretion of SAP precursor was enhanced 2–3-fold. Moreover, SAP-C could be localized to lysosomal structures by indirect immunofluorescence in normal and in I-cell disease fibroblasts. Mannose 6-phosphate was not found to interfere significantly with endocytosis of SAP precursor. Normal fibroblasts internalized SAP precursor secreted from I-cells nearly as efficiently as the protein secreted from normal cells. To our surprise, deglycosylated SAP precursor was taken up by mannose 6-phosphate receptor double knock out mouse fibroblasts more efficiently than the glycosylated protein. We propose that intracellular targeting of SAP precursor to lysosomes is only partially dependent on mannose 6-phosphate residues, whereas its endocytosis occurs in a carbohydrate-independent manner.

The lysosomal degradation of most glycosphingolipids with short oligosaccharide chains by acidic hydrolases is dependent on the action of small, nonenzymic glycoproteins, so-called sphingolipid activator proteins (SAPs)

- B, -C, and -D, also called saposins A–D, derive from a common precursor protein by proteolytic cleavage (2–4). Besides being the precursor protein of SAPs, there is increasing evidence that SAP precursor exhibits a function of its own. A portion of the precursor protein escapes from intracellular degradation. It is found in various human secretory fluids, predominantly in cerebrospinal fluid, milk, semen, bile, and pancreatic juice (5). High concentrations of the SAP precursor are found in brain and muscle (6), whereas the individual SAPs are present mainly in liver, kidney, and spleen.

An in vitro study using both recombinant and native protein revealed that SAP precursor combines some functions of mature SAPs, e.g. as a ganglioside-binding protein (7). Moreover, SAP precursor was also identified as a neurotrophic factor in vivo (8, 9). However, detailed studies on the mechanisms of endocytosis and intracellular processing of SAP precursor are still missing.

Early investigations of the biosynthesis and processing of SAP-B and -C have been performed by Fujibayashi and Wenger (10, 11) in cultured human fibroblasts. In both studies the authors found that a precursor glycoprotein of 65 kDa is initially synthesized, which is further processed to a 70-kDa polypeptide. A part of the latter is converted by proteolytic cleavage within lysosomes into 8–13-kDa mature SAP-B and SAP-C, respectively, whereas another part of the 70-kDa protein is secreted into the culture medium.

In I-cell disease (ICD) fibroblasts, that fail to phosphorylate mannose residues on newly synthesized lysosomal proteins, only half of the normal SAP-C concentration (12) and very low levels of SAP-B were found (13). Simultaneously, increased amounts of the precursor protein could be immunoprecipitated from the media of ICD fibroblasts (11). These results suggest that the transport of SAP precursor to lysosomes of human fibroblasts is predominantly mediated by the mannose 6-phosphate (Man-6-P)-dependent pathway (for review, see Ref. 14).

However, many tissues such as brain, liver, spleen, and kidney from ICD patients have normal levels of lysosomal enzymes (15) and SAPs (16), indicating a Man-6-P-independent pathway of lysosomal enzyme targeting in various cell types of these organs. Most data on the Man-6-P-independent targeting of a lysosomal protein have been derived from studies of cathepsin D. Experiments with ICD lymphoblasts revealed that the lysosomal targeting of cathepsin D is not dependent on carbohydrate residues, but is probably mediated by a peptide.
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determinant in the cathepsin D carboxyl-terminal lobe (17). Moreover, in human promonocytes the cathepsin D propeptide is also essential for targeting of cathepsin D to lysosomes (18). Rijnboutt et al. (19) showed a membrane association of β-glucosylceramidase, procathepsin D, and a 73-kDa SAP precursor protein in HepG2 cells, which was partially Man-6-P-independent. The Man-6-P-independent membrane association of 73-kDa SAP precursor and procathepsin D is presumably accompanied by a transient association of these proteins to each other during their passage from the Golgi to dense lysosomes in HepG2 cells (20). In the same study these authors demonstrated an early association of a 68-kDa SAP precursor form and procathepsin D within the ER and Golgi that was not combined with an association to either membrane.

In order to elucidate the significance of Man-6-P residues for the targeting of the SAP precursor, we studied biosynthesis and processing of the protein in normal and ICD fibroblasts. We demonstrate that intracellular targeting of SAP precursor is partly dependent on Man-6-P residues. In contrast, endocytosis of the protein is not mediated by a carbohydrate recognition marker.

EXPERIMENTAL PROCEDURES

Materials—[35S]Methionine (Trans35S-S-lABEL™, 41.77 TBq/nmol) and [35S]P9aHPO4 (3.7–37 GBq/nmol of P) were from ICN, [35S]Na2SO4 (0.9–1.5 TBq/mg of S) was from Amersham Buchler. β-Endo-N-acetylglucosaminidase H and peptide-N-glycosidase F were from New England Biolabs.

Baflozym A, 3-bromoladene, tunicamycin, mannose 6-phosphate, glucose 6-phosphate, fructose 6-phosphate, mannos e, and mannos e from Sigma. Asialo α1,3-acidglycoprotein was a kind gift of Dr. G. Schwarzmann (Institut für Organische Chemie und Biochemie der Universität Bonn, Germany) and had been prepared as described previously (21). Brefeldin A was from Boehringer Mannheim. All other chemicals were used of reagent grade and were purchased from various commercial sources.

Antiserum—Rabbit anti-human (SAP-C) antiserum was a kind gift of Dr. H. Christomanou (Laboratory of Neurochemistry and Molecular Biology, University of Athens, Greece) (22). Goat anti-human aroylsulfatase A antiserum was a generous gift of Dr. V. Gieselmann (Biochemisches Institut der Universität Kiel, Germany). The preparation of rabbit anti-human (SAP-A, SAP-B, and SAP-D) antiserum was described previously (23). Goat anti-human SAP-D antiserum was raised against a carboxyterminally expressed SAP-D (24). Rabbit anti-human (SAP-A) antiserum was raised against a peptide corresponding to the C terminus of the 50-kDa SAP-A purified from spleen of a Gaucher patient (25). Rabbit anti-human β-glucosylceramidase antiserum was a kind gift of Dr. Hans Aerts (E. J. Meuleman Institute, Belgium). Mouse anti-human SAP-C antiserum was a kind gift of Dr. Hans Aerts.

Media and pretreatment cell lysates were then incubated with 10 μl of rabbit-anti-SAP-A, -B, or -C antiserum or 3 μl of goat-anti-SAP-D antiserum overnight. Cross-reactive antiserum was precipitated with protein A-Sepharose (for rabbit IgG) or protein G-agarose (for goat IgG) (Calbiochem-Novabiochem). The immunocomplexes were then washed with PBS, pH 7.2, containing 0.8 M KCl, 0.5% Triton X-100, 0.25% BSA, and with PBS, pH 7.2, containing 0.1 M NaOH. (0.01% Nonidet P-40, 0.1% bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.4 kDa. Labeled polypeptides were detected by fluorography. Radioactivity of SAP proteins was quantified by densitometry of the fluorograms or by use of a PhosphorImager (Fuji-Raytest BAS 1000).

Glycosidase Treatment—Washed immunoprecipitates were boiled for 10 min in 60 μl of gel electrophoresis sample buffer (26). The supernatants were divided into three aliquots. Two aliquots were adjusted to 200 μl with 50 mM sodium citrate, pH 7.5, containing 1% Nonidet P-40, 0.5% SDS, and 1% β-mercaptoethanol, 2000 New England Biolabs units of peptide-N-glycosidase F (PNGase F) were added only to one aliquot, the other serving as a control sample. The third aliquot was adjusted to 200 μl with 50 mM sodium citrate, pH 5.5, containing 0.5% SDS and 1% β-mercaptoethanol and was incubated with 50 New England Biolabs units of β-Endo-N-acetylglucosaminidase H (RpoH1). After incubation for 20 h at 37 °C the protein was precipitated by addition of an equal volume of 10% ice-cold trichloroacetic acid, washed twice with ice-cold acetone, and dried under air. The pellets were solubilized in gel electrophoresis sample buffer and subjected to SDS-PAGE as described above.

For treatment of [35P]PO4- and [35S]SO4-labeled immunocomplexes, the procedure was modified to prevent release of the inorganic residues by trichloroacetic acid. Washed immunoprecipitates were boiled for 15 min in 60 μl of denaturing buffer (0.5% SDS, 1% β-mercaptoethanol), divided into three aliquots. Two aliquots were adjusted with 60 μl of 25 mM sodium phosphate, pH 7.2, 50 mM EDTA, 0.5% octylglucoside with or without PNGase F. The third aliquot was supplemented with buffer and Endo H as described above. After incubation at 37 °C for 20 h, the samples were supplemented with 20 μl of 5-fold concentrated gel electrophoresis sample buffer, boiled for 10 min, and subjected to SDS-PAGE.

Endocytosis—Confluent human skin fibroblasts or I-cell disease skin fibroblasts (75-cm2 flask) were labeled with 25 μCi of 35S-methionine for 1 h (0.5–1.0 μCi/ml) of [35S]methionine in 4 ml of methionine-deficient DMEM, containing 5% dialyzed fetal calf serum and 5% fetal calf serum, and added to fresh cells in the absence or presence of various reagents as indicated in Fig. 8, A and B. After the times indicated the cells were harvested, and cells and media were processed for immunofluorescence as described above.

Immunofluorescence—Human fibroblasts cultured on glass coverslips were washed with PBS at 4°C and fixed with 4% freshly prepared paraformaldehyde at room temperature for 30 min. After washing with PBS, cells were permeabilized by incubation for 7 min with 0.05% saponin (ICN Biochemicals, Cleveland, OH) at room temperature. Then the cells were treated for 60 min at 37 °C with 3% BSA (Serva Bio-
In the presence of BFA, processing of the 65-kDa form into the 73-kDa form was considerably slowed, but not completely abolished. These findings are consistent with previous observations that Golgi enzymes, which are redistributed to the ER in the presence of BFA, remain active (29). As expected, the secretion of the 73-kDa precursor into the culture medium and the proteolysis of the precursor into mature SAP-C was completely blocked by BFA.

These observations indicate that SAP precursor is synthesized as a 65-kDa polypeptide within the ER and that conversion into 73-kDa SAP precursor occurs in the Golgi apparatus. In accordance with previous findings (11), cleavage into mature SAPs has to take place in a compartment distal to the TGN, i.e. within endosomes or lysosomes.

**Proteolytic Processing of SAP Precursor Is a Late Endosomal/Lysosomal Event**—The pH-dependent sorting of lysosomal proteins to lysosomes is affected by weak bases. Indeed, NH₄Cl strongly stimulated the secretion of SAP precursor, whereas only 10–20% of SAP precursor were processed into mature SAP-C (Fig. 3). However, weak bases are rather non-specific reagents that may effect parameters other than pH, that are important for vesicular protein transport. A specific inhibitor of vacuolar H⁺-ATPases (30) is the macrolide antibiotic bafilomycin A₁ (BA₁), which is known to neutralize the pH of endosomes and to block the transport from endosomes to lysosomes (31, 32). In the presence of BA₁, formation of mature SAP-C was completely blocked, whereas secretion of SAP precursor was further enhanced compared to the cells incubated with NH₄Cl. In addition, there are 44–50-kDa intermediate forms appearing in the culture medium in the presence of NH₄Cl and BA₁. These polypeptides presumably derive from extracellular limited proteolysis of SAP precursor.

In order to exclude the possibility that the effect of BA₁ was caused by an inhibition of acidic proteases by the neutral environment in the endosome, we studied the processing of SAP precursor in the presence of 3-methyladenine. This agent blocks the transport from late endosomes to lysosomes by a yet unknown mechanism without affecting the pH of intracellular organelles (33). Under 3-methyladenine treatment, nearly normal amounts of SAP-C were formed (Fig. 3). Thus, processing of SAP precursor presumably begins in late endosomes. The higher molecular mass (14 kDa) of the mature protein under 3-methyladenine compared to the normal mature SAP-C (9–11 kDa) possibly reflects the lack of further processing events taking place within the lysosomes.

Our attempts to block proteolytic cleavage of SAP precursor in vivo with 100 μM leupeptin alone or together with pepstatin (100 μM each) during metabolic labeling led to a slowed, but still efficient proteolysis (data not shown), suggesting that there is no specific serine, aspartyl, or cysteiny protease solely responsible for cleavage of SAP precursor.

**Glycosylation of SAP Precursor**—PNGase F digestion of immunoprecipitates from normal fibroblasts leads to conversion of 73- and 65-kDa SAP precursors to a 53-kDa polypeptide, whereas the apparent molecular mass of mature SAP-C is reduced from 9–11 kDa to about 7 kDa (10).

In order to distinguish between complex and high mannose or hybrid carbohydrate structures, we performed Endo H digestion of anti-SAP-C cross-reactive material from normal fibroblasts (Fig. 4). Endo H treatment resulted in conversion of all of the 65-kDa SAP precursor and a minor part of the intracellular 73-kDa form into a 53-kDa polypeptide, whereas the 73-kDa precursor precipitated from medium remained unaltered. Consequently the 73-kDa form possesses predominantly complex carbohydrate chains, whereas the 65-kDa form contains only high mannose or hybrid sugars. Only 20–30% of
mature 9–13-kDa SAP-C was converted by Endo H into the unglycosylated 7-kDa form. In contrast, SAP-C from ICD fibroblasts showed a higher molecular mass (14 kDa) and Endo H-resistant carbohydrate chains.

Phosphorylation and Sulfatation of the SAP Precursor—In fibroblasts, the targeting to lysosomes of most soluble lysosomal proteins is mediated by Man-6-P residues. In order to investigate whether there are phosphate residues present on SAP polypeptides, we metabolically labeled human fibroblasts with Na₂H₃[³²P]PO₄ for 5 h.

Both 65- and 73-kDa SAP precursor forms bear phosphate residues on N-linked oligosaccharide chains linked to both the SAP-C (Fig. 5) and the SAP-D domain (data not shown). In contrast, no carbohydrate-bound phosphate residues could be detected on SAP-A and -B. Compared to the amounts of SAP precursor labeled with [³⁵S]methionine (Fig. 5), phosphate labeling of the secreted precursor protein appeared to be up to 10-fold weaker than that of the intracellular SAP precursor.

In addition, the 73-kDa SAP precursor, SAP-C (Fig. 5), and SAP-A (data not shown), could be labeled efficiently with Na₂[³⁵S]SO₄ after a 5-h pulse, whereas no evidence was found for a sulfate residue on SAP-B. Sulfate labeling of SAP-D was strong in ICD cells, but only barely or not detectable in several experiments with normal fibroblasts (not shown). The inorganic residue on SAP-A and -C is bound to complex N-linked sugar chains, since PNGase F, but not Endo H, released all detectable radioactivity from these proteins. Incorporation of the SO₄ radiolabel was abolished in the presence of tunicamycin (data not shown).

Targeting of SAP Precursor to Lysosomes Is Only Partially MPR-dependent—As shown in Fig. 6, ICD fibroblasts synthesized a 65-kDa form of SAP precursor in amounts comparable to those of the 65-kDa form in control cells within 75 min. The time course of proteolytic processing into mature SAP-C appeared to be normal, and the amounts of mature SAP-C in ICD fibroblasts were slightly reduced (20–40%) compared to normal cells. On the other hand, ICD fibroblasts showed a 2–3-fold enhanced secretion of high molecular mass SAP precursor (75 kDa), clearly indicating a missorting of the protein.

Mature SAP-C was also detectable in normal fibroblasts treated with 10 µg/ml tunicamycin. However, in drug-treated cells only 15% (versus 50% in controls) of total cross-reactive material was found as mature SAP-C after a pulse of 5 h, whereas secretion of the 53-kDa SAP precursor into the medium was enhanced 1.5–2-fold (Fig. 3). These data point toward an alternative route for targeting of SAP precursor to lysosomes besides the Man-6-P-dependent pathway.

Subcellular localization of SAP-C in normal and ICD fibroblasts (each 3 different cell lines) support the results obtained by metabolic labeling and immunoprecipitation. Immunofluorescence studies with a rabbit- and a goat-anti-SAP-C antiserum revealed a vesicular pattern typical for endosomal/lysosomal structures (the same results were obtained with an anti-SAP-A, -B, and -D antiserum, data not shown). In both cell types SAP-C is mainly localized in lysosomes, as determined by double immunofluorescence microscopy with a monoclonal anti-lamp-1 antibody (Fig. 7 a–d). Moreover, SAP-C could be colocalized with β-glucosyleramidase in normal and ICD fibroblasts (Fig. 7, e–h).

Endocytosis of 73-kDa SAP Precursor Is MPR-independent—The best known pathway for internalization of soluble lysoso-
mal proteins by cultured human fibroblasts is the endocytosis via the mannose 6-phosphate/insulin-like growth factor II receptor (MPR 300) (14, 34). If endocytosis of SAP precursor depends on the action of MPR 300, it should be blocked by the presence of Man-6-P in the culture medium. Moreover, the lack of Man-6-P residues on SAP precursor secreted from ICD fibroblasts should prevent its uptake.

NH₄Cl-induced secretion of [³⁵S]methionine-labeled normal and ICD fibroblasts was dialyzed against DMEM and fed to unlabeled cells of the same type. Within 24 h of endocytosis, both control and ICD fibroblasts converted endocytosed SAP precursor to the mature SAPs (9–11 kDa), consistent with the pulse-chase experiments (Figs. 2 and 6), which had shown that processing to the mature SAPs takes only a few hours. After 24 h of endocytosis, normal fibroblasts internalized about 4.3 ± 2.0% (mean ± S.D. from seven experiments) of normal SAP precursor present in the medium, whereas ICD cells internalized 6.6 ± 1.5% (mean ± S.D. from three experiments) of extracellular SAP precursor derived from ICD cells. The rates of endocytosis of ICD cells after 1 h were 3-fold higher than that of normal cells, decreasing to 2-fold after 4–16 h and approaching the levels of normal fibroblasts after 24 h. The surprisingly efficient uptake of SAP precursor secreted from ICD cells by the same cell type points toward a Man-6-P-independent mechanism for endocytosis of SAP precursor in human fibroblasts.

In order to investigate whether there are other carbohydrate-specific receptors involved in endocytosis of SAP precursor, the [³⁵S]methionine-labeled proteins secreted from normal cells were added to normal cells in the absence or presence of various sugars and sugar conjugates, respectively. As shown in Fig. 8A, after 24 h of endocytosis anti-SAP-C immunoprecipitates of the recipient cells contained minor quantities of the unprocessed labeled 73-kDa SAP precursor. The major part of the precursor protein (>95%) had been processed into mature SAPs as demonstrated by the bands at 9–11 kDa, referring to labeled mature sphingolipid activator protein C. Under these conditions, intermediate forms of processed SAP precursor were not detectable in the cells. In the presence of 10 mM Man-6-P the amount of endocytosed SAP polypeptides (SAP precursor at 73 kDa and mature SAP-C at 9–11 kDa) decreased by only 20% (compared to untreated cells). Simultaneously, uptake of labeled arylsulfatase A (Fig. 8A) and β-hexosaminidase B (data not shown) was completely blocked by 10 mM Man-6-P. Furthermore, neither glucose 6-phosphate, fructose 6-phosphate, mannose, asialoglycoprotein, and mannan, respectively nor a combination of Man-6-P, mannan, and asialoglycoprotein was sufficient to reduce internalization of labeled SAP precursor significantly, whereas uptake of labeled arylsulfatase A was completely blocked by 10 mM Man-6-P.
secreted from ICD cells. When NH₄Cl-induced secretions of effects and influence uptake of the SAP precursor indirectly.
components in the conditioned medium could alter secondary ability that an exogenous carbohydrate together with undefined SAP precursor uptake, although we cannot exclude the possi-
dencethatneithermannoseorgalactoseresiduescontributeto phosphate receptors. Furthermore, the experiments provide evi-
discussing the defect within these cells.

Based on these data, we asked whether the carbohydrate structures of SAP precursor do play a role in endocytosis of the protein at all. For this purpose we took advantage of the fact that tunicamycin induces an increased secretion of nonglyco-
sylated SAP precursor into the culture medium of human fibroblasts. Surprisingly, the uptake of those tunicamycin-in-
duced secretions was up to 2-fold more efficient than uptake of the carbohydrate-bearing protein (not shown).

The above results are supported by experiments with fibroblasts from mice with a targeted disruption of both mannose 6-phosphate receptor alleles (MPR 46/300⁺) (35) and wild type mouse fibroblasts (MPR 46/300⁻) (Fig. 9). SAP precursor secreted from normal human fibroblasts was taken up rather efficiently by MPR 46/300⁻ fibroblasts (76% of control cells after 16 h of endocytosis). Again, carbohydrate-deficient SAP precursor secreted from normal human fibroblasts in the pres-
ence of tunicamycin was internalized easily by both cell lines, with rates being 130% (MPR 46/300⁻) and 150% (MPR 46⁻/300⁻) of the rates in the experiments with the carbohydrate-bearing protein. In MPR 46/300⁻ fibroblasts the internalized protein was only poorly degraded, presumably due to the lack of responsible proteases and glycosidases in this cell line. Taken together, our data indicate, that endocytosis of SAP precursor is not dependent on its carbohydrate structures.

**DISCUSSION**

The present study demonstrates that targeting of SAP pre-
cursor from the Golgi to lysosomes is only partially dependent on mannose 6-phosphate residues, whereas endocytosis occurs in a carbohydrate-independent manner.

We found that SAP precursor from normal fibroblasts bears phosphate residues linked to carbohydrate chains of the SAP-C and SAP-D domain. The considerably decreased phosphate incor-
prception of the secreted precursor protein versus the intra-
cellular SAP precursor points toward a decisive role of phos-
phate residues for intracellular targeting. This hypothesis was confirmed by experiments with ICD fibroblasts. In I-cell dis-
ease fibroblasts, secretion of SAP precursor is enhanced 2–3-
fold compared to normal cells, clearly indicating a missorting of the protein. On the other hand, these cells produce nearly as much SAP-C in acidic compartments as normal fibroblasts do. In immunofluorescence studies mature SAP-C could be local-
ized within lysosomes of ICD cells. Consequently, the precursor reaches the lysosomes, even if it is lacking phosphorylated mannose residues. In addition, even in the presence of tunicamycin at concentrations completely inhibiting N-glycosylation of proteins, normal fibroblasts produce mature SAP-C. The amounts of SAP-C are 2–3-fold reduced in relation to normal untreated cells, but nevertheless considerable amounts of the mature protein indicate that the presence of sugar chains is not essential for targeting of SAP precursor to lysosomes.

It is likely that the SAP precursor reaches lysosomes in I-cells by a mechanism of secretion and increased reuptake via the plasma membrane, since this cell type showed an enhanced rate of endocytosis of SAP precursor compared to normal fibro-
blasts. Together with the high amounts of SAP precursor pres-
ent in the culture medium of I-cells, endocytosis might compen-
sate the insufficient rates of intracellular transport to lysosomes. Additional support for this hypothesis comes from experiments with mouse fibroblasts that lack both mannose 6-phosphate receptors. In these cells about 15% of all lysosomal enzymes reach the lysosomes (35, 36). Interestingly, the effi-
ciciency of enzyme transport to lysosomes returns to nearly nor-
mal values when these cells are transfected with the MPR 300 gene (37). In contrast, the transfection with the gene of the MPR 46, that is not active in endocytosis of lysosomal enzymes, corrects the missorting only partially (37, 38). Thus, the secreted lysosomal enzymes seem to be recaptured by endocytosis.

The above proposition seems to be contradictory to the fact that there is a dramatic loss of intracellular mature SAP-C.
in NH4Cl-treated cells despite the high amounts of SAP precursor present in the medium. However, lysosomotropic agents such as NH4Cl cause a depletion of receptors on the cell surface by impairing receptor recycling, thereby inhibiting receptor-mediated endocytosis (39–41).

As supported by the present work, SAP precursor is a member of a growing group of lysosomal enzyme precursors such as those of cathepsin D, β-glucosylceramidase, and α-glucosidase (42–44), that are sorted via Man-6-P-independent mechanisms in ICD fibroblasts. One of these targeting mechanisms involves membrane association, as demonstrated for SAP precursor, cathepsin D, and β-glucosylceramidase in HepG2 cells by Rijnboutt et al. (19) and for α-glucosidase in transfected COS-1 cells by Wisselaar et al. (45). According to the results of Zhu and Conner (20), the membrane association of cathepsin D and SAP precursor is accompanied by the transient formation of a complex of the two precursor proteins during their way from the late Golgi to dense lysosomes in HepG2 cells. However, we did not find any coprecipitation of cathepsin D by immunoprecipitation of extracts from cross-linked fibroblasts with an anti-SAP-C antiserum (data not shown). In addition, the amounts and the cellular distribution of cathepsin D polypeptides in SAP-deficient fibroblasts were normal (as determined by immunoprecipitation and immunofluorescence microscopy, data not shown). Therefore, the association of SAP precursor and cathepsin D plays a minor role for targeting of SAP precursor to lysosomes in fibroblasts. It remains an open question which determinants account for the Man-6-P-independent pathway to lysosomes in human fibroblasts.

Additionally, the function of the carbohydrate-linked SO4 residues on SAP precursor remains unclear. There are several other lysosomal proteins bearing a sulfate residue on precursor and mature forms, among them cathepsin D, the α-chain of β-hexosaminidase, and arylsulfatase A (46). The sulfate resi-
FIG. 9. Endocytosis of carbohydrate-deficient SAP precursor by normal and MPR-deficient mouse fibroblasts. Normal human fibroblasts were labeled with [35S]methionine in the presence of 10 mM NH4Cl or 10 μg/ml tunicamycin for 20 h. The media were diazoyed against DMEM and given to wild type (MPR 46/300) and MPR double knock out mouse fibroblasts (MPR 46/300) for 16 h. Immunoprecipitation of SAP polypeptides from whole cell lysates and aliquots (1/15) of media was performed with a rabbit-anti-SAP-C antisem, followed by analysis by SDS-PAGE (5–20%) and fluoroography.

The considerable amounts of nonglycosylated SAP precursor present in cells and media after metabolic labeling in the presence of tunicamycin indicate that stability and correct folding of SAP precursor does not require carbohydrate chains. A recent study shows that the lack of a 48-amino acid peptide corresponding to the mRNA levels in human fibroblasts have been determined to be 72:4:24 (55), but only the protein corresponding to the mRNA species lacking exon 8 has been detected so far. In an actual study, the three human SAP precursor isoforms have been expressed in baby hamster kidney cells (25). No difference in the processing of those SAP precursor proteins and their ability to produce functional active SAP-B isoforms was found. Furthermore, Henseler and co-workers (25) demonstrated that the amino acids encoded by exon 8 are not decisive for endocytosis of SAP precursor. All three expressed isoforms were endocytosed by human fibroblasts in similar amounts within 24 h.

Another mechanism of endocytosis of SAP precursor could occur via an association of the protein with glycosphingolipids of the cell surface mediated by its four lipid binding domains, since SAP precursor has been shown to bind to gangliosides in vitro as efficiently as individual SAPs (7). However, these experiments had been performed at acidic pH (pH 4.0), whereas the extracellular milieu is neutral (pH 7.0–7.4). A recent study (56) demonstrates that a decrease in pH down to pH 4.0 induces conformational changes of individual SAPs that increase the hydrophobicity of SAP-A, -C, and -D considerably. Simultaneously, the affinity of individual SAPs, in particular that of SAP-B, to phospholipid membranes increases. Therefore, the ganglioside binding properties of SAP precursor at neutral pH could differ significantly from those at acidic pH. We are attempting to clarify the Man-6-P-independent pathway of SAP precursor to lysosomes by further experiments.

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