Prosaposin is a multifunctional protein encoded at a single locus in humans and mice. The precursor contains, in tandem, four glycoprotein activators or saposins, termed A, B, C, and D, that are essential for specific glycosphingolipid hydrolase activities. Prosaposin appears to be a potent neurotrophic factor. To explore the proteolytic processing from prosaposin to mature activator proteins, metabolic labeling was done with human prosaposin expressed in insect cells, human fibroblasts, neuronal stem cells (NT2) and retinoic acid-differentiated NT2 neurons. In all cell types, the major processing pathway was through a tetrasaposin, A-B-C-D, from which saposin A was then removed. In mammalian cells monosaposins were derived from the trisaposin B-C-D by cleavage to the disaposins, B-C and C-D, that were processed to monosaposins. In insect cells the major end products were the disaposins, A-B and C-D derived from the tetrasaposin, A-B-C-D, or with B-C and C-D derived from the trisaposin, B-C-D. In insect and mammalian cells, the nonsignalNH₂-terminal peptide preceding saposin A (termed Nter) was usually removed prior to saposin A cleavage. In NT2-derived differentiated neurons, precursor tetrasaposins containing A-B-C-D were secreted with and without Nter. Immunofluorescence studies using prosaposin-specific antisera showed large steady state amounts of uncleaved prosaposin in Purkinje cells, cortical neurons, and other specific cell types in adult mice. These studies indicate that prosaposin processing is highly regulated at a proteolytic level to produce prosaposin, tetrasaposins, or mature monosaposins in specific mammalian cells.

The prosaposin cDNA encodes a 508- or 511-amino acid mature polypeptide chain (4, 5) and a 16-amino acid signal sequence (Fig. 1) (4). The four saposins contain ~80 amino acids and have highly similar sequences. The six cysteines in each saposin have nearly identical placement. The complete disulfide structures of saposin B and C have been defined and predict a tight "hairpin-like" structure (17). This structure and the predicted four α-helical bundles (18) are probably conserved in saposins A and D and likely will account for the great thermostability of the saposins and some of their biological activity. In human prosaposin, intersaposin amino acid sequences range from 16 to 54 residues in length (Fig. 1). In murine prosaposin an additional 30 amino acids are incorporated in the region between saposins C and D (6, 19, 20). Proteolytic cleavage of the mature prosaposin glycoprotein within the lysosome produces the mature saposins (21, 22). Saposins B, C, and D contain single occupied N-glycosylation sites, and saposin A contains two (23–27).

The saposins are intralysosomal, whereas prosaposin can be secreted into the medium of cultured cells and into body fluids (28, 29). Prosaposin has high concentrations in seminal fluid, breast milk, and cerebrospinal fluid (30). Although the precise physiological function of intact prosaposin has not been defined, it functions in vitro as a glycosphingolipid transfer protein (31, 32) and ex vivo as a neurotrophic agent (32, 33). Administration of prosaposin to cultured mammalian cells or in fluid surrounding injured sural nerves leads to neurite outgrowth (32, 33) or regeneration (34), respectively.

Fujibayashi and Wenger (21, 22) used anti-B or -C antisera to characterize the time courses for proteolytic processing of prosaposin to mature saposin B or C in cultured skin fibroblasts. Several saposin-containing intermediates were found (21, 22), but the saposin contents and the distributions between the intracellular and extracellular compartments were not described. While these studies provided the general outline for the time courses, the order and timing of liberation of the individual saposins from prosaposin during proteolytic processing is not known. Using the baculovirus expression system, several prosaposin-derived intermediates were found in the media of insect cells infected with virus containing the prosaposin cDNA. The processing pattern was suggested to be sequential removal of individual saposins proceeding from the NH₂- to COOH terminus (35).
The mature human prosaposin is 508 (4, 46) or 511 (5) amino acids in length excluding a 16-amino acid signal sequence. The variation in amino acid sequence results from alternative splicing of a 9-base pair exon in the saposin B genomic sequence. Nter refers to the amino acid sequence that follows the signal sequence and precedes the mature saposin A NH2-terminus. The intersaposin sequences refer to amino acids that are proteolytically clipped during the processing to mature saposins and are shown in approximate proportion to their length. The individual saposins are ~80 amino acids in length. The intersaposin C-D amino acid sequence is present in the mouse and contains 30 amino acids that are 80% homologous to the human protein. Saposin A, B, C, and D proteins were derived from the human prosaposin cDNA sequence, and the Nter and C-D proteins were from the respective mouse sequences.

In this communication, we characterized the proteolytic processing of prosaposin to various intermediates in cultures of insect cells, skin fibroblasts, human neuronal stem cells, and mature differentiated neurons (NT2 cells). In addition, immunofluorescence microscopy, using antisera specific for saposin or prosaposin, showed cell-specific proteolytic processing of prosaposin in adult mouse tissues.

EXPERIMENTAL PROCEDURES

Materials—The following were from commercial sources: Spodoptera frugiperda (SF9) donor cells and wild-type AcMNPV1 virus (Invitrogen, San Diego, CA); NT2 (Ntera 2/D1) cells (Stratagene, La Jolla, CA); [35S]cysteine (specific activity 1075 Ci/mmol) (Du Pont NEN); Sf 900 (San Diego, CA); NT2 (Ntera 2/D1) cells (Stratagene, La Jolla, CA); E. coli host strain (BL21 (DE3)) and His60 (San Diego, CA); HSF, cultured human skin fibroblasts; VL-prosap, recombinant AcMNPV derived from homologous recombination of PVL-{35S}cysteine pulse, the confluent HSF or NT2 cells were rinsed once with cysteine-free DMEM and incubated for 1 h under fresh cysteine-free DMEM, containing 10% dialyzed FBS. This medium was replaced with prewarmed (4 °C, 37 °C) cysteine-free DMEM containing 10% dialyzed FBS and 150 μCi/ml [35S]cysteine. After incubation for the indicated time, the cells were rinsed twice with phosphate-buffered saline (150 mM NaCl, 25 mM NaHPO4, 25 mM K2HPO4, pH 7.5) and harvested with trypsin-EDTA. In some experiments, after incubation with [35S]cysteine, the cells were washed three times with DMEM, and nonradioactive chase medium was added. Cells were washed three times with 0.3% bovine serum albumin in phosphate-buffered saline and then lysed. After the indicated chase periods, the harvested cells were lysed in 250–500 μl of lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride). After incubation for 30 min on ice with shaking, these mixtures were centrifuged at 10,000 × g (20 min), and an equal volume of radioimmunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5) was added to the supernatants. For SF9 cells, the procedure was the same, except that cysteine-free Grace insect cell culture medium, containing 150 μCi/ml [35S]cysteine was used for labeling and SF900 medium was used for chase experiments. Cells were grown in 25-cm2 flasks and lysed in 1 ml of lysis buffer. Spent media was clarified by centrifugation twice at 13,000 × g (10 min) and then 10,000 × g (30 min).

Preparation and Characterization of Antisera—The coding regions for the individual human saposins A, B, C, and D, and the mouse NH2-terminal prosaposin (Nter, amino acid residues Pro18 to Lys345), or the mouse intersaposin C-D region (residues Arg389 to Gly436) (4) were cloned into the pET vector, overexpressed in E. coli, and purified to homogeneity on nickel column essentially as described (16). Polyclonal antibodies were raised in New Zealand white rabbits (40). The specificity of each antiserum was determined by Western blot analysis (16) and immunoprecipitation experiments using purified saposins expressed in E. coli (data not shown). No cross-reactivities were detected.

Immunoprecipitation—Nonspecific components that might bind to immunoprecipitates were removed from cell lysates and media by treatment with S. aureus cells (1:10, v/v) for 1.5 h. After centrifugation (10,000 × g, 20 min), the test supernatants were incubated with 1% preimmune serum overnight (4 °C). Nonspecific complexes were precipitated with protein A-Sepharose CL-4B beads. The clarified supernatants were incubated (4 °C) overnight with antisaposin antisera. The antigen-antibody complexes were removed from the test supernatants by treatment with protein A-Sepharose CL-4B beads and washed three times with radioimmune precipitation buffer at 4 °C. This procedure was repeated 5 times with radioimmune precipitation buffer by centrifugation and once with 0.1% Nonidet P-40 in 5 mM Tris-HCl, pH 7.5. The washed immuno-precipitates were denatured (95 °C, 7 min) in 4-fold-diluted (v/v) SDS-electrophoresis buffer. Radioactivity (cpm) was determined by scintillation spectrometry. Aliquots were electrophoresed in 20% SDS-PAGE (PhastSystem, Pharmacia Biotech Inc.). Autoradiography was performed by exposure of the dried gels to phosphor screens (Molecular Dynamics, Sunnyvale, CA) for 2–4 days. For fluorography, x-ray films were exposed to the gels (−80 °C, 3–4 weeks). Control experiments omitting antisera. Controls for SF9 experiments showed no reactivity of antisapossin antisera with infected (AcMNPV) or uninfected SF9 cells. N-Glycanase was used to remove N-linked oligosaccharides according to the manufacturer’s instructions (16). Exhaustive immunoprecipitation was used to completely deplete all saposin-associated immunoreactive materials (prosaposin, intermediates, and forms, saposins A, B, C, or D). Repeated immunoprecipitation of the supernatants with one antisapossin antiserum was done until no further radioactivity was precipitated. This was monitored by fluorography of SDS-PAGE and scintillation spectrometry for radioactivity in precipitates. Some lysates or media were treated sequentially with all five antisera, i.e. anti-Nter and anti-A, B, C, and D. Incubation with each successive antiserum was done only after no further radioactive material was immunoprecipitated. To eliminate contamination of the immunoprecipitable material due to remaining soluble immunocomplexes, the following was done. After the last treatment with the specific antisapossin, the supernatants were treated twice with protein A-Sepharose.

Immunofluorescence in Tissue Sections—Three-month old C57 black
although the general pattern of labeled species was similar in intermediates on SDS-PAGE during sequential immunoprecipitation, suggested by the differences in signals from various intermediates. This was also probably due to competition for antiserum by the very large amounts of unlabeled saposin intermediates. This was also confirmed by the diminishing immunoprecipitation of the supernatant with anti-D antiserum. This was particularly important in Sf9 cells, since antiserum were quantitatively depleted before adding the next antiserum. These selective effects differed with each antiserum, e.g., with anti-A all species were immunoprecipitated nearly equally from Sf9 cells in successive steps even though prosaposin was predominant (data not shown). The relationships between the immunoprecipitated labeled species were determined by sequential exhaustive immunoprecipitations using each antiserum and the same lysates. Systematic varying of antiserum addition order allows for identification of the saposin contents of the various species (Fig. 2). For example, disaposins containing A-B, B-C, and C-D have similar molecular weights but could be distinguished since A-B could be precipitated by anti-A, and B-C and C-D could be immunoprecipitated from A-B-depleted supernatants with anti-C. Conversely, if anti-B was used first, no anti-A precipitable species would remain in this molecular weight range.

RESULTS

Studies in Sf9 Cells—To compensate for different avidities and titers of the various antisera and the varying levels of prosaposin-derived forms, we developed an exhaustive immunoprecipitation procedure (see "Experimental Procedures") (Fig. 2). This procedure ensured that all prosaposin-derived forms could be immunoprecipitated with a particular antiserum were quantitatively depleted before adding the next antiserum. This was particularly important in Sf9 cells, since the proteins were greatly overexpressed. For example (Fig. 3), nine sequential immunoprecipitations with anti-D antiserum were needed to deplete all of the saposin D-containing species from VL-prosap infected Sf9 cells. The decreasing signal on SDS-PAGE gels was confirmed by the diminishing immunoprecipitable radioactivity. The increasing SDS-PAGE signal and radioactivity in the first three immunoprecipitations are probably due to competition for antiserum by the very large amounts of unlabeled saposin intermediates. This was also suggested by the differences in signals from various intermediates on SDS-PAGE during sequential immunoprecipitation, although the general pattern of labeled species was similar in all lanes. In Fig. 3a, lanes 3–6, the M, ~43,000–48,000 (arrow) and ~16,500 species were more intense than in lanes 1 and 2. This result suggests that this antiserum has greater avidity for intact prosaposin (M, ~60,000) and/or that this precursor competes with other saposin D-containing species for the antisera. These selective effects differed with each antiserum, e.g., with anti-D all species were immunoprecipitated nearly equally from Sf9 cells in successive steps even though prosaposin was predominant (data not shown).

The relationships between the immunoprecipitated labeled species were determined by sequential exhaustive immunoprecipitations using each antiserum and the same lysates. Systematic varying of antiserum addition order allows for identification of the saposin contents of the various species (Fig. 2). For example, disaposins containing A-B, B-C, and C-D have similar molecular weights but could be distinguished since A-B could be precipitated by anti-A, and B-C and C-D could be immunoprecipitated from A-B-depleted supernatants with anti-C. Conversely, if anti-B was used first, no anti-A precipitable species would remain in this molecular weight range.

In a typical experiment (Fig. 4a), Sf9 cells were pulse-labeled (1 h) and chased (3 h). Sequential exhaustive immunoprecipitations with antiserum to Nter, saposin A, and saposin D showed that numerous immunoprecipitations were needed to completely remove anti-Nter or anti-A immunoreactive material. Lanes 1–3 or 4–6 (Fig. 4a) show reductions in Nter- or saposin A-containing species, respectively, after 4 and 3 successive immunoprecipitations. A single major species at M, ~60,000 was precipitated with anti-Nter. This was consistent with the full-length mature prosaposin containing Nter-saposin A-B-C-D. A low level diffuse signal was at M, ~50,000 (lanes 1 and 2). This was consistent with the presence of small amounts of a Nter-saposin A proteolytic fragment.

The Nter-depleted specimens contained intermediates with saposin A components (the trisaposin A-B-C (M, ~45,000, Fig. 4a, lane 4, asterisk); the disaposin A-B (M, ~31,000), and saposin A (M, ~20,000)). Subsequent immunoprecipitations reduced these intermediates (Fig. 4a, lane 5). A dense band near the molecular weight of a precursor (M, ~60,000) persisted until the fourth anti-A immunoprecipitation (lanes 4–6). This most likely represents the tetrasaposin, A-B-C-D, after Nter had been removed. Subsequent immunoprecipitation with anti-D (Fig. 4a, lane 7) identified two major species with M, ~40,000 (lane 7, arrow) and M, ~17,000. These were consistent

**Fig. 2.** Schematic diagram of the protocol for the exhaustive immunoprecipitation of cell lysates and the potential immunoprecipitable intermediates. This procedure was generalized to cellular and media sources. For example, exhaustive immunoprecipitation of a cell lysate with anti-A antiserum could deplete the supernatant of a tetrasaposin (A-B-C-D), a trisaposin (A-B-C), a disaposin (A-B), and a monosaposin (A). The order of treatment with the different antisera will determine the species that are precipitated. If anti-B was used before anti-saposin A and the disaposins A-B and B-C were present, anti-B would deplete the supernatant of both these disaposins. Subsequent treatment of the supernatant with anti-A would precipitate any remaining saposin A-containing intermediates.
the antiserum treatments. The decreasing presence of a virus. After infection (36 h), Sf9 cells were pulse-labeled with \[\text{[35S]}\text{cysteine}\] labeling. Following \[\text{[35S]}\text{cysteine}\] labeling, different species of trisaposin containing the saposin compositions A–C and B–D were detected. These forms could not be completely resolved.

Disaposins A–B and C–D were detected occasionally. The Nter-saposin was detected up to 24 h. In media, the amounts of prosaposin and disaposins were at lower levels than the precursors or disaposins. The disaposins were detected at 0–4 h of chase. The disaposins were detected up to 24 h. In media, the amounts of prosaposin and disaposin increased between 0 and 4 h of chase and decreased by 24 h (data not shown). Fig. 4b shows the effect of deglycosylation on these species (4 h of pulse, no chase). The mobilities of all species were increased following deglycosylation. The faster migration of the C–D (lane 5, ●), compared with the A–B (lane 3, asterisk), disaposin is explained by the heavier glycosylation of saposin A and the potential presence of greater amounts of intersaposin sequences (see Fig. 1).

To determine the rate of prosaposin processing, VL-prosap-infected Sf9 cells were pulse-labeled (10 min) and chased for up to 24 h (Fig. 4c, anti-A). Prosaposin, trisaposins, or disaposins were present in cell lysates at 0 min of chase. Other uncharacterized transient species were present (lanes 1–3, asterisks). The trisaposins were detected at 0–4 h of chase. The disaposins were detected at 24 h. In media, small amounts of proteins with Mr, < 15,000 occasionally were detected in the media. The results were similar with all anti-saposin antisera.

Prosaposin, tetrasaposins, trisaposins, and disaposins were in spent media of VL-prosap-infected High-5 cells. The trisaposins were at lower levels than the precursors or disaposins. At least two distinct species of tri- and disaposins were present. 1) Trisaposins M, ~45,000–48,000, A–B–C (Fig. 5, lanes A, B, and C), and M, ~43,000–45,000, B–C–D (lanes B, C, and D) were detected. These forms could not be completely resolved. The broad diffuse bands at M, ~43,000–48,000 in lanes A, B, and C and the tighter band at M, ~43,000 in lane D support the existence of two trisaposin species. 2) Disaposins M, ~31,000, A–B (lanes A and B), and M, ~27,000, C–D (lanes C and D), were detected. The differences in migration and the diffuseness of the disaposin bands in lane B suggested that the anti-B antiserum recognized disaposins containing A–B and B–C. This was verified by differing sequential immunoprecipitations in pulse-chase studies (data not shown). Small amounts of saposins A, B, C, or D were detected occasionally. The Nter-saposin

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**Fig. 4.** Immunoprecipitation of VL-prosap-infected Sf9 cells following \([\text{[35S]}\text{cysteine}\) labeling. a. Insect cells were labeled for 20 h following infection with VL-prosap and then immunoprecipitated with anti-Nter (N), anti-A (A), or anti-D (D) antiserum. Analyses were by SDS-PAGE. The superscripts of N, A, and D refer to the number of sequential immunoprecipitations, and the arrows indicate the order of the antiserum treatments. The decreasing presence of a Mr, ~60,000 species with sequential immunoprecipitations with anti-Nter (lanes 1–3) and anti-A (lanes 4–6) show the presence of two tetrasaposins containing saposins A, B, C, and D with (lanes 1–3) or without (lanes 4–6) the Nter sequence. The presence of a Mr, ~43,000 and ~40,000 species in lane 4 (anti-A) and lane 7 (anti-D), respectively, indicate two different species of trisaposin containing the saposin compositions A–B–C and B–C–D, respectively. The disaposins A–B (M, ~31,000, lane 4) and C–D (M, ~27,000, lane 7, ●) differ in Mr, due to glycosylation differences (Fig. 1) and, potentially, intersaposin sequences. In panel b, Sf9 cells were pulse-labeled for 4 h and sequentially and exhaustively immunoprecipitated with the indicated antisera. Immunoprecipitates were untreated (−) or treated (+) with N-glycanase prior to SDS-PAGE. All of the detected labeled species increased their mobility after treatment with N-glycanase. The asterisk (lane 3) and circle (lane 5) indicate the disaposins A–B and C–D, c, Sf9 cells infected with the VL-prosap virus. After infection (36 h), Sf9 cells were pulse-labeled with \([\text{[35S]}\text{cysteine}\) for 10 min and chased fresh, nonradioactive media for the indicated times. The immunoprecipitates were obtained with anti-A and were typical for the other antisaposin antisera. The asterisks in panel c refer to unidentified transient intermediate species.

**Fig. 5.** Western blots of partially purified prosaposin-processing intermediates from media of High-5 cells. Media from High-5 cells were harvested and subjected to partial purification on concanavalin A-Sepharose. The fractions enriched in prosaposin and processing intermediates were collected and subjected to SDS-PAGE followed by transfer to nitrocellulose membranes and treatment with the indicated antisera. Alkaline phosphatase-conjugated second antibody was used for detection. NT, A, B, C, and D refer to the specific antisera used (i.e. NT represents anti-Nter).

With the trisaposin, B–C–D (lane 7, arrow) and saposin D, respectively. The trisaposin B–C–D was consistently present in all tissue sources (see below) and migrated slightly faster than the trisaposin A–B–C (lane 4, asterisk). A minor species at Mr, ~27,000 (lane 7, ●) was the disaposin C–D (see below and Fig. 4b, lanes 5 and 6). This band was more dense with anti-C due to better reactivity in this system (data not shown). Fig. 4b shows the effect of deglycosylation on these species (4 h of pulse, no chase). The mobilities of all species were increased following deglycosylation. The faster migration of the C–D (lane 5, ●), compared with the A–B (lane 3, asterisk), disaposin is explained by the heavier glycosylation of saposin A and the potential presence of greater amounts of intersaposin sequences (see Fig. 1).
A species suggested in Fig. 4a, lanes 1 and 2, was confirmed in lane NT (Fig. 5).

Studies in Human Skin Fibroblasts—HSF were labeled (20 h) with [35S]cysteine, lysed, and exhaustively immunoprecipitated with each of the four saposin-specific antisera in different sequences of treatment as follows: 1) anti-A, followed by anti-B, anti-C, and then anti-D (Fig. 6a); 2) anti-B, followed by anti-A, anti-D, and then anti-C (Fig. 6b); 3) anti-C followed by anti-D, anti-A, and then anti-B (Fig. 6c); and 4) anti-D followed by anti-C, anti-B, and then anti-A (Fig. 6d). Two major species initially precipitated by each of these antisera had apparent molecular weights of the mature saposins (Mᵦ, ~12,000–16,000) and prosaposin (Mᵦ, ~65,000–68,000). Saposin A had Mᵦ ~16,000 (panel a, lane 1; panel b, lane 2; panel c, lane 3, and panel d, lane 4). Saposins B, C, and D migrated at Mᵦ ~12,000.

The saposin content of each of the other radiolabeled species was determined as in methods 1–4 described above. These results are shown in Fig. 6, a–d, where the label at the bottom of each panel indicates the order of immunoprecipitation with each antiserum. With anti-A, four radiolabeled species were precipitated, Mᵦ ~65,000–68,000, 48,000, 27,000, and 16,000 (Fig. 6a). These correspond to the mobilities of prosaposin, trisaposin A-B-C (arrow), Nter-saposin A (asterisk), and saposin A, respectively. In panel a, lane 2 is the subsequent immunoprecipitation with anti-B. A major form with Mᵦ ~45,000 corresponds to the mobility of a trisaposin, B-C-D. This form was in larger amounts than the slower migrating trisaposin species A-B-C (Fig. 6a, lane 1, arrow). A smaller amount of a Mᵦ ~29,000 species corresponds to the disaposin, B-C (lane 2, ●). This conclusion was supported by the presence of a similar Mᵦ band in panel d, lane 2 (●) (anti-D- and then anti-C-treated) and its absence in panel d, lane 3 (anti-D, anti-C, and then anti-B). In panel a, lane 3 (●), a similar Mᵦ band is observed following immunoprecipitation with anti-C. This band was immunoprecipitated with anti-C after anti-B treatment, so that saposin B-containing intermediates were depleted (panel a, lane 2). Thus, the disaposin bands in lanes 2 and 3 (panel a) represent different disaposins; i.e. B-C (lane 2, ●) or C-D (lane 3, ●). The absence of a disaposin band in the anti-D immunoprecipitations (panel a, lane 4; panel c, lane 2) after immunoprecipitation with anti-C and its presence in panel d, lane 1 (●) support the composition of a disaposin C-D. Also, the presence of such a band in immunoprecipitations with anti-D preceding treatment with anti-C (panel b, lane 3, ●) confirms the composition as C-D.

These data were made more accessible in Fig. 6, e–h, by rearranging the lanes from Fig. 6, a–d, so that all the immunoprecipitations with a single saposin antisera are consolidated into one panel. For example, in Fig. 6b, lane 1, a disaposin (Mᵦ ~29,000 (●)) band was present when precipitated with anti-D or sequentially with anti-B, anti-A, and then anti-D (lane 3, ●). This band is absent in lanes 2 or 4, which had been precipitated, respectively, first with anti-C, or with anti-A, anti-B, and then anti-C. This result indicates that this is disaposin C-D. The presence of a dense band with Mᵦ ~45,000 in all immunoprecipitates obtained with anti-B, -C, or -D (lane 1 in panels f, g, and h; below arrows) identifies this species as trisaposin B-C-D. This band did not contain saposin A components, since it was not eliminated by anti-A treatment (panel f, lane 2). A low level of trisaposin A-B-C was detected as a Mᵦ ~48,000 species in samples precipitated with anti-A (panel e, lane 1, arrow) before, but not after, treatment with anti-B (panel f, lane 1, arrow) or anti-C (panel g, lane 1, arrow). Trisaposin A-B-C was confirmed by treatment with anti-D and then anti-C (panel g, lane 2) that showed the presence of a Mᵦ ~48,000 species that could not be precipitated by anti-D. A Mᵦ ~27,000 species was present in all anti-A (Fig. 6e, asterisk) or anti-Nter immunoprecipitates. This shows that Nter-saposin A was liberated from prosaposin following proteolytic clipping of the leader sequence. A few of the species in Fig. 6 were not
identified and may represent various other saposin intermediates containing larger or smaller amounts of intersaposin sequences or with varying glycosylation (Fig. 1). We cannot exclude the existence of the precursors containing larger or smaller amounts of intersaposin sequences and may represent various other saposin intermediates.

NT2/D1 Neuronal Cells—NT2, neuronal precursor cells were labeled (4 h) with [35S]cysteine. Cell lysates were exhaustively immunoprecipitated with anti-A, -B, -C, or -D. In contrast to HSF, very small amounts of the M₃₀,000 band precipitated with anti-C (panel b). This band was present in HSF and does not change in migration following deglycosylation within N-glycanase (Fig. 7, lanes 5 and 6). With this exception, all visualized intermediates appear to be glycosylated, since their mobilities change significantly on treatment with N-glycanase (Fig. 7).

NT2/D1 Neuronal Cells—NT2, neuronal precursor cells were labeled (4 h) with [35S]cysteine. Cell lysates were exhaustively immunoprecipitated with anti-A, -B, -C, or -D. In contrast to HSF, very small amounts of the M₃₀,000 band precipitated with anti-C (panel b). This band was present in HSF and does not change in migration following deglycosylation within N-glycanase (Fig. 7, lanes 5 and 6). With this exception, all visualized intermediates appear to be glycosylated, since their mobilities change significantly on treatment with N-glycanase (Fig. 7).

Fig. 7. Deglycosylation of immunoprecipitates from cultured skin fibroblasts following exhaustive immunoprecipitation with anti-saposin antisera. HSF were pulse-labeled for 20 h with [35S]cysteine, immunoprecipitated with the indicated antisaposin antiserum. The immunoprecipitates were untreated (-) or treated (+) with N-glycanase and then subjected to SDS-PAGE and fluorography. In these experiments the cell lysates were treated directly with the specific antisera and were not subject to sequential, exhaustive immunoprecipitation.

Prosaposin Processing

PROSAPOSIN PROCESSING

Fig. 8. Pulse labeling of NT2 neuronal precursor cells. NT2 cells were pulse-labeled for 4 h with 20 h of chase (a) or no chase (b) prior to harvesting and sequential immunoprecipitation with the indicated antisera. N, A, B, C, and D refer to antisera to Nter, saposin A, saposin B, saposin C, and saposin D, respectively. In panel c, spent media from NT2-derived differentiated neurons following 4 h of labeling were individually, not sequentially, immunoprecipitated with the indicated antisera, and the immunoprecipitates were analyzed by fluorography.

DISCUSSION

Prosaposin is unique among putative "lysosomal proteins" since it has housekeeping functions (43) and growth-promoting effects (32–34). Only the protector protein may have similar intra- and extracellular functions (44, 45). The prosaposin locus is temporally and spatially regulated with the highest levels of mRNA expression in specific cellular types of adult animals (20). Although not unique to prosaposin, this "lysosomal protein" has tissue-specific proteolytic processing leading to quantitative differences in the level of the precursor compared with the mature saposins in various tissues (20). However, the order and specificity of proteolytic processing events from the precursor, prosaposin, to mature saposins have only been described in broad outline (21, 22). The current studies indicate that the proteolytic machinery for cleavage of the prosaposin precursor to disaposins is partially shared in species as evolutionarily divergent as insects and humans. The final processing of pathways and end points from the precursors varies across species and, also, in different cellular types within a single species.

Fig. 10 provides a schematic diagram for the major proteolytic processing pathways of prosaposin in insect and mamma-
saposin D is detectable, as is A-B-C. 2) Disaposin A-B could result from cleavage of saposin C from A-B-C. Saposin C was detected infrequently in very small amounts. The flux through the trisaposin A-B-C pathway in mammalian cells was very low, since A-B-C and A-B were found only occasionally. In mammalian cells, the major processing pathway is through the tetrasaposin A-B-C-D and the trisaposin B-C-D. Quantitatively, we were unable to distinguish between the pathway that liberates saposin D or saposin B from the trisaposin. In insect cells the significant pathway is directly from the tetrasaposin A-B-C-D to disaposins A-B and C-D. In insect cells saposin D could frequently be detected, whereas other saposins could not. These results suggest that the insect proteases needed for the deavage of the disaposins are present at very low levels or that the disaposins are inefficient substrates. In comparison, in HSF, neuronal stem cells, or differentiated neurons, the monosaposins are generated rapidly from the precursor. These results imply the presence of a powerful proteolytic machinery for the production of the saposins that may vary with cell type.

Previously, a prosaposin precursor form was shown to be secreted following the treatment of HSF with ammonium chloride or from skin fibroblasts from patients with I-cell disease (22). This finding showed that prosaposin proteolytic processing to the monosaposins occurred in lysosomes of mammalian cells. Similarly, a precursor form of prosaposin was present in the cytoplasm of Sertoli cells and in large quantities in media surrounding primary cultures of these cells (6). Western blotting studies detected prosaposin forms in a variety of body fluids including plasma, breast milk, cerebrospinal fluid, and seminal fluid (6, 29, 30). The present studies show the form and amount of secreted prosaposin precursor can vary with the tissue and cellular type. For example, spent media from HSF or NT2 cells contained very little if any prosaposin-like precursor, whereas the media from NT2-derived differentiated neurons contained two forms of prosaposin. These two forms were composed of the tetrasaposin A-B-C-D or Nter-A-B-C-D. Evidently, these neuronal cells have a nonsyosomal protease that clips the Nter peptide. Similar findings were obtained in VL-prosaposin-infected insect cells (Sf9 or High-5) where the Nter-tetrasaposin was in spent media. Recently, we have detected a protease in media of insect cells with significant hydrolytic activity toward prosaposin only at pH <4.0. At pH 6.0, this protease has little activity. These results also indicate that the Nter peptide cannot be the primary signal for secretion of prosaposin from the cell, since quantitatively A-B-C-D is the major component in media. This result differs from previous data, where prosaposin isolated from media of Sf9 cells had the NH2-terminal sequence of Nter (35).

Previously, our in situ antisense mRNA hybridization studies showed transcriptional regulation of prosaposin expression during development and in specific cellular types (20). Using antisera that specifically recognize either mature saposins or only the prosaposin precursor (i.e. anti-Nter or anti-intersaposin C-D), we detected highly specific tissue processing. Most surprisingly, a high steady-state concentration of prosaposin precursor was present in Purkinje cells of the cerebellum, whereas mature monosaposins were present in Purkinje cells and the surrounding Bergmann glial cells. Both prosaposin and mature saposins were present in cerebral neurons. The latter would be expected, since saposins are required for glycosphin-golipid hydrolysis. The precise nature of the prosaposin-containing neurons throughout the brain is the subject of on-going studies, as is the role of the cell-specific processing of prosaposin in other tissues (Table I).

\[\text{A. Bencosme and E. Ponce, unpublished observation.}\]
Prosaposin's repertoire of functions have now extended from essential roles in lysosomal glycosphingolipid hydrolysis to lipid transfer functions and, possibly, neurite outgrowth and/or regrowth (34). In addition to these multifunctional properties, expression of the locus is spatially regulated, and only recently has the promoter been cloned and several of its functional elements and transcription factors identified. This complexity of biological function and expression has now been shown to be mirrored at the cellular level with proteolytic processing differences.

3 Y. Sun, P. Jin, and G. A. Grabowski, unpublished observation.

**TABLE I**

| Tissue Source | Anti-intersaposin C-D or anti-Nter | Anti-saposin A, B, or D |
|---------------|-----------------------------------|------------------------|
| Cerebellum    | ++                                 | +                      |
| Purkinje cell bodies | ++                                    | ++ (granular organelles) |
| Bergmann glial cells | -                                    | -                      |
| Cerebrum      | ++                                 | ++ (granular organelles) |
| Cortical neurons | ++                                 | ++ (granular organelles) |
| Testes        | ++                                 | ++ (granular organelles) |
| Sertoli cells | ++ (cytoplasm)                     | ++ (granular organelles) |
| Seminiferous tubule lumen | ++ (spermatids) | -                      |
| Epididymis lumen | ++ (spermatids)                  | ++ (epithelial cells) |
| Leydig cells  | +                                  | + (zona reticularis)   |
| Adrenal gland |                                    |                        |
| Medulla       | +                                  |                        |
| Cortex        |                                    |                        |
| Ovary         |                                    |                        |
| Corpus Luteum | ++                                 | -                      |

**FIG. 10.** The proteolytic processing pathways of prosaposin in insect and mammalian cells. The open rectangles refer to the mature saposin amino acid sequences indicated by the contained letter. The thinner black rectangles refer to the intersaposin sequences including Nter. The signal sequence (SS) is clipped during passage of the nascent polypeptide through the endoplasmic reticulum. For the intermediates, the amounts of intersaposin sequence shown refer only to potential, not actual, additional amino acids in the tetra-, tri-, and disaposins. The major and minor pathways in the lower part of the figure differ quantitatively by the initial step. The initial step in the minor pathway (lower) for both mammalian and insect cells is the cleavage of Nter-saposin A, whereas that in the major pathway is cleavage of Nter. The trisaposin containing B-C-D is a major product in mammalian or insect cells. From the trisaposin, either saposin B or D is removed to generate the disaposins B-C or C-D. These disaposins are processed to monosaposins in mammalian cells. In insect cells another significant pathway is through A-B-C-D, leading directly to the disaposins A-B and C-D that are the major end products. Only small quantities of mature saposins are produced in insect cells. Minor quantitative differences in the pathway were observed between SF9 and High-5 cells. Another minor, but consistent pathway, in insect cells is the removal of saposin D from the tetrasaposin A-B-C-D. In mammalian cells this pathway is detectable but is very minor. The proteolytic removal of Nter from Nter A-B-C-D occurs prior to the lysosomal compartment, since Nter-A-B-C-D is secreted into the media of insect cells (SF9 or High-5) and of some mammalian cells. In the latter cells the proteolytic processing of the tri- and disaposins probably occurs intralysosomally, as does the cleavage of the Nter-A fragment.
ences between and among tissues and cellular types. The highly specific expression and processing of this locus is clearly at the root of its biological and physiological functions that remain to be defined.

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REFERENCES
1. Inui, K., Kao, R.-T., Fujibayashi, S., Jones, C., Morse, H. G., Law, M. L., and Wenger, D. A. (1985) Hum. Genet. 69, 197–200
2. Fujibayashi, S., Kao, R.-T., Jones, C., Morse, H., Law, M., and Wenger, D. A. (1985) Am. J. Hum. Genet. 37, 741–748
3. O'Brien, J. S., Kretz, K. A., Dewji, N., Wenger, D. A., Esch, F., and Fluharty, A. L. (1988) Science 241, 1098–1101
4. Gavriel-Roman, E., and Grabowski, G. A. (1989) Genomics 5, 486–492
5. Nakano, T., Sandhoff, K., Stumper, J., Christomanou, H., and Suzuki, K. (1989) J. Biochem. (Tokyo) 105, 152–154
6. Collard, M. W., Sylvester, S. R., Tsuruta, J. K., and Griswold, M. D. (1988) Biochemistry 27, 4557–4564
7. Reiner, O., Dagan, O., and Horowitz, M. (1989) J. Md. Neuroscience 1, 225–235
8. Schnabel, D., Schröder, M., and Sandhoff, K. (1991) FEBS Lett. 284, 57–59
9. Zhang, X., Rafi, M. A., DeGala, G., and Wenger, D. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1426–1430
10. Schnabel, D., Schröder, M., Furst, W., Klein, A., Hurwitz, R., Zerk, T., Weber, J., Harzer, K., Paton, B. C., Poulos, A., Suzuki, K., and Sandhoff, K. (1992) J. Biol. Chem. 267, 3312–3315
11. Zhang, X., Rafi, M. A., DeGala, G., and Wenger, D. A. (1991) Hum. Genet. 87, 211–215
12. Rafi, M. A., DeGala, G., and Wenger, D. A. (1990) Biochem. Biophys. Res. Commun. 166, 1017–1023
13. Rafi, M. A., DeGala, G., Zhang, X. L., and Wenger, D. A. (1993) Somat. Cell Mol. Genet. 19, 1–7
14. Klein, A., Henseler, M., Klein, C., Suzuki, K., Harzer, K., and Sandhoff, K. (1994) Biochem. Biophys. Res. Commun. 205, 1440–1448
15. Furst, W., and Sandhoff, K. (1992) Biochim. Biophys. Acta 1126, 1–16
16. Qi, X., Leonova, T., and Grabowski, G. A. (1994) J. Biol. Chem. 269, 16746–16753
17. Vaccaro, A. M., Salvioli, R., Barca, A., Tatti, M., Claffoni, F., Maras, B., Sicilliano, R., Zappacosta, F., Amoresano, A., and Pucci, P. (1995) J. Biol. Chem. 270, 9953–9960
18. Munford, R. S., Shippard, P. O., and O'Hara, P. J. (1996) J. Lipid Res. 36, 1653–1663
19. Sprecher-Leyvi, H., Orr-Urtreger, A., Loni, P., and Horowitz, M. (1993) Cell Mol. Biol. 29, 297–299
20. Sun, Y., Witte, D. P., and Grabowski, G. A. (1994) Am. J. Pathol. 145, 1390–1398
21. Fujibayashi, S., and Wenger, D. A. (1986) Biochim. Biophys. Acta 875, 554–562
22. Fujibayashi, S., and Wenger, D. A. (1986) J. Biol. Chem. 261, 15339–15343
23. Hiroi, M., Soeda, S., Furst, W., Paton, B. C., Suzuki, K., Harzer, K., O'Brien, J. S., and Kishimoto, Y. (1993) Arch. Biochem. Biophys. 303, 326–331
24. Sano, A., Mizuno, T., Kondoh, K., Hineno, T., Ueno, S., Kakimoto, Y., and Morita, N. (1992) Biochim. Biophys. Acta 1120, 75–80
25. Morimoto, S., Martin, B. M., Yamamoto, Y., Kretz, K. A., O'Brien, J. S., and Kishimoto, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3389–3393
26. Morimoto, S., Martin, B. M., Kishimoto, Y., and O'Brien, J. S. (1988) Biochim. Biophys. Res. Commun. 156, 403–410
27. Furst, W., Madhiedt, W., and Sandhoff, K. (1988) Biol. Chem. Hoppe Seyler 369, 317–328
28. Morimoto, S., Yamamoto, Y., O'Brien, J. S., and Kishimoto, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3493–3497
29. Kondoh, H., Kishimoto, Y., Sano, A., and Kakimoto, Y. (1991) Biochim. Biophys. Res. Commun. 181, 286–292
30. Hineno, T., Sano, A., Kondoh, K., Ueno, S., Kakimoto, Y., and Yoshida, K. (1991) Biochim. Biophys. Res. Commun. 176, 668–674
31. Hiraiwa, M., Soeda, S., Kishimoto, Y., and O'Brien, J. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11254–11258
32. Qi, X., Qin, W., Sun, Y., Kondoh, K., and Grabowski, G. A. (1996) J. Biol. Chem. 271, 6874–6880
33. O'Brien, J. S., Carson, G. S., Seo, H., Hiraiwa, M., Weiler, S., Tomich, J. M., Barranger, J. A., Kahn, M., Azuma, N., and Kishimoto, Y. (1995) FASEB J. 9, 681–685
34. Kotani, Y., Matsuda, S., Sakanaka, M., Kondoh, K., Ueno, S., and Sano, A. (1996) Neurochem. in press
35. Hiraiwa, M., O'Brien, J. S., Kishimoto, Y., Galdzicki, M., Fluharty, A., Ginns, I. E., and Martin, B. M. (1993) Arch. Biochem. Biophys. 304, 110–116
36. Summers, M. D. and Smith, G. E. (1987) Tex. Agric. Exp. Stn. Bull. 241, 5–55
37. Andrews, P. W. (1984) Dev. Biol. 103, 285–293
38. Lee, V. M. Y. and Andrews, P. W. (1980) J. Neurosci. 6, 514–521
39. pleasure, S., page, C., and lee, v. M. Y. (1992) J. Neurosci. 12, 1802–1815
40. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
41. Xu, Y., Ponce, E., Sun, Y., Lepo, T., Bove, K., Witte, D. P., and Grabowski, G. A. (1996) Pediatr. Res. 39, 313–322
42. Kondoh, K., Sano, A., Kakimoto, Y., Matsuda, S., and Sakanaka, M. (1993) J. Neurosci. Comp. Neurol. 334, 590–602
43. Sandhoff, K., Harzer, K., and Furst, W. (1995) In The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., and Valle, D., eds) pp. 2427–2442, McGraw-Hill, Inc., New York
44. Morreau, H., Galjart, N. J., Willemsen, R., Gillemans, N., Zhou, X. Y., and d'Azzo, A. (1991) J. Biol. Chem. 266, 15339–15346
45. Galjart, N. J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E. J., and d'Azzo, A. (1991) J. Biol. Chem. 266, 14754–14762
46. Gavriel-Roman, E., Scheinker, V., and Grabowski, G. A. (1992) Genomics 13, 312–318