Biochimica et Biophysica Acta 1783 (2008) 1480–1489

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

The secretion and maturation of prosaposin and procathepsin D are blocked in embryonic neural progenitor cells

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ARTICLE INFO

Article history:
Received 20 December 2007
Accepted 30 January 2008
Available online 20 February 2008

Keywords:
Prosaposin
Procathepsin D
Human neural stem cells
Post-translational modifications
Protein secretion
Alternative mRNA splicing

ABSTRACT

The notion that prosaposin (Prosap) is likely involved in brain development and regeneration led us to explore its expression in stem/progenitor neural cells and its fate after cell differentiation. The expression of procathepsin–cathepsin D (proCath–Cath D), an endoprotease that plays an important role in the processing and sorting of Prosap, has been concomitantly examined. Our data evidenced that in embryonic human neural progenitor cells (eHNPCs) intact and high molecular weight intermediate forms of Prosap and intermediate forms of Cath D accumulated inside the cells, while the formation of saposins and mature Cath D was impaired. Furthermore, neither Prosap nor proCath D were secreted from eHNPCs. The block of the processing and secretion shared by Prosap and proCath D was overcome during the course of differentiation of eHNPCs into a mixed population of astrocytes and neuronal cells. Upon differentiation, large amounts of Prosap and proCath D were secreted from the cells, while saposins and mature Cath D were produced inside the cells. The dramatic accumulation of Prosap (an antiapoptotic factor) and reduction of mature Cath D (a proapoptotic factor) in the undifferentiated eHNPCs most likely play a role in the molecular mechanisms regulating the resistance to apoptotic signals of these cells and might represent a critically important issue in HNPCs biology.

1. Introduction

Prosaposin (Prosap) is a glycoprotein of 65–73 kDa mass proteolitically cleaved in late endosomes/lysosomes to give rise to four small glycoproteins, saposin (Sap) A, B, C and D [1,2]. Saps play an important role as intracellular activators of several lysosomal enzymes involved in the degradation of specific sphingolipids; Sap A favours the degradation of galactosylceramide [3], Sap B that of sulfatide [4,5], Sap C that of glucosylceramide [6] and Sap D that of ceramide [7]. Recently, the involvement of Saps in lipid antigens presentation has been also demonstrated [8,9].

Besides being the precursor of Saps, Prosap is also a secretory protein present in several biological fluids such as cerebrospinal fluid, milk and seminiferous tubule fluid [10–12]. The sorting of Prosap to intracellular organelles or to extracellular spaces is specifically regulated in different human and animal tissues by as yet not clearly defined signals. It has been proposed that sphingomyelin-containing raft and the Sap D domain with its adjacent COOH-terminal region play a crucial role in the transport of Prosap to lysosomes [13,14]. Moreover, sortilin, a transmembrane protein with multiple ligand-binding domains, possibly mediates the Prosap sorting in specific tissues [15]. After reaching lysosomes, Prosap is converted to Saps by lysosomal proteases. Several lanes of evidence have demonstrated the involvement of cathepsin (Cath) D in the lysosomal Prosap proteolysis [16].

Prosap is expressed in all cell types and its expression is temporally and spatially regulated at transcriptional and post-translational levels [17,18]. Prosap is present at high concentration in brain, being localized in specific neurons and nerve fibres [19,20]. Abundant Prosap expression has been found in the epithelial cells of the choroid plexus and in various gray matter areas, including cortex and hippocampus [17,21].

While some of the physiological roles of Saps, the lysosomal proteolytic products of Prosap, are well defined, the actual function of intact Prosap is still uncertain. A pivotal role for Prosap in development and regeneration of brain has been proposed [22]. It has been observed that Prosap functions 'ex vivo' as a neurotrophic and antiapoptotic factor and that exogenous addition of Prosap to neuronal cells leads to neurite outgrowth, prevents cell death and promotes the nerve regeneration processes [23–27]. Furthermore, the expression of Prosap markedly increases in response to brain injury, after ischemia and following sciatic nerve crush injury [22,28,29]. In this context it is worthwhile to note that recent studies have shown that after a brain lesion neural progenitor cells (NPCs) are activated, migrate from their niche towards lesion areas, where differentiate into neuronal and glial cells [30,31]. The parallel increase in the brain injured areas of the NPCs population and of the Prosap expression strongly suggests a relationship between these two events.

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doi:10.1016/j.bbamcr.2008.01.033

The ability to promote proliferation and prevent cell death makes Prosap an ideal candidate as a protein factor able to modulate the well known high regenerative capacity of NPCs and their high resistance to apoptotic signals. Although the discrete distribution of Prosap in animal brains has been previously described, no information is available about its expression in the human NPCs population. To further extend the search for proteins that might contribute to determine the biological responses of human neural progenitor cells (HNPCs), we have thus investigated the expression and properties of Prosap in these cells and its fate after differentiation. Since proCath–Cath D plays an important role in the traffic and processing of Prosap [32], the expression and properties of this endoprotease has been simultaneously examined in details. The study of the Cath D behaviour in HNPCs is of particular interest not only for its relationship with Prosap, but also because Cath D is considered an important mediator in the control of cell death [33]. Herein we uncovered a dramatic intracellular accumulation of Prosap (a protein with neurothophic and antiapoptotic effects) and a markedly reduced level of mature Cath D (a protein with proapoptotic effect) in embryonic HNPCs (eHNPCs). The altered expression and post-translational modifications of these two proteins most likely have a role in modulating the eHNPCs proliferation and resistance to apoptotic signals.

2. Materials and methods

2.1. Cell culture

2.1.1. Human neural progenitor cells

Adult human neural progenitor cells (aHNPCs) were obtained from the olfactory bulb of patients undergoing particularly invasive neurosurgery as previously described [34,35]. Embryonic human progenitor cells (eHNPCs) were purchased from Cambrex (Walkersville, MD). HNPCs were cultured in DMEM-F12 (1:1) (Invitrogen, Carlsbad, CA) supplemented with 20 ng/ml of human recombinant epidermal growth factor (EGF), 10 ng/ml of human recombinant basic fibroblast growth factor (bFGF), 2 mM glutamine, 0.6% glucose, 9.6 μg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, and 0.1 mg/ml transferrin. The cells were used from passage 2 to 5 throughout the study.

HNPCs were differentiated by plating them on Matrigel (Becton Dickinson) in DMEM-F12 (1:1) medium lacking growth factors but added with 5% fetal bovine serum (FBS). The cells were allowed to differentiate for up to 21 days. Differentiation was evaluated by cell morphology and by confocal microscopy analysis of neuronal marker β-III tubulin and astrocyte marker glial fibrillary acidic protein (GFAP).

2.1.2. Fibroblasts

Fibroblast cultures were established from skin explants of normal individuals. Fibroblasts were cultured in DMEM (Invitrogen, Carlsbad,CA) supplemented with 10% FBS, 2 mM glutamine, 100 units/ml of penicillin and 100 μg/ml streptomycin. The cells were harvested after reaching confluency.

2.1.3. Neuroblastoma cells

Human neuroblastoma cells SHSY5Y were cultured in MEM/Ham's F12 (1:1 mixture) (Invitrogen, Carlsbad,CA) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml streptomycin. The cells were harvested after reaching confluency.

2.2. Antibodies

Rabbit polyclonal anti-human Sap C antibody was prepared in our laboratory [36]. It recognizes both Sap C and Prosap. Rabbit polyclonal anti-human Cath D antibody was obtained from DakoCytomation (Glostrup, DK) and mouse monoclonal anti-Cath D antibody was obtained from Sigma Aldrich (St. Louis, MO). Mouse monoclonal anti-Cath B antibody was obtained from Calbiochem (Darmstadt, Germany). Mouse monoclonal anti-human lysosome-associated membrane protein type 1 (LAMP 1) antibody, was obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa (Iowa City, IA). Anti-actin monoclonal antibody was obtained from Oncogene Research Products (Boston, MA).

2.3. Western blot analysis

Cell pellets were washed twice with cold PBS and resuspended in 50 mM phosphate buffer, pH 6.5, containing 0.5% (v/v) Triton X-100 and one tablet of protease inhibitor mixture (Roche Applied Science)/50 ml (lysis buffer). After brief sonication the homogenates were centrifuged for 30 min at 20,000 ×*g* at 4 °C. Lysates were resolved on NuPAGE 4–12% Bis–Tris gels using a mini-gel apparatus (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc. Hercules, CA). Membranes were blocked for 1 h with 2% nonfat milk powder in PBS containing 0.1% Tween-20 and incubated for 1 h with specific antibodies. Primary and secondary antibodies were diluted in blocking solution. Immunoreactive proteins were visualized using an ECL Western blotting kit, according to the manufacturer's instructions (Amersham Bioscience, Buckinghamshire, UK).

2.4. Glycosidase treatment

An aliquot of cell lysate was adjusted to pH 7.5 with 50 mM sodium phosphate, while another aliquot was adjusted to pH 5.5 with 50 mM sodium citrate. The first sample was incubated with peptide-*N*-glycosidase F (PNGase F) and the second with β-endo-*N*-acetylglucosaminidase H (Endo H) in the presence of appropriate reagents supplied together with the glycosidases by New England BioLabs Inc. (Beverly, MA). The samples were incubated according to the manufacturer's instructions and then loaded on NuPAGE 4–12% Bis–Tris gels, subjected to electrophoresis, transferred to PVDF membranes and analyzed by Western blotting utilizing the anti-Sap C antibody as described above.

2.5. Real-Time PCR and analysis of Prosap exon 8 isoforms

Total RNA was extracted with TRIzol® Reagent (Invitrogen). 1 μg of RNA was reverse transcribed in a 20 μl reaction mixture using M-MLV Reverse Transcriptase (Invitrogen). The relative quantification of human Prosap mRNA was performed by TaqMan technology, using the ABI PRISM 7900 DNA sequence detection system (Applied Biosystems). Commercial ready-to-use primers/probe mixes were used (Assay-on-Demand Gene Expression products, Hs00358165_m1; Applied Biosystems). Amplification was performed with 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Original input RNA amounts were calculated with a relative standard curve for Prosap and GAPDH mRNA. Gene expression values were reported as the normalized percentage obtained by dividing the copy numbers of specific gene by GAPDH.

To examine Prosap isoforms, cDNA was used in a PCR reaction to amplify a region encompassing the Exon-8 sequence of the human Prosap gene. A set of primers previously described [22] were utilized, namely hB1 (5′-AAGAACTATATCAGCCAGTAT-3′) and hB2 (5′-TTTCACCTCATCACAGAACCC-3′). PCR reaction was performed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C. PCR products were separated by electrophoresis on 8% polyacrylamide gel.

2.6. Metabolic labeling of cells

Prior to being labeled eHNPC or fibroblasts (grown until near confluency) were washed twice with ice-cold PBS supplemented with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Fibroblasts were then starved for 2 h in methionine/cysteine-deficient DMEM supplemented with 4% FBS, 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml streptomycin, while eHNPCs were starved for 2 h in a medium prepared as described in the cell culture section except that DMEM-F12 (1:1) was substituted with methionine/cysteine-deficient DMEM. For pulse experiments PRO-MIX L-[^{35}S] (Amersham Biosciences, Buckinghamshire, UK) 150 μCi/ml was added to the medium and the cells (eHNPCs or fibroblasts) were further cultured for the indicated periods. For experiments including chase periods, after 1 h of pulse the cells were washed three times with PBS and then cultured with their medium devoid of PRO-MIX L-[^{35}S] and supplemented with 5 mM methionine. For pulse and pulse-chase experiments on differentiated cells, the eHNPCs were previously allowed to differentiate for up to 14 days as described in the cell culture section. The cells were then starved for 2 h in methionine/cysteine-free medium and than pulsed or pulse-chased for the indicated periods.

2.7. Immunoprecipitation

The cells, labeled as described above, were harvested and disrupted in lysis buffer. The suspensions were subjected to brief sonication and centrifuged at 20,000 ×*g* for 30 min at 4 °C. To remove DNA and histones, the supernatants were incubated with 0.03% protamine sulfate for 45 min at 4 °C and centrifuged as above. After addition of 0.1% BSA, the cell lysates were incubated with pre-immune serum overnight at 4 °C and non-specific complexes were precipitated with Protein A-Sepharose CL-4B (Sigma Aldrich, St. Louis, MO). The clarified supernatants were then incubated overnight with anti-Sap C or anti-Cath D antiserum. Cross-reacting material was precipitated with Protein A-Sepharose CL-4B. The immunocomplexes were washed four times with PBS containing 1% BSA, 1% Triton X-100, 1% SDS, 0.4% sodium deoxycholate and finally with only PBS. The washed precipitates were analyzed by SDS-PAGE. Labeled proteins were detected by fluorography.

2.8. Cath D activity

Cell pellets (HNPCs and fibroblasts) were lysed in water containing 0.5% CHAPS (w/v) for 30 min on ice. The suspension was centrifuged at 15,000 ×*g* for 20 min at 4 °C. The activity of Cath D was assayed on the supernatants using the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dpb)gamma-NH2 (Peptide International) as described by Yasuda et al. [37].

2.9. Microscopy

eHNPCs (undifferentiated and differentiated) and human fibroblasts were grown on polylysine-coated glass coverslips for microscopy. After a fixing step in Bouin's

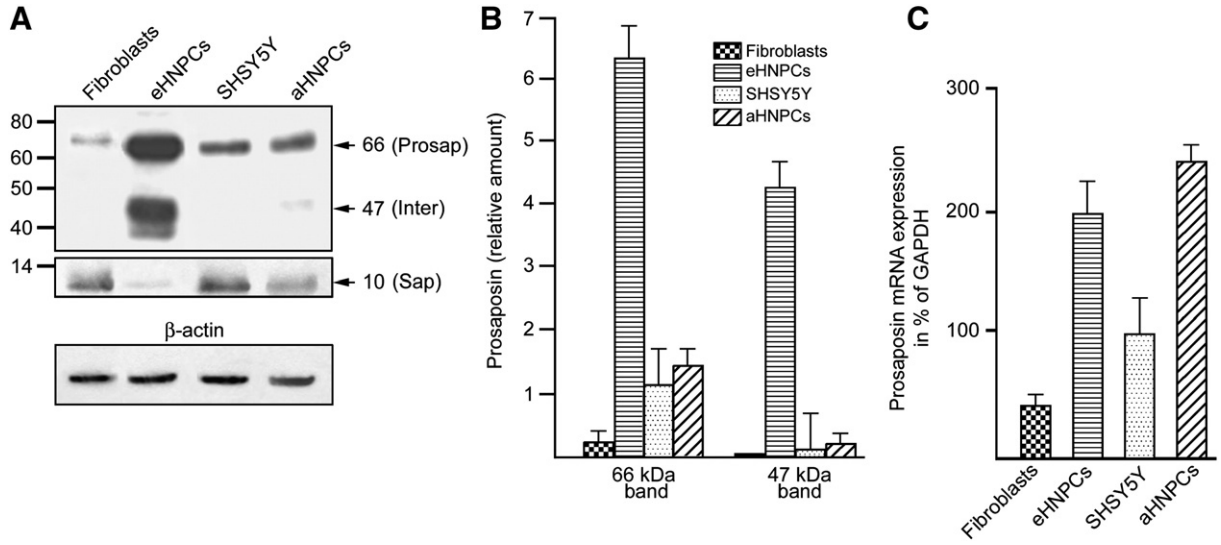

Fig. 1. Comparison of the Prosap and Saps levels in HNPCs, neuroblastoma cells and human fibroblasts. (A) Western blotting of Prosap and Saps in homogenates of human fibroblasts, eHNPCs, neuroblastoma cells (SHSY5Y) and aHNPCs. Identical amounts of homogenates (5 μg of protein) were loaded in each lane and analyzed by SDS-PAGE/Western blot with an anti-Sap C antibody that recognizes Prosap and Sap C. The blots were reprobed for ß-actin as a loading control. The number on the left refers to molecular mass standards (in kDa). The arrows on the right indicate the molecular weights of the intact and intermediate forms of Prosap and of mature Sap C. The experiment was repeated more than three times giving similar results. (B) Relative levels of intact and intermediate forms of Prosap (MW 66 kDa and 47 kDa, respectively) were evaluated by densitometric analysis of the Prosap bands normalized against the ß-actin bands. (C) Prosap mRNA expression in fibroblasts, eHNPCs, neuroblastoma cells (SHSY5Y) and aHNPCs. Prosap mRNA amounts were normalized to and presented as percentages of GAPDH for each sample. The data represents the means+S.E.M. of three independent experiments.

solution (Sigma Aldrich) for 20 min at room temperature, the cells were washed once with PBS and incubated 2 h with 3% BSA.

For the immunofluorescence analysis of the antigenic markers expression in astrocytes and neurons, slides were incubated for 1 h at 37 °C with antineuron-specific ß-III tubulin-specific antibody (mouse IgG_1; Serotec Inc., Munich, Germany) and anti-glial fibrillary acid protein (GFAP) (rabbit polyclonal; Dako Cytomation). Nuclei were counterstained with TOTO-3 iodide (Molecular Probes). After two washes in PBS, slides were incubated with secondary antibodies for 45 min at 37 °C. Secondary antibodies, including FITC-conjugated goat anti-mouse IgG and Texas red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were used at 2.5 μg/ml.

For double immunostaining, the cells were incubated for 1 h at room temperature with a specific rabbit polyclonal antibody (anti-Sap C) together with a specific mouse monoclonal antibody (anti-Cath D or anti-Lamp1). Cells were then rinsed twice with PBS, and incubated for 1 h with a secondary anti-rabbit antibody conjugated with Alexa Fluor 594, together with a secondary anti-mouse antibody conjugated with Alexa Fluor 488 (Molecular Probes). Cells were finally rinsed twice with PBS and mounted with ProLong antifade reagent (Molecular Probes). Images were collected with a laser scanning microscope (IX81; Olympus, Suffolk, UK).

3. Results

3.1. Expression of Prosap in HNPCs

To investigate whether neural stem/progenitor cells might contribute to the Prosap overexpression previously observed in specific regions of the brain [17,19,20], we have analyzed the intracellular

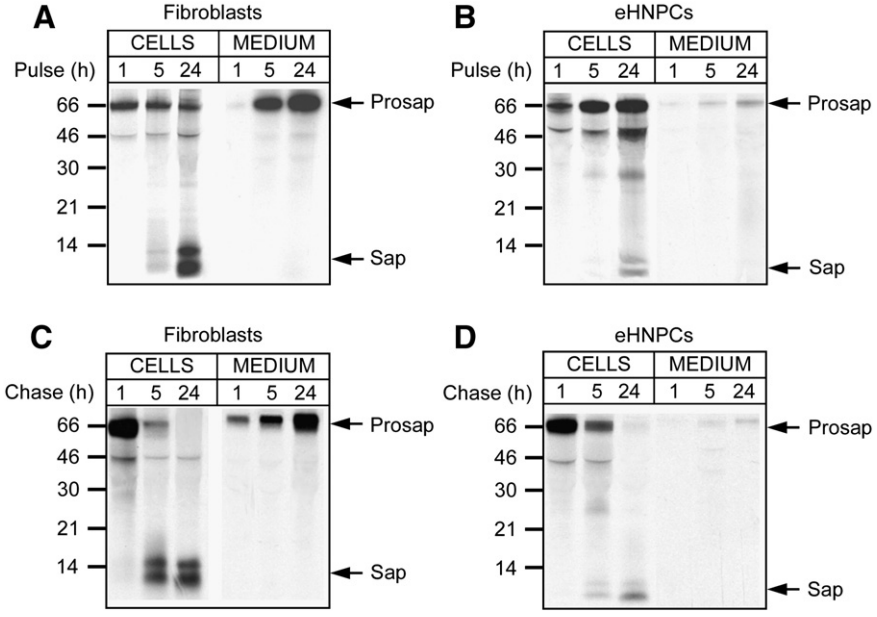

Fig. 2. Prosap processing and secretion. Human fibroblasts (A and C) and eHNPCs (B and D) were labeled with [^{35}S] methionine/cysteine for the indicated times (A and B, pulse experiments) or, alternatively, labeled for 1 h and subsequently chased for the indicated times (C and D, pulse-chase experiments). Both the cell-lysates and the medium were immunoprecipitated with anti-Sap C antibody that recognizes Prosap and Sap C. Immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. The number on the left refers to the molecular mass of standards (in kDa). The experiments were repeated more than three times giving similar results.

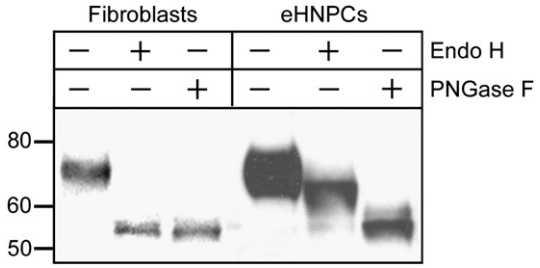

Fig. 3. Endoglycosidase treatment of Prosap. Cell extracts from fibroblasts (100 μg) and from eHNPCs (10 μg) were treated either with PNGase F or with Endo H as described in Materials and methods. The samples were then analyzed by SDS-PAGE/Western blot with the anti-Sap C antibody. The number on the left refers to the molecular mass of standards (in kDa). The experiments were repeated more than three times giving similar results.

Prosap level in eHNPCs and aHNPCs. Western blot analysis revealed a much higher level of intact (about 66 kDa) and partially processed forms (about 47 kDa) of Prosap in eHNPCs when compared with aHNPCs or with a neuronal-like cell type such as SHSY5Y or with human fibroblasts (Fig. 1A). Conversely, the band corresponding to mature Sap C (about 10 kDa) was clearly detected in all the examined cell types except than in eHNPCs. The densitometric analysis showed that the intensity of the intact Prosap band at 66 kDa in eHNPCs was at least 4 times stronger than the corresponding band in aHNPCs and SHSY5Y and at least 20 times stronger than that observed in fibroblasts. Moreover, the Prosap band at about 47 kDa observed in eHNPCs was almost absent in the other cells (Fig. 1B).

In order to evaluate whether the accumulation of Prosap in eHNPCs was related to an increase of the Prosap mRNA expression, Real-Time PCR analysis was performed. For correction of minor variations in input amounts of RNA samples, mRNA levels were normalized to the amount of GAPDH for each sample (Fig. 1C). A comparison between Fig. 1B with Fig. 1C shows that there was no direct correlation between Prosap mRNA and protein level in the examined cells. For example, the dramatic difference in the Prosap protein amount between eHNPCs and fibroblasts did not correspond to an analogous difference at mRNA level. These results indicate that the accumulation of the Prosap protein in eHNPCs, was not solely due to mRNA overexpression, but also to post-translational events.

In an effort to define such events, we have compared the Prosap processing and sorting in eHNPCs and in human fibroblasts, a cell type where processing, trafficking, secretion and post-translational modifications of Prosap/Sap have been extensively characterized [1,2]. The cells were labeled for different periods of time and both the cell-lysates and the growth medium were immunoprecipitated with an antibody which recognizes Prosap and Sap C. Fig. 2A shows that in human fibroblasts Prosap was synthesized as a protein with an apparent molecular mass of 66 kDa. Within approximately 5 h, two bands corresponding to small Saps (MW about 10 kDa) became detectable in the fibroblast extract. After 24 h the intensity of the bands at ~10 kDa overcame that of intact Prosap, that was also exported as such into the external medium. Conversely, in eHNPC the Prosap forms at high molecular weights (the 66 kDa intact and the 47 kDa intermediate forms) were the predominant ones also after 24 h of pulse, when faint bands corresponding to mature Saps became visible. Moreover, only trace amounts of intact Prosap were immunoprecipitated from the eHNPCs medium (Fig. 2B). To further confirm the block of Prosap processing and sorting, pulse-chase experiments were performed. The cells were pulse-labeled for 1 h and then chased for different times (Fig. 2C and D). After 5 h of chase most of Prosap was proteolitically

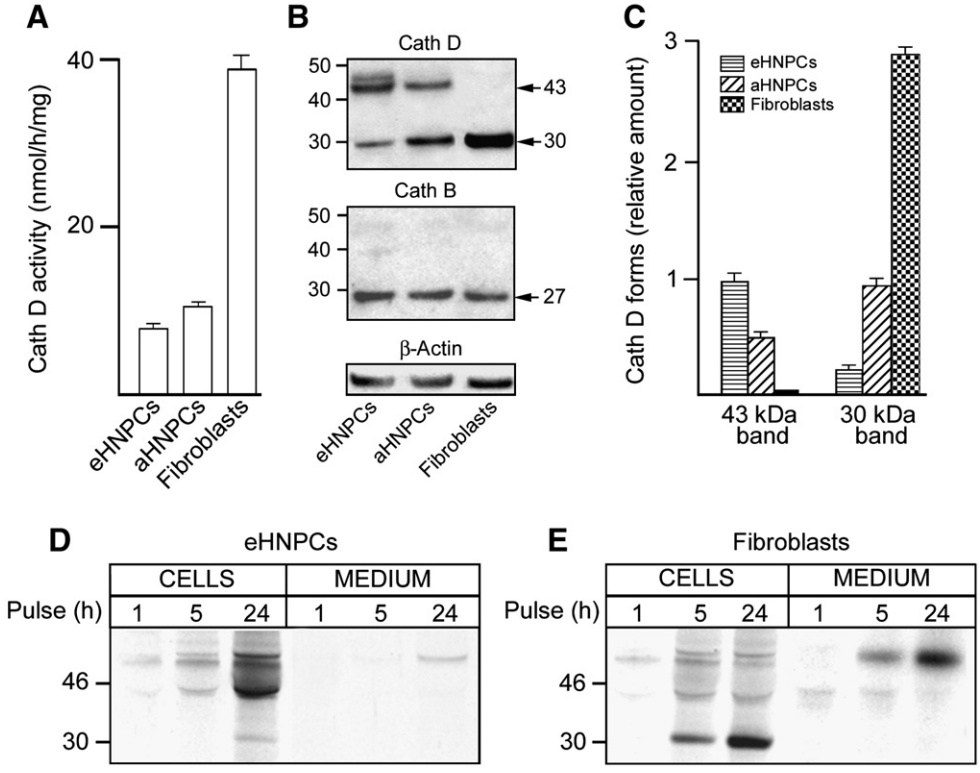

Fig. 4. ProCath-Cath D expression. (A) Cath D activity was determined in extracts of eHNPCs, aHNPCs and fibroblasts. (B) Western blot analysis of Cath D and Cath B isoforms in the extracts. The blots were reprobed for ß-actin as a loading control. The number on the left refers to molecular mass of standards (in kDa). The arrows on the right indicate the molecular weights of Cath D and Cath B isoforms. (C) Densitometric analysis of the Cath D bands normalized against the ß-actin bands. (D) eHNPCs and (E) human fibroblasts were labelled with [^{35}S] methionine/cysteine for the indicated times. Both the cell lysates and the medium were immunoprecipitated with an anti-Cath D antibody that recognizes the precursor, intermediate and mature isoforms. Immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. The number on the left refers to the molecular mass of standards (in kDa). The experiments were repeated more than three times giving similar results.

processed into Saps in fibroblasts, while poor proteolysis (less than 10%) was observed in eHNPCs. After 24 h of chase, the conversion of Prosap produced an equivalent amount of Saps in fibroblasts, but only trace amounts in eHNPCs. Concomitantly, the analysis of the cell medium revealed an abundant secretion of intact Prosap from fibroblasts, but not from eHNPCs. These results indicate that the Prosap transport to extracellular spaces and its intracellular conversion to Saps are inhibited or are not activated at all in eHNPCs.

3.2. Endoglycosidase treatments of Prosap

It has been previously observed that the sugar chains linked to intracellular Prosap differ from those linked to the Prosap form exported outside the cells, suggesting a relation between oligosaccharide processing and sorting [14,38]. To investigate the molecular factors determining the intracellular retention of Prosap in eHNPCs, we have examined the structure of its N-linked carbohydrates. To this aim homogenates of eHNPCs were digested either with Endo H, which hydrolyzes high mannose oligosaccharides or with PNGaseF, which hydrolyzes asparagines-linked glycans of both high mannose and complex types [39,40]. For comparison, fibroblasts homogenates were also incubated with the two endoglycosidases. As shown in Fig. 3, both endo H and PNGase F treatment of fibroblasts resulted in the total conversion of Prosap into the 55 kDa deglycosylated form, confirming that intracellular Prosap contains only high mannose sugars in these cells. Conversely, treatment with Endo H of eHNPCs homogenate produced diffuse bands between 65 and 55 kDa, presumably corresponding to partially deglycosylated forms (Fig. 3). This result indicates that in eHNPCs Prosap contains both types of oligosaccharides (high mannose type and complex asparagine-linked glycans) in one protein molecule.

3.3. Expression of Cath D in HNPCs

In most cell types intracellular Prosap is almost completely converted into Saps by the action of lysosomal proteases, especially of the aspartyl endoproteinase Cath D [12,16]. The observed intracellular accumulation of Prosap in eHNPCs and, to a lesser extent, in aHNPCs, prompted us to investigate whether the delayed/abolished Prosap proteolysis might be related to low Cath D activity. The fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dpb)gamma-NH2 was utilized to evaluate the proteinase activity in cell homogenates [37]. As shown in Fig. 4A, a markedly reduced Cath D activity (15–20% of that in fibroblast homogenate) was measured in eHNPCs and aHNPCs.

To assess whether the low activity was related to the presence of a low amount of Cath D protein, Western blot analysis using an anti-Cath D antibody that recognizes unprocessed and processed Cath D

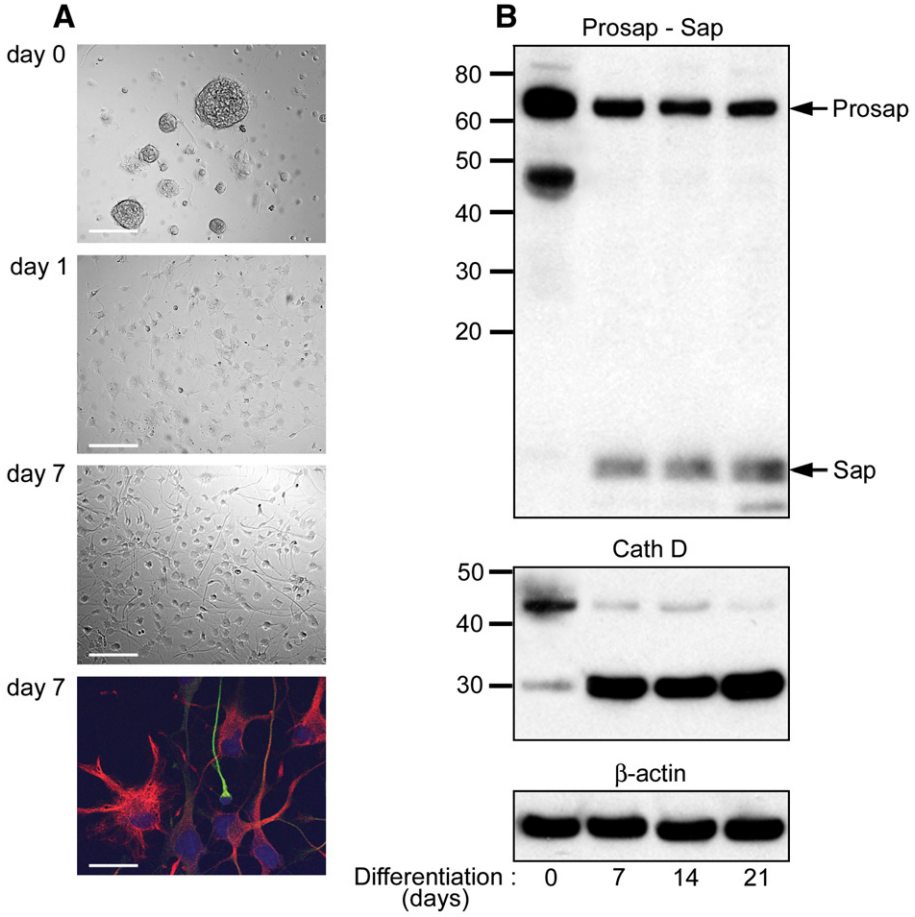

Fig. 5. Detection of Prosap-Sap and Procath-Cath D isoforms in eHNPCs during their differentiation into astrocytes and neurons. (A) Representative images of eHNPCs neurospheres before differentiation (day 0) and after 1 and 7 days of culture in the differentiation medium (see Materials and methods). Cells on day 7 of differentiation were also immunostained with ß-III tubulin-specific antibody (green) and anti-GFAP antibody (red) to confirm the differentiation into GFAP-positive astrocytes and tubulin-positive neurons. Nuclei were counterstained with TOTO-3 iodide (blue). Scale bars in the micrographs and in the immunostained fluorescent image indicate 500 µm and 100 µm, respectively. (B) Equal amounts (10 µg) of cell lysates from undifferentiated eHNPCs (day 0) and from eHNPCs cultured for 7, 14 and 21 days respectively in differentiation medium (see Materials and methods) were analyzed by Western blot with antibodies against Sap C, Cath D and ß-actin (as loading control). A representative Western blot out of three independent experiments is shown. The number on the left refers to the molecular mass of standards (in kDa).

isoforms was performed. Cath D can exist as precursor (53–50 kDa), intermediate (48–45 kDa) and mature (33–30 kDa) isoforms [41]. Fig. 4B and C show that the relative abundance of the different Cath D isoforms in eHNPCs, aHNPCs and fibroblasts is markedly different. The intensity of the 30 kDa band, corresponding to the mature double-chain form was much weaker in HNPCs (especially in eHNPCs) than in fibroblasts. Conversely, a band at about 43 kDa, presumably representing an intermediate Cath D form, was detected in HNPCs, but not in fibroblasts. Cath B, another lysosomal cathepsin evaluated as a control, was present as a mature 27 kDa form in both HNPCs and fibroblasts homogenates (Fig. 4B).

To confirm the reduced formation of the mature 30 kDa form of Cath D in eHNPCs and to evaluate the secretion of the precursor form, pulse experiments have been performed. As shown in Fig. 4D, the main intracellular forms found after 24 h of continuous labeling were the precursor (~50 kDa) and the intermediate (~43 kDa) forms, while the mature form (30 kDa) was hardly visible. On contrast, in fibroblasts homogenates the mature form of Cath D was already the predominant one after 5 h of pulse (Fig. 4E). Concomitantly, the amount of the precursor form (~50 kDa) increased in the medium of fibroblasts in a time-dependent manner, while only trace amounts were detected in the media of eHNPCs. These results indicate that in eHNPCs proCath D, likewise Prosap, is poorly processed and secreted.

3.4. Expression of Prosap and proCath D during differentiation of eHPNCs

To investigate whether the observed behaviour of Prosap and proCath D was related to the undifferentiated state of eHNPCs, we examined the expression of these two proteins during differentiation. The cells were induced to differentiate by cultivating them in the presence of FBS. Under this experimental condition, eHNPCs mainly differentiated into astrocytes and neurons (Fig. 5). Semiquantitative evaluation of the antigenic markers expression indicated that differentiated cultures contained about 80% GFAP-positive astrocytes, about 10% of β-III tubulin-positive neurons and rare astroglia (Fig. 5A). Western blot analysis revealed that differentiation caused a decrease of the 66 kDa intact Prosap, the disappearance of the intermediate 45 kDa form and a marked increase of the mature forms of Saps (about 10 kDa) already evident after a week of culture in the differentiation medium. Simultaneously, the level of the mature form of Cath D (30 kDa) dramatically increased (Fig. 5B).

The time-dependent formation of mature Saps and mature Cath D in the differentiated cells was confirmed by pulse and pulse-chase experiments (Fig. 6). Cells, that were cultivated for two weeks in FBS-containing medium, were pulse-labeled with [^{35}S]methionine/cysteine and harvested after various time periods. After 5 h of labeling two bands at about 10 kDa corresponding to Saps appeared and their intensity dramatically increased after 24 h (Fig. 6A). A similar increase in the formation of the mature form of Cath D (30 kDa) was observed (Fig. 6C). Moreover, the differentiated cells were able to export large amounts of the intact form of Prosap and proCath D in the medium. (Fig. 6A and C). Pulse-chase experiments (1 h of pulse and 5 and 24 h of chase) confirmed the conversion of Prosap into Saps and of proCath D into mature CathD (Fig. 6B and D). These results indicate that the differentiation process of eHNPCs promotes the intracellular lysosomal proteolysis and the extracellular sorting of both Prosap and proCath D.

3.5. Subcellular localization of Prosap–Sap and proCath–Cath D in undifferentiated and differentiated eHNPCs

The above results show that, while high molecular forms of Prosap–Sap and of proCath–Cath D are the main forms present in undifferentiated eHNPCs, large amounts of mature Saps and Cath D are generated after differentiation. The precursors and mature forms of these proteins presumably reside in different compartments of the cells. To compare their subcellular localization before and after differentiation, immunostaining experiments were carried out utilizing anti-Sap C and anti-CathD antibodies that recognize the precursor and mature forms. Confocal microscopy revealed that in the undifferentiated eHNPCs Prosap did not colocalize with the lysosomal marker Lamp1 but was located on and near the cell surface (Fig. 7A). This result indicates that intact and high molecular intermediate

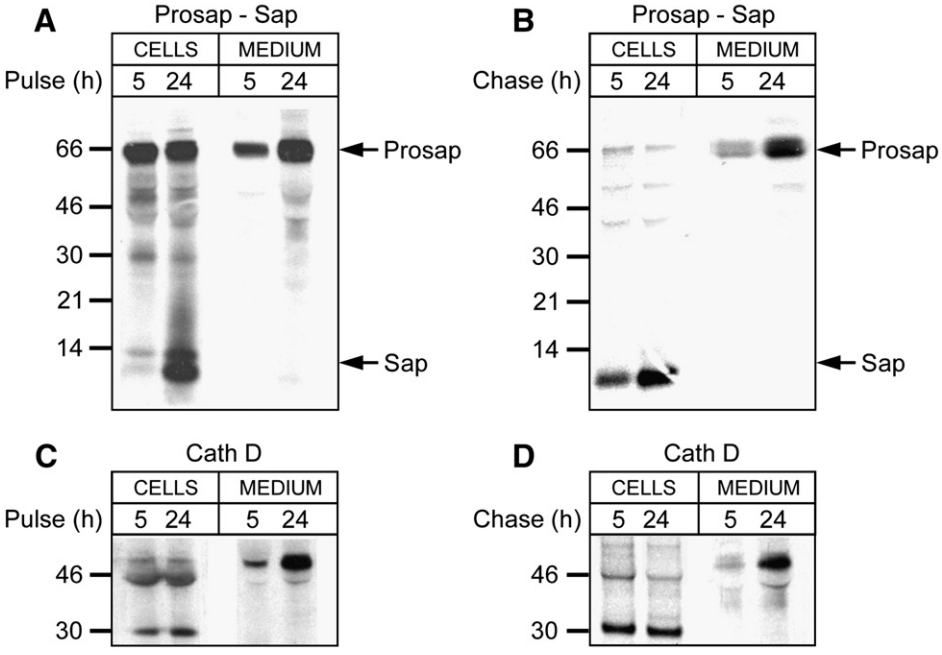

Fig. 6. Processing and secretion of Prosap and proCath D after differentiation of eHNPCs. eHNPCs were cultured for 14 days in the differentiation medium and then metabolically labelled with [^{35}S] methionine/cysteine for 5 h or 24 h (A and C, pulse experiments) or, alternatively, labeled for 1 h and chased for the indicates times (B and D, pulse-chase experiments). Prosap–Sap (A, B) and proCath–cath D (C, D) were immunoprecipitated from cell lysates and medium with the respective specific antibodies. Immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. The number on the left refers to the molecular mass of standards (in kDa). The experiments were repeated more than three times giving similar results.

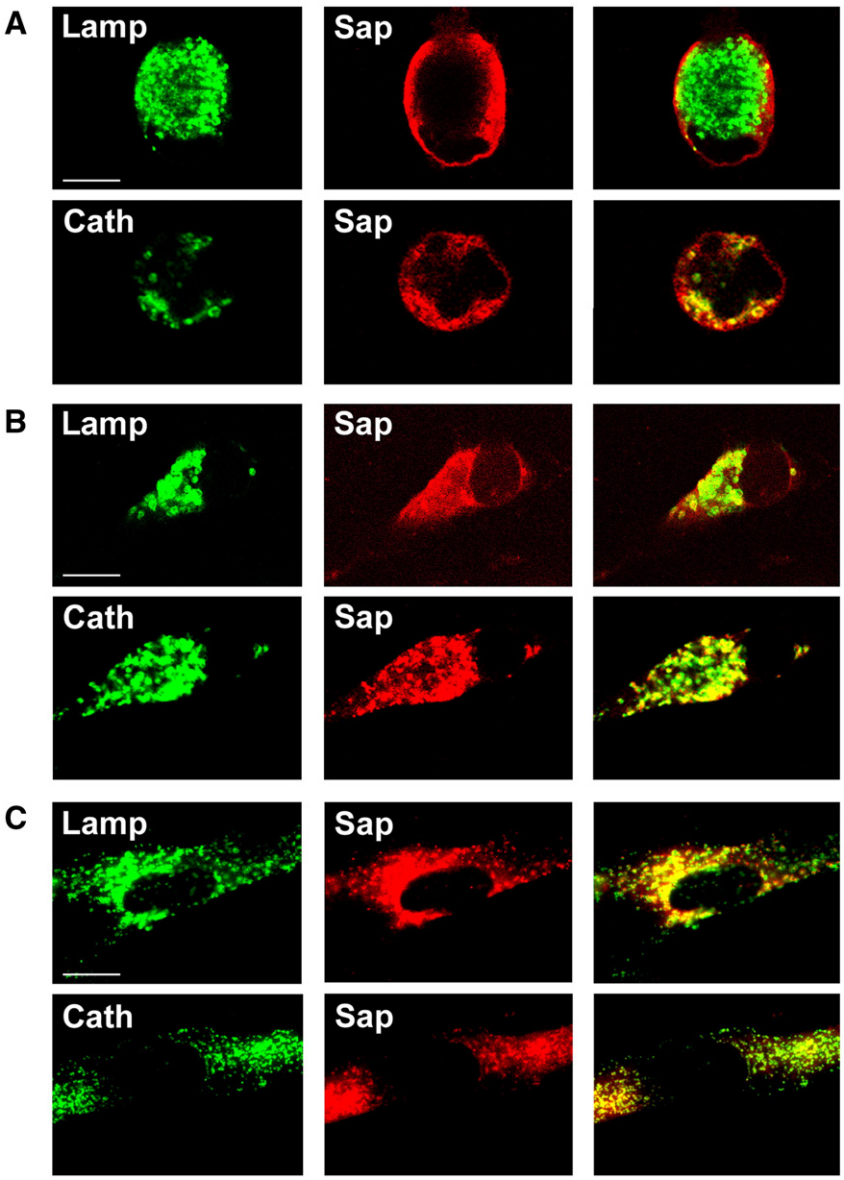

Fig. 7. Confocal microscopy of Prosap–Sap and proCath–Cath D in undifferentiated and differentiated eHNPCs. Undifferentiated eHNPCs (A) or eHNPCs after 14 days of culture in the differentiation medium (B) or human fibroblasts (C) were double-immunostained for Lamp1 and Prosap–Sap or for proCath–Cath D and Prosap–Sap. The right panels show the merged images. Bars, 10 μm (A and B), 20 μm (C).

forms of Prosap (the only forms present in eHNPCs) reside in a specific compartments of the cells distinct from late endosomes/lysosomes. Fig. 7A shows that also proCath–Cath D is localized near the cell plasma membrane, partly colocalizing with Prosap.

When eHNPCs were induced to differentiate, Cath D and Sap C were detected in vesicles that also contained Lamp1 (Fig. 7B), indicating that the mature forms of the two proteins mostly reside in the late endosomes/lysosomes of the differentiated cells. As a control human fibroblasts were examined under the same experimental conditions; a complete colocalization of Sap C, Cath D and Lamp1 was observed in these cells, as expected (Fig. 7C).

3.6. Alternative Prosap mRNA splicing during differentiation of eHPNCs

It has been previously shown that in human and animal brains Prosap mRNA is present as two major isoforms differing in the inclusion of 9 bps of exon 8 within the Sap B domain [42]. In normal rat brain the two Prosap mRNA species with and without the 9 base insertion are expressed at a ratio of 85:15 and this equilibrium is reverted to 5:95 following ischemic injury [22]. In order to evaluate whether the differentiation process might also affect the Prosap mRNA splicing, we assessed the abundance of Prosap mRNA with and without exon 8 in eHNPCs and in their differentiated progeny. The experimental approach described by Hiraiwa et al. [22] was followed. As shown in Fig. 8, the abundance of Prosap mRNAs with exon 8 was markedly higher than that without exon 8 in the undifferentiated

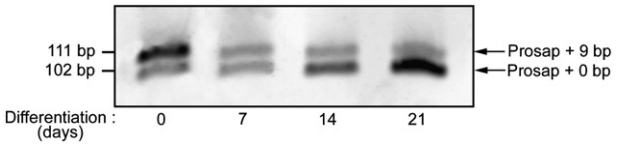

Fig. 8. Alternative 9-bp splicing of Prosap mRNA during eHNPCs differentiation. Total RNA from eHNPCs before and after differentiation for 7, 14 and 21 days was subjected to RT-PCR and the products were separated by gel electrophoresis on 8% acrylamide gel as described in Materials and methods. A representative result was shown in the Figure. Note the presence of a 111 bp and a 102 bp band representing transcripts with or without the 9-bp insertion sequence (exon 8) [22].

eHNPCs. Upon differentiation the isoform without exon 8 increased in a time-dependent manner, becoming the predominant one after 14 days of differentiation treatment. Thus, the alternative splicing of Prosap mRNA, that has been previously observed following brain injury [22], occurs also upon eHNPCs differentiation (Fig. 8).

4. Discussion

Although neural stem/progenitor cells have recently received much attention for their therapeutic potential in the treatment of various neurological diseases or injuries [43], several aspects of their biological behaviour are ill defined. In the present investigation we have studied in HNPCs the expression of Prosap, a protein proposed to play important roles in the nervous system, and of Cath D, a lysosomal aspartyl endoproteinase involved in the transport and proteolytic conversion of Prosap into Saps [16]. We have found that eHNPCs accumulate inside the cells large amounts of intact and high molecular weight intermediate forms of the Prosap protein, while the formation of mature Saps was blocked. Concomitantly, accumulation of an intermediate form of Cath D and a minute amount of mature Cath D were detected. Moreover, eHNPCs did not export neither Prosap nor proCath D outside the cells.

Quantitative real-time PCR showed that HNPCs contained more Prosap mRNA than other cell types such as neuroblastoma cells (SHSY5Y) or fibroblasts, but the different gene expressions did not correlate with the observed differences in the level of intact Prosap protein within these cells. The accumulation of Prosap at protein more than at mRNA level in eHNPCs strongly suggested that this phenomenon was due to post-translational events such as the inhibition of Prosap maturation and secretion.

The intracellular proteolytic conversion of Prosap to mature Saps has been well characterized in human fibroblasts [38]. Prosap is synthesized as a ~55-kDa protein, post-translationally modified to a 65-kDa glycosylated form and further glycosylated in the Golgi compartment to a 70-kDa product. The 65 kDa form is sorted to the lysosomes where it is almost completely converted into four small glycoproteins, Sap A, B, C, and D, while the 70-kDa form is exported as such into the external medium. In eHNPCs the intracellular Prosap is hardly converted into mature Saps, strongly suggesting an impaired transport from the Golgi to the late endosomes/lysosomes where Saps are usually produced [14,38,44]. Most of the lysosomal proteins are targeted to the lysosomes via mannose-6-phosphate (M6P) receptors, after acquiring M6P residues while traversing the cis-compartment of the Golgi apparatus [45]. However, recent studies have indicated that Prosap and other lysosomal proteins such as the GM2 activator and acid sphingomyelinase poorly interact with the M6Preceptors, but rather utilize alternative mechanisms and alternative receptors such as sortilin, for lysosomal sorting [15,46]. Sortilin, also called neurotensin receptor 3, is a member of a family of Vps10p-domain receptors, expressed in a variety of tissues, notably brain, spinal cord and muscle [47]. The sortilin role as a mediator of lysosomal trafficking of Prosap was confirmed by the finding that sortilin inhibition or structural modifications prevents the transport of Prosap from the trans Golgi to the lysosomes [15]. By Western blot analysis we have found that the sortilin protein was present in eHNPCs homogenate (data not shown). This observation suggests that the altered Prosap transport in eHNPCs is not due to the lack of sortilin expression.

In several cell types a specific relationship between the glycosylation state of Prosap and its intra or extracellular destination has been observed. In fact, the secreted form usually contains complex carbohydrate chains, being endo H-resistant, while the intracellular Prosap form, assigned to be converted into Saps in late endosomes/lysosomes, contains only high mannose sugars, being endo H-sensitive [14,38]. Conversely, the present findings indicate that in eHNPCs intracellular Prosap contains both high mannose and complex type oligosaccharide chains, suggesting that Prosap, after reaching the cis region of the Golgi apparatus, where it is glycosylated with high mannose type chains, proceeds towards the trans cisternae acquiring also complex sugar chains. The difference in the N-linked oligosaccharides indicates a different transport through the Golgi compartments of intracellular Prosap contained in eHNPCs or in other cell types such as human fibroblasts.

The present study shows that in eHNPC also the sorting of proCath D, a protein that has been proposed to travel together with Prosap [32], is altered. It is known that the intracellular conversion of the inactive precursor proCath D (52 kDa) into a single-chain intermediate form (48–45 kDa) take place in a late-Golgi compartment, while the successive limited proteolysis of the intermediate form to give rise to mature two-chain Cath D (30 kDa) occurs in mature, dense lysosomes [48]. In eHNPCs proCath D was mainly processed to the intermediate rather than to the mature form, suggesting that the transport of Cath D to the lysosomes was inhibited likewise that of Prosap. In some cell lines such as HepG2 or breast and ovarian cancer cells the formation of a complex between Prosap and proCath D in their way from the Golgi to the lysosomes has been shown [49,50]. It has been proposed that the formation of this complex might play a role in the intracellular and extracellular trafficking of the two precursors [32]. The shared block of the sorting/processing machinery of Prosap and proCath D in eHNPCs indicates that their complex (if it is formed at all) cannot reach its final destination/s, namely the lysosomes and/or the medium outside the cells.

The incapacity of Prosap to reach lysosomes in eHNPCs was confirmed by confocal microscopy, which revealed that Prosap was rather located on and near the cell surface in the undifferentiated cells. Interestingly, the occurrence of Prosap as a surface membrane component has been previously observed in mouse neuroblastoma cells [51]. It was concluded that Prosap can follow three different pathways; one to the lysosomes as precursor of mature saposins, another one to the extracellular space as a secreted form, and a third one as a membrane component of neuronal plasma membranes. While neuronal cells have been reported to have all three Prosap pools [51], eHNPCs appear to have only the pool located near the cell surface.

The sorting/processing block of Prosap and proCath D appears to be overcome during the course of differentiation of eHNPCs into a mixed population of astrocytes and neurons. The analysis of the protein expression profiles revealed that the lysosomal conversion of Prosap to Saps and of proCath D to mature Cath D only occurred when the cells were differentiated. The confocal microscopy confirmed the lysosomal localization of the mature forms of Sap C and Cath D in the differentiated cells. Concomitantly, a parallel increase of the Prosap and proCath D secretion was observed, consistent with the existence of a common, cell differentiation-regulated, sorting mechanism.

The expression of Prosap–Saps and ProCath–Cath D in undifferentiated eHNPCs most likely has a physiological significance. The complex mechanisms controlling the survival, proliferation and regenerative capacity of neural stem cells are still poorly understood. eHNPCs have been shown to be completely resistant to apoptosis induced by death receptor ligands such as TNF-α [35]. In this context it is interesting to note that Prosap treatment is able to protect neuronal and non neuronal (U937) cells from TNF-α-induced cell death [52]. It is thus reasonable to hypothesize that the accumulated intracellular Prosap might constitute an additional protective factor involved in the antiapoptotic machinery of eHNPCs.

On the other hand, also the extremely low level of mature Cath D protein assessed in eHNPCs might protect these cells from apoptosis. Several reports have demonstrated that Cath D is a central mediator of the apoptotic response and participates in apoptosis execution. A role of Cath D in cell death pathways is well documented, with evidence that Cath D is a mediator of caspase activation [53,54]. Antisense-mediated inhibition of Cath D expression and activity inhibits apoptosis caused by interferon-gamma, Fas/APO-1 and TNF-α [33].

Down-regulation of Cath D by small interfering RNA inhibits early apoptopic events [54]. In eHNPCs the low expression and activity of mature Cath D might contribute to the resistance of these cells to apoptotic stimuli. It must be noted that the low Cath D activity here measured at pH 4.5 in the cell homogenate might be even an overestimation of the actual activity expressed 'in vivo', since the predominant intermediate form of Cath D present in eHNPCs is presumably localized in less acidic organelles than mature lysosomes, where the low pH can fully promote the potential protease activity.

The Prosap gene has a 9 bp exon (exon 8) that can be alternatively spliced generating three mRNAs, having a stretch of 9 or 6 or 0 additional bases within the Sap B coding region [42]. The expression of mRNA containing exon 8 is tissue specific, its level being high in brain, heart and skeletal muscle [22,42]. Although it remains unclear which is the specific function of the different mRNA forms, past data have shown that the splicing of Prosap mRNA is crucially regulated during nerve regenerative processes and that the percentage of Prosap mRNA without exon 8 dramatically increases in rat brain in response to brain injury, the change being mostly restricted in the lesion area [22]. A similar increase of this Prosap mRNA form has been observed following differentiation of eHNPCs (see Fig. 8) into a mixed neural population in which astrocytes predominate. The analogous alternative Prosap mRNA splicing after brain injury and after eHNPCs differentiation strongly suggests that differentiation of endogenous neural stem/progenitor cells might contribute to the elevation of the Prosap mRNA form without exon 8 occurring in the brain lesion area. This hypothesis is supported by recent finding showing that neural precursors significantly proliferate in response to traumatic brain injury and most of the proliferating cells differentiate into activated astrocytes [55].

In conclusion, the present results have shown for the first time that in eHNPCs the maturation and secretion of Prosap and proCath D are blocked and that these biological processes only occur after cell differentiation. Although our findings relate to studies in cultured cells, most likely they reflect the 'in vivo' situation. Recently, protein profiles have been carried out on several stem cell populations [56,57], most of the studies being concentrated on the relative abundance of proteins. However, another important aspect of proteomic analyses is the study of post-translational modifications, which can affect the activity and function of proteins. This is particular true for those proteins whose final destination are lysosomes, where the low pH promotes the last steps of their maturation and regulates their activity and function [36]. Prosap–Saps and proCath–Cath D have been shown to have several potential functions that are modulated not only by their level but also by their degree of maturation, by the pH of the environment and by their localization inside or outside the cells. Our results on the distinct behaviour of these two multifunctional proteins in eHNPCs and in their differentiated progeny provide new basis for a more detailed understanding of neural stem cell biology.

Acknowledgements

This study was partially supported by a research grant on the use of stem cells from the Italian Ministry of Health and by the Association 'Vaincre les maladies lysosomales' (VML). We thank Vincenzo Raia for technical assistance.

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