Prolyl Endopeptidase Is Involved in Cellular Signalling in Human Neuroblastoma SH-SY5Y Cells

M. José Moreno-Baylach^a Katja A. Puttonen^b Jofre Tenorio-Laranga^a
Jarkko I. Venäläinen^b Markus Storvik^b Markus M. Forsberg^b
J. Arturo García-Horsman^a

^aDivision of Pharmacology and Toxicology, University of Helsinki, and ^bSchool of Pharmacy, Pharmacology, Faculty of Health Sciences, University of Eastern Finland, Kuopio Campus, Kuopio, Finland

Key Words

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Kinase cascades

Abstract

Prolyl endopeptidase (PREP), probably acting through the inositol cycle, has been implicated in memory and learning. However, the physiological role of PREP is unknown. It has been shown that PREP expression, regulated in cerebellar granule cells, has probably a role in cell proliferation and differentiation. Here, we report the levels and subcellular distribution of PREP in human neuroblastoma SH-SY5Y cells in proliferating conditions and under differentiation induced by retinoic acid (RA). We analysed the levels of cell signalling intermediates, growth behavior and gene expression, and differentiation morphology changes, upon PREP inhibition. After induction of differentiation, PREP activity was found decreased in the nucleus but increased to high levels in the cytoplasm, due in part to increased PREP transcription. The levels of inositol (1,4,5)-trisphosphate revealed no correlation with PREP activity, but phosphorylated extracellular signal-regulated kinases 1 and 2 were decreased by PREP inhibition during early stages of differentiation. Morphologi-

cal evaluation indicated that PREP inhibition retarded the onset of differentiation. PREP activity regulated gene expression of protein synthesis machinery, intracellular transport and kinase complexes. We conclude that PREP is a regulatory target and a regulatory element in cell signalling. This is the first report of a direct influence of a cell signalling molecule, RA, on PREP expression.

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Introduction

Prolyl endopeptidase (PREP, EC 3.4.21.26, also known as POP, PO or PEP) is a serine protease which cleaves short proline-containing peptides. PREP was first found in the uterus as an oxytocin-cleaving enzyme in the early 70s [1]. During the following decades, it was found that PREP was able to cleave a number of neuropeptides and hormones [2]; it was postulated that PREP was involved in the metabolism of neuropeptides, such as substance P, thyrotropin-releasing hormone, gonadotropin-releasing hormone, arginine-vasopressin, angiotensin, bradykinin, β -endorphin, neurotensin and α -melanocyte-stimulating hormone among others, and thus involved in regulating the neurotransmitter or hormonal action of these

peptides. This function of PREP was substantiated when it was found that specific PREP inhibitors could influence memory and learning, e.g. reversing amnesia induced by drugs or by specific brain lesions [2]. PREP has also been shown to have neuroprotective effects in certain experimental conditions [3]. However, one of the main criticisms raised against the role of PREP in the control of neuropeptide response is the fact that PREP is mainly found within the cytoplasm, with no apparent interaction with its potential extracellular substrates. Furthermore, conflicting results have been reported on the effect of PREP inhibitors on neuropeptide levels [3]. Recent studies have indicated that the levels of certain neuroactive peptides, such as angiotensin II, substance P and arginine-vasopressin, are not modified by PREP inhibition, at least in rat heart, kidney, and brain [4].

There are previous reports indicating that circulating levels of PREP are altered in bipolar disorder [5] and it has been claimed that this peptidase is a modulator of Li^+ sensitivity [6–8]. Subsequent studies demonstrated that PREP was a negative regulator of inositol (1,4,5)-trisphosphate (IP_3) synthesis, a Li^+ sensitive intracellular signalling molecule [9, 10]. It was shown in *Dictyostelium* and in cultured human cells that PREP could act via the enzyme multiple inositol polyphosphate phosphatase (Mipp1) to control gene expression and hence affect the level of the second-messenger IP_3 [9]. This was an indication of the existence of a novel gene regulatory network modulating inositol metabolism and hence Li^+ sensitivity. On the other hand, brain distribution studies showed that PREP is not clearly associated with a specific neurotransmitter pathway, but instead, it seems to be involved in multiple neuronal systems, both inhibitory and excitatory. Despite all the research performed on PREP, the physiological role of this peptidase is still a matter of controversy.

In order to elucidate the biological relevance of PREP in the central nervous system, we have previously studied the levels of PREP expression during the lifespan of cerebellar granule cells, and furthermore, we monitored the distribution of PREP within different intracellular compartments. It was observed that PREP activity was regulated during the neuronal life cycle and that it was localized in both cytoplasm and neurite projections [11]. During early developmental phases, PREP was found to be located inside the nucleus. It was shown that PREP was strictly regulated during cerebellar granule cell differentiation, maturation and ageing, indicative of a role in the cell signalling system. Nonetheless, no detectable effects of PREP inhibitors were observed on the onset of neuro-

nal differentiation, cell maturation or ageing in that system. However, no studies aimed to identify the mechanism of PREP modulation or the cellular signalling system involved in these processes have been done. In the present study, the role of PREP during proliferation and differentiation has been further investigated in human neuroblastoma SH-SY5Y cell cultures. Here, we assessed PREP expression during cell proliferation as well as during differentiation induced by retinoic acid (RA). Additionally, in an attempt to elucidate the cellular role of PREP, we examined the effects of PREP inhibitors on these processes and the changes in the levels of cell signalling intermediates.

Materials and Methods

All general reagents were purchased from Sigma Aldrich (St. Louis, Mo., USA). Cell culture media and reagents were from Invitrogen (Carlsbad, Calif., USA). Peptidase assay substrate (Suc-Gly-Pro-AMC) and Z-Pro-Prolinal were from Bachem (Weil am Rhein, Germany), and molecular biology reagents were purchased from BioRad (USA). PREP inhibitors JTP-4819 ((S)-2-[[S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N(phenylmethyl)-1-pyrrolidinecarboxamide) and KYP-2047 (4-phenylbutanoyl-L-prolyl-2(S)-cyanopyrrolidine) were synthesized in the School of Pharmacy, University of Eastern Finland (formerly Department of Pharmaceutical Chemistry, University of Kuopio).

Cell Culture

SH-SY5Y human neuroblastoma cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's F12 (1:1) supplemented with 15% of inactivated fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids and 200 U/ml of penicillin and 200 µg/ml of streptomycin. Cells (0.5×10^6) were plated in 6-well plates and, when indicated, 10 µM (final concentration) of JTP-4819, KYP-2047 or Z-Pro-Prolinal (PREP inhibitors) was added. The inhibitors used in this study are based on the Pro-Pro scaffold, and all have a reactive group which enables tight binding to the reactive serine in PREP catalytic site, i.e. they possess the same mode of action and selectivity profile [2, 3] (also see online suppl. material, www.karger.com/doi/10.1159/000326342). The potency of all three inhibitors is very similar and the crystals show the same reactivity [Fülöp, pers. commun.]. These concentrations of inhibitors (10–100 times higher than their EC_{50}) were sufficient to achieve more than 95% inhibition of endogenous PREP activity in SH-SY5Y cells (online suppl. material) as reported before [11–13, and A.M. Lambeir, pers. commun.].

Cell differentiation was induced with 10 µM RA after 24 h of plating. To induce proliferation in RA-treated cultures, cells were washed twice with fresh medium and incubated in RA-free medium (with or without PREP inhibitor). Cells were incubated for 20–35 days. In all experiments, the cells were used between 17 and 27 passages. Cell culture curves were constructed by cell counting (Bürker chamber) and plotted against number of days in vitro (DIV).

Lysis and Cell Fractionation for PREP Activity Assay

The lysis and fractionation protocols were performed according to Moreno-Baylach et al. [11]. Briefly, the cells were rinsed with 2 ml PBS, and subjected to hypotonic shock (300 μ l of 50 mM KH_2PO_4 , 1.5 mM MgCl_2 , 10 mM NaCl, 1 mM DTT, 1 mM EDTA; pH 7.4, 5 min on ice). The cells were scraped, transferred to a 1.5-ml tube and kept for 20 min on ice. The homogenate was centrifuged (800 g, 10 min at 4°C), and then the supernatant (cytosolic fraction) was frozen at -80°C. Pellets containing the nuclei were washed with hypotonic buffer and centrifuged (1,000 g, 5 min, 4°C) and extracted with 100 μ l of cold hypertonic buffer (50 mM KH_2PO_4 , 1.5 mM MgCl_2 , 250 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100; pH 7.4) for 30 min on ice. Samples were homogenized by sonication (Branson Sonifier 150; Danbury, Ct., USA) twice for 5 s, centrifuged (20,000 g, 25 min, 4°C), and the supernatant (nuclear protein fraction) was frozen at -80°C. Fractions were verified for cross-contamination by immunoblotting with lamin B, a nuclear marker, and β -actin, a cytoplasmic marker.

PREP Activity Assay

PREP activity assay was done in 48-well plates in triplicate as reported before [11], using 100 μ M Suc-Gly-Pro-AMC as substrate in 0.1 M potassium phosphate, pH 7 (final volume 500 μ l) at 37°C. The formation of AMC was determined fluorometrically in a micro-plate fluorescence reader (Victor2; Perkin-Elmer, Boston, Mass., USA) with excitation beam at 360 nm and emission recorded at 460 nm. The volumes of protein samples were 1:10 to 1:20 compared with the total assay volume; control experiments were performed to take into account any differences in salt or detergent concentrations. No effects were found due to these small changes in the conditions. To verify that the formation of AMC was specifically due to PREP action, and not due to other enzymes which could cleave the same substrate Suc-Gly-Pro-AMC, e.g. α -fibroblast-activated protease (FAP or seprase), we tested the sensitivity of the measured PREP activity of three PREP specific inhibitors (JTP-4819, KYP-2047 and ZPP). Since over 98% of the activity measured was sensitive to the inhibitors, it was concluded that the activity reported here is due to PREP.

PREP Western Blotting

Protein samples were acetone precipitated and resuspended in 1 $\times$ loading buffer (50 mM Tris-HCl pH 6.8, 35% glycerol, 1% SDS, 0.002% bromophenol blue, 5 mM β -mercaptoethanol). Western blots were done as described before [14] using a purified chicken anti-human POP [14] as the first antibody at 1:5,000 dilution, and goat anti-chicken IgY-HRP conjugate (Sigma-Aldrich) as a secondary antibody at 1:10,000 dilution. Standard SDS-PAGE, transfer, blocking and blotting techniques were used. One to 5 fmol of purified recombinant porcine POP, prepared as described before [14], was used as the positive control.

Morphological Analysis

A volume containing 0.1×10^6 cells was plated into each well of 6-well plates containing 2 ml of medium. After 4 h, a PREP inhibitor (10 μ M of JTP-4819, KYP-2047 or Z-Pro-Prolinal) was added into the corresponding wells. After 24 h, RA (10 μ M) was added into the wells where differentiation was induced. On day 6, cells were observed with a phase contrast microscope (Nikon Eclipse TE300; Garden City, N.Y., USA) by an investigator who

was blinded to the identity of the wells in terms of PREP inhibition or RA. Each well was divided into 5 areas and each area into 3 subareas. From each subarea, one photograph was taken, i.e. 15 photographs were taken from each well at random. From each photograph, the following parameters were counted: number of differentiated neurons, number of neurites per neuron, length of the neurites, and number of connections per neuron. A neuron was assessed as being differentiated when the cell displayed neurite projections. Relative neurite length was calculated with reference to cell body width. Neuron connectivity was assessed as the number of neurite connections between two or more cells. The results of the counting were provided to the statistician along with the keys for the codes of different conditions (control vs. PREP inhibited) for statistical analysis (online suppl. material).

Prep mRNA Quantitation

A qRT-PCR protocol was used to determine the levels of *Prep* transcription. Absolute levels of *Prep* mRNA were assayed referring to a standard curve of *Prep* cDNA. Cells (0.5×10^6) from different stages of culture in the presence or absence of RA were harvested. Total RNA was extracted with Aurum Total RNA Mini Kit (Biorad), according to the manufacturer's instructions. *Prep* cDNA was synthesized by a reverse transcriptase reaction using the iScript™ cDNA Synthesis Kit (Biorad) on 10 μ l of total RNA (around 60–50 ng). cDNA was amplified by PCR (forward primer 5'-ATG CTG TCC CTT CAG TAC C-3'; reverse primer 5'-GCT GAA CGC ATA ACC TCG G-3') using MiniOpticon cyclor (BioRad) according to the manufacturer's instructions. The size of the amplified fragment was 360 pb. The C_t values were compared with a standard curve of *Prep* cDNA plasmid fragment (*Bam*HI/*Eco*RI fragment from pBADHumPREP plasmid as described in Venäläinen et al. [15]). The determinations were made in triplicate.

IP₃ Determination

The assay used is based on competitive binding between an IP_3 -[^3H] tracer and unlabelled IP_3 from cell lysates or standards to an IP_3 receptor preparation (rat cerebellar membranes) according to an IP_3 radioreceptor assay kit (Perkin-Elmer). Supernatants containing cellular IP_3 were prepared extracting the cells for 20 min with 20% HClO_4 , neutralizing (1.5 M KOH, 60 mM HEPES), and centrifuging (2,000 g). Binding was conducted in tubes containing 165 μ l of incubation buffer (0.1 M Tris-HCl, pH 9.4 mM EDTA), 10 μ l IP_3 -[^3H] (18 Ci/mmol) 200 μ l (250 μ g) of an IP_3 receptor preparation and 125 μ l of each standard (6.25–100 pmol IP_3), or sample extract. Tubes were incubated on ice for 10 min and then 2 ml scintillation liquid (Optiphase HiSafe3; Perkin-Elmer) was added. Radioactivity was measured by liquid scintillation counting (1414 Winspectral; Wallac, Mass., USA).

The cerebellar membrane suspension used in this assay was prepared by homogenizing rat cerebella in 10 vol of 12 mM HEPES, pH 7.4 containing 300 mM mannitol supplemented with protease inhibitor cocktail (cOmplete; Roche, Mannheim, Germany) and centrifuged for 15 min at 3,000 g. The supernatant was centrifuged for 45 min at 150,000 g and the pellet was resuspended in PBS containing a protease inhibitor cocktail.

Determination of Extracellular Signal-Regulated Kinases 1 and 2

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) were determined by Western blotting. The cells (0.5×10^6) were lysed

Fig. 1. PREP activity levels in cytoplasm (**a**) or nuclei (**b**) of SH-SY5Y cells in culture under control proliferative conditions (●), after induction of differentiation by RA (10 μ M; ○) and after RA removal (▲), plotted against DIV. The levels of PREP protein as assayed by Western blot for DIV 4 and DIV 20 in control cells (C) and differentiation-induced cells (RA) are presented in the insets. **c** SH-SY5Y growth curve under proliferative conditions (●, ○), RA conditions (■, □) and conditions when RA had been removed (▲, △) in the absence (—) or presence (---) of JTP-4819.

as described above in the presence of phosphatase inhibitor cocktail (PhosSTOP; Roche). SDS-polyacrylamide electrophoresis (12% acrylamide-bisacrylamide) and Western blotting were performed under standard conditions. Two identical gels were blotted and transferred. One blot was incubated with anti-ERK1/2 antibody (1:1,000, rabbit poly-clonal anti-human, Santa Cruz, Calif., USA) and the other with anti-phospho-ERK1/2 (1:1,000, monoclonal anti-human, Danvers, Mass., USA). After washing ($3 \times$ TTBS), the secondary antibody (1:3,000, goat anti-human IgG-peroxidase complex, Pierce, Mass., USA) was added and incubated for 2 h. Detection was performed with SuperSignal West Femto (Pierce, Thermo Fisher Scientific Inc., USA). The signal was recorded by Syngene Bio Imager and the density was measured with Genesnap software (Syngene, Cambridge, UK). Phosphorylated kinases (p-ERK1/2) were determined by calculating the ratio of p-ERK to ERK band densities. At least four different experiments per condition were done and the average of band densities is reported.

Affymetrix DNA Array

One-day-old SH-SY5Y cells (seed 0.5×10^6) under standard conditions were incubated with 10 μ M Z-Pro-Prolinal, or vehicle (0.1% DMSO in Dulbecco's Modified Eagle's Medium) for 24 h corresponding to the condition of DIV 2 in figure 1. Total RNA was extracted using RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The integrity of RNA was verified by denaturing gel electrophoresis. The Affymetrix array (GeneChip® Human Genome U133A; Affymetrix, High Wycombe, UK) was performed by The Finnish Microarray and Sequencing Centre (FMSC) at Turku Centre for Biotechnology in Finland. Each sample was hybridized at +45°C overnight (16 h) according to GeneChip® Expression Analysis technical manual. GeneChip Scanner 3000 with AutoLoader was used to scan the arrays. GeneChip Command Console® (AGCC) software version 1.0 was used to control GeneChip Fluidics Stations and Scanner. Cell files were automatically generated by the AGCC. After hybridization, quality control was done with Affymetrix Expression.

Console

The RMA pre-processed data were then normalized by using a quantile normalization method. Samples were analysed in triplicate. The significant alterations in gene expression values between treatments were observed after ANOVA statistical evaluation with a false discovery rate, $p < 0.05$, and fold-change ± 1.3 -fold between the treated and control samples.

Bioinformatic and Enrichment Analysis

The genes displaying altered expression in the microarray experiment were classified according to the enriched canonical pathways of the gene ontology (GO) terms (cellular compartment and biological processes) by using DAVID Bioinformatic Resources [16, 17] and FatiGo application from the Babelomics web portal [18–21] from the resources <http://david.abcc.ncifcrf.gov/> and <http://babelomics.bioinfo.cipf.es/>.

Statistics

Statistical analyses were performed using GraphPad Prism (version 4.03; GraphPad Software, Inc., San Diego, Calif., USA). One-way ANOVA followed by Tukey's test was performed to assess the differences in cell count and activity. Two-way ANOVA followed by Bonferroni's test was performed to analyse RNA transcripts, IP₃ levels and p-ERK1/2 band densities. Standard errors of the mean (SEM) are shown as error bars. Statistical analysis for the morphological study was carried out with the R project for statistical computing (<http://www.R-project.org>) using generalized linear models [22]: Poisson regression for differentiated neuron and neurite counts, gamma regression for neurite length and multinomial logistic regression for number of connections between neurons were performed (online suppl. material). Statistically significant differences were considered to be $p < 0.05$.

Results

PREP Activity Levels during Proliferative and Differentiated Culture of SH-SY5Y Cells

We measured the levels of PREP in cytoplasm and nuclei of SH-SY5Y cells during proliferative culture from 0 to over 30 DIV. It was observed that cytoplasmic PREP activity decreased slowly and steadily from DIV 0 throughout the growth curve until reaching confluence (between DIV 10 and 15; fig. 1a). PREP activity remained at low levels from DIV 15 until the end of the experiment. On the other hand, PREP activity inside the nucleus (fig. 1b) increased 3-fold at DIV 1 compared with its initial levels (DIV 0). This higher nuclear activity, over 200% greater than that measured in the cytoplasm, was maintained until DIV 5. As soon as cells entered into fast logarithmic growth (DIV 5 onwards; fig. 1c), PREP nuclear activity declined dramatically until the cells reached confluence (right after DIV 10). At this point, nuclear PREP activity was at the same level as that detected in the cytoplasm. We then recorded POP activity in a culture where

Fig. 2. *Prep* mRNA levels in SH-SY5Y cultured cells under control conditions (●), after induction of differentiation by RA (○) or after RA removal (▲) along the cell culture (in DIV). mRNA was measured by qRT-PCR using total RNA extracted from 0.5×10^6 cells in all cases as described in the Methods section. Levels of PREP protein as determined by Western blot are represented below the curve of *Prep* mRNA expression levels for DIV 1, DIV 8 and DIV 15, respectively, for control cells (C), differentiation-induced cells (RA) and from cells where the RA was removed (-RA).

differentiation was induced by the addition of RA at DIV 1. Under these conditions, the cytoplasmic activity was similar to the activity registered for the control cells up to DIV 5 (fig. 1a). However, when cell growth became arrested at around DIV 5 (fig. 1c), a steady increase in the cytoplasmic PREP activity was observed. Cytoplasmic activity levels reached a plateau from around DIV 10 to 23, but this abruptly began to rise again after DIV 25 when the cells became senescent. It was observed that the nuclear PREP activity declined considerably at DIV 2, i.e. at 24 h after the addition of RA, to levels comparable to those measured in the cytoplasm. Subsequently, nuclear PREP activity remained low until the end of the experiment. On the other hand, upon RA removal at DIV 9, cytoplasmic PREP activity was restored to control levels (fig. 1a). Under these conditions, activity within the nuclei was also restored to control levels (fig. 1). However, after DIV 10, there was a transient increase in nuclear activity which coincided with proliferation recovery (fig. 1c). The activity returned to control levels after DIV 25 upon reaching confluence. The changes in the levels

Fig. 3. Steady state IP₃ levels in SH-SY5Y cells cultivated under proliferative conditions (white), or after induction of differentiation (RA, grey), in the absence (**a**) or presence of 10 μ M JTP-4819 (**b**, stripes). The difference between the levels of IP₃ in different conditions (control vs. RA) was significant in each case (DIV5, $p < 0.005$; DIV7, $p < 0.05$; and DIV11, $p < 0.01$).

of activity correlated with changes in the protein levels of PREP assayed by Western blotting as shown in the insets in figure 1a, b.

We monitored the cell count (Bürker chamber) daily under control conditions, after RA addition (at DIV1), and after subsequent RA removal (at DIV8) as shown in figure 1c. The effect of PREP inhibition (10 μ M JTP-4819, shown in fig. 1c; 10 μ M KYP-2047 or 10 μ M Z-Pro-Prolinal, not shown) was also tested on these curves. Even though these concentrations of inhibitors are sufficient to inhibit PREP activity quantitatively in these cells, we observed no effects on the cell count in control, RA-treated or RA-depleted cultures (fig. 1c). It is important to note that these inhibitors were used at concentrations at which they exert no measurable inhibition of any related proteases (online suppl. material).

Prep Transcript Changes upon Induction of Differentiation by RA

To clarify the origin of the cytoplasmic increase in PREP activity observed after RA-induced differentiation, we measured the levels of transcription of the *Prep* gene. Accordingly, we measured the levels of *Prep* mRNA in control proliferating conditions, under RA-induced differentiation, and also after RA removal (fig. 2). *Prep* mRNA levels did not change during the culture period under control conditions (fig. 2). However, a significant increase of *Prep* mRNA level was observed after the addition of RA; about 60–100% increase versus control lev-

el ($p < 0.0005$) was seen at DIV 5, 9 and 15 (fig. 2). The removal of RA from the media of cultures undergoing differentiation produced a concomitant decrease in the *Prep* mRNA to the control level (fig. 2). This variation was consistent with the changes observed in Western blot experiments (fig. 2).

PREP and the Cell Signalling System in SH-SY5Y Cells

Inositol Triphosphate. It is known that PREP is involved in the modulation of the inositol cycle, an action believed to be mediated by interference with the enzymes that feed phosphoinositol into the cycle or by controlling the gene expression levels of the intermediates as Mippl1 and inositol monophosphatase [9]. We explored the steady-state levels of IP₃ in SH-SY5Y cells after induction of differentiation by RA, and compared with those levels in proliferating control conditions in the absence and presence of PREP inhibitors. Figure 3a shows the cellular levels of IP₃ at DIV 5, DIV7 and DIV11 from cells cultured under proliferating conditions or after induction of differentiation by RA. During proliferation, we observed a bell-shaped change in IP₃ levels with a peak at DIV 7, a time point that corresponds to the onset of cell growth (fig. 1c) and also to a substantial decline in PREP nuclear activity (fig. 1b). Under these conditions, the IP₃ levels decreased again at DIV 11, which coincided with the reach of confluence (fig. 1c) when PREP activity in nuclei or cytoplasm is minimal. Under differentiation conditions, a similar change in the IP₃ levels was observed but

Fig. 4. Phosphorylation ratios (p-ERK/ERK) for ERK1/2 at DIV 5 and DIV 9 in SH-SY5Y cells cultured under control conditions (white), in the presence of JTP-4819 (grey), and in the presence of RA (stripes). * $p < 0.05$.

the overall levels were elevated at all time points, by almost 3-fold. It is interesting to note that in the presence of RA at DIV 7, where the IP_3 levels reached their peak, cell growth had been completely arrested (fig. 1c) and the overall PREP activity was low. At DIV 11, when the IP_3 level was again low, increasing cytoplasmic PREP activity reached a plateau. PREP inhibition by JTP-4819 (10 μ M) from DIV 0 had no effect on the IP_3 levels in these experiments (fig. 3b).

Extracellular Signal-Regulated Kinases. ERK1/2 are kinases of the MAPK family involved in the regulation of many cellular processes, especially in cell proliferation and differentiation [23]. It has also been reported that RA switches on a genetic program involving ERK1/2 activation [24]. We measured ERK1/2 phosphorylation ratios at DIV5 and DIV9 in SH-SY5Y cells under control conditions, after induction of differentiation by RA and after PREP inhibition with 10 μ M JTP-4819 (fig. 4) or Z-Pro-Prolinal (not shown). We observed that RA induced a decrease in the phosphorylation levels of ERK1/2 at early stages (DIV 5) which was significant for ERK2. However, at later stages, DIV 9, we observed that the ratios of both phosphorylated kinases were substantially increased. During the early stages of proliferative growth (DIV 5), PREP inhibition produced a decrease in the phosphorylation levels of the kinases but especially of ERK2 (fig. 4). However, under RA conditions, PREP inhibition did not have any effect on the already reduced levels of phosphorylation of ERK2 and only marginally increased p-ERK1

to control levels. At a later stage during the culture period, at DIV9, JTP-4819 did not have any effect on the levels of p-ERK1/2 in RA-treated cells. Analogous results were obtained using Z-Pro-Prolinal (not shown).

Morphological Changes in Differentiated SH-SY5Y Cells upon PREP Inhibition

The sprouting of neurites and neurite trees are key morphological features characterizing neuronal differentiation. Neurite outgrowth is important for neuronal plasticity as well as for neuronal regeneration. The differentiation into neuron-like cells induced by RA in SH-SY5Y cultures has been used as a model to study the molecular processes involved in neurite formation [25]. We studied the morphological changes of SH-SY5Y cells under differentiation conditions by light microscopy at DIV6, and recorded the number of neurites, their length and the frequency of neuron-neuron connections. In order to study the possible relationship between PREP activity and the morphological changes, the same analysis was undertaken in the presence of 10 μ M JTP-4819 in the culture medium. Figure 5 shows the statistical frequencies of finding a given number of differentiated neurons, a given number of neurites and also a given size of neurites per field in control cultures or on those in the presence of PREP inhibitor. The frequency of finding non-differentiated cells was significantly higher when PREP was inhibited, as well as the frequency of finding only one differentiated neuron. The frequencies of finding two or

Fig. 5. Statistical frequency of finding a number of neuron-like cells (**a**), a number of neurites per cell per field (**b**) and a certain neurite length per field (**c**) in RA-treated cells in the absence (black, blue in the online version) or presence (grey, red in the online version) of JTP-4819. Total counts appear in the insets. * $p < 0.05$. Statistical analysis is described in supplemental material.

more differentiated neurons were generally decreased, but there did not seem to be any relationship between the changes in these frequencies with the presence of JTP-4819 (fig. 5a). However, counts of the total number of differentiated neurons revealed that this number was significantly higher under control conditions than when the cells were incubated with the PREP inhibitor.

The statistical frequency of finding no neurites per field was significantly higher in JTP-4819-treated cells than in control cells. In general, the likelihood of detecting a few neurites (less than 4) was also higher in cultures where PREP was inhibited than under control condition (fig. 5b). It was also observed that the frequency of detecting a given size of neurites was higher in control cultured cells compared to the condition where JTP-4819 was present (fig. 5c).

In the case of neuronal connectivity (fig. 6), similar results to those illustrated in figure 5 were found, i.e. there were more cells showing no connections at all in the presence of JTP-4819 than in control cells, whereas there

were significantly more cases where one connection was observed in the control cells. There was no difference between JTP-4819 and control cells in the frequency of finding 2 or 3 connections between 2 neurons. However, connections of higher order (3–4) between 3 cells were significantly increased in JTP-4819-treated cells (fig. 6).

Gene Expression Profiling of PREP-Inhibited SH-SY5Y Cells

Since we observed that neuronal differentiation was in some way retarded in the presence of PREP inhibitors, it was hypothesized that these changes were, at least in part, due to differences in gene expression after PREP inhibition. An Affymetrix DNA chip was screened with the RNA extracted from PREP-inhibited cells and levels of gene expression were compared to control cells. Out of the 50,000 genes present on the microarray, we found 85 upregulated genes with ratios above 1.3, and 114 genes downregulated with ratios below –1.3 of statistical significance ($p < 0.05$; online suppl. table IS).

Fig. 6. The occurrence of connections between neurons measured at DIV6 under RA-induced differentiation of SH-SY5Y cells under control conditions (black bars, blue in the online version) or in the presence of JTP-4819 (grey bars, red in the online version). Frequencies per plate are depicted and represent the counting of 6 randomly chosen fields per plate in three different plates. Statistical analysis is described in the supplemental material.

Fig. 7. Cellular localization of transcripts found to be affected by PREP inhibition after 24 h incubation with 10 μ M Z-Pro-Prolinal. Clustering was done by using DAVID Bioinformatic Resources (see Materials and Methods).

Among the highly upregulated genes (ratios from 2 to 5), there were several coding for ribosomal proteins or RNA. After those, transcripts from a variety of proteins were found to be moderately increased (ratios from 1.3 to 2), which included mainly mitochondrial respiratory complexes, regulatory proteins and nuclear machinery components, like elongation or transcription factors. With respect to the downregulated genes, the changes were smaller, the maximum ratio observed was not lower than -1.54 .

In order to try to understand if there was any specific functional meaning of the data on the microarrays, we performed a functional analysis to classify all the genes whose expression was modified according to the biological process tag. Table 1 shows the changes in the expression of genes modified after PREP inhibition classified by function. We found three groups with a statistically significant number of genes. The first group consisted of genes that are involved in protein synthesis and all of these genes were upregulated. A second group of upregulated genes after PREP inhibition coded for proteins involved in intracellular transport. It was also notable that the downregulated genes mostly belonged to the class of protein kinase cascades. Some of the changes observed in the gene expression profile were re-evaluated by qRT-PCR, and these results confirmed the microarray data (online suppl. material).

When classifying the genes whose expression was modified under PREP inhibition according to the location of their protein products, a similar distribution was observed between those up- or downregulated (fig. 7). In both cases, a major proportion of the genes could be classified as coding for cytosolic proteins. Moreover, another important set of genes could be grouped as coding for proteins located in intracellular organelles other than the nucleus or mitochondria. Nuclear proteins represented a third important category. Only a few genes which were up- or downregulated by PREP inhibition coded for proteins being exported out of the cells (fig. 7).

Discussion

PREP is able to digest a large variety of neuropeptides in vitro [2]. However, there has been some debate about the physiological role of PREP in the regulation of active neuropeptide levels in vivo mainly because the enzyme has a cytoplasmic localization and active peptides and hormones are located extracellularly [26]. On the other hand, increased PREP expression has been found in aged mice [27, 28] and PREP inhibitors have been reported to have memory-preserving activities (reviewed in Männistö et al. [3]). However, it has not been established whether these effects result from modulation of neuro-

Table 1. Biological processes associated with gene expression effect of PREP inhibition in SH-SY5Y cells

Probe set	Gene	Fold
<i>Upregulated genes</i>		
Translation: p value = $1.8 \cdot 10^{-12}$ Benjamini = $1.5 \cdot 10^{-9}$		
200908_s_at	ribosomal protein, large, P2	3.98
213350_at	ribosomal protein S11	3.91
202648_at	ribosomal protein S19	3.84
214001_x_at	ribosomal protein S10	2.07
213642_at	ribosomal protein L27	2.05
212044_s_at	ribosomal protein L27a	2.02
219138_at	ribosomal protein L14	1.72
213087_s_at	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	1.59
213757_at	eukaryotic translation initiation factor 5A	1.56
214041_x_at	ribosomal protein L37a	1.41
216246_at	ribosomal protein S20	1.41
222487_s_at	ribosomal protein S27-like	1.39
209972_s_at	aminoacyl tRNA synthetase complex-interacting multifunctional protein 2	1.35
234225_at	eukaryotic translation initiation factor 4H	1.34
221997_s_at	mitochondrial ribosomal protein L52	1.34
218808_at	DALR anticodon binding domain containing 3	1.32
Intracellular transport: p value = $1.1 \cdot 10^{-3}$ Benjamini = $2.5 \cdot 10^{-1}$		
M10098_M_at	G protein-coupled receptor 34	2.59
213550_s_at	transmembrane and coiled-coil domains 6	2.26
213757_at	eukaryotic translation initiation factor 5A	1.56
213296_at	RER1 retention in endoplasmic reticulum 1 homolog	1.54
216962_at	RPA interacting protein	1.46
206352_s_at	peroxisomal biogenesis factor 10	1.46
202211_at	ADP-ribosylation factor GTPase activating protein 3	1.32
232210_at	B-cell CLL/lymphoma 2	1.32
1555895_at	dynamin 2	1.31
221915_s_at	similar to RAN binding protein 1	1.3
<i>Downregulated genes</i>		
Protein kinase cascade: p value = $2.4 \cdot 10^{-4}$ Benjamini = $2.1 \cdot 10^{-1}$		
206943_at	transforming growth factor, beta receptor 1	-1.41
210973_s_at	fibroblast growth factor receptor 1	-1.38
227812_at	tumor necrosis factor receptor superfamily, member 19	-1.36
204633_s_at	ribosomal protein S6 kinase	-1.33
244486_at	PTEN-induced putative kinase 1	-1.32
1552264_a_at	mitogen-activated protein kinase 1	-1.32
204325_s_at	neurofibromin 1	1.32
229114_at	GRB2-associated binding protein 1	-1.31
211214_s_at	death-associated protein kinase 1	-1.31
204036_at	lysophosphatidic acid receptor 1	-1.3

peptide levels. There are also studies indicating that PREP inhibitors are neuroprotective [13, 29–32], and that PREP possesses intracellular roles in axonal transport [33] and in the regulation of the inositol cycle [9, 34].

We have previously studied the expression of PREP in cerebellar granule cells in order to clarify its role in differentiation, maturation and ageing [11]. In that study, we found that PREP expression was modulated at all stages of neuron development. PREP activity was located within the nuclei of pre-neurons, but it was located only in the cytoplasm during maturation and ageing. However, PREP inhibitors did not appear to exert any effect on neuron development. We speculated that PREP may have other roles in the processes associated with differentiation, maturation and ageing.

As SH-SY5Y neuroblastoma cells can be grown in conditions where proliferation and differentiation processes can be easily controlled, we employed this model to try to assess the role of PREP in these cellular processes. In this paper we report (1) the effect of PREP inhibitors on the onset of differentiation induced by RA; (2) the localization of intracellular PREP during proliferation and differentiation; (3) the correlation of PREP inhibition with cell signalling intermediates, and (4) the effect of PREP inhibition on gene expression in SH-SY5Y cells. As far as possible, we carefully selected the conditions for PREP inhibition to ensure that the effects were specific for this protein rather than being mediated by secondary targets (online suppl. material, section 6).

In agreement with our previous findings, we observed that PREP is located mainly in the cellular nuclei during the early stages of proliferative growth, but some levels of activity were also found in the cytoplasm (fig. 1). However, at the onset of the logarithmic growth phase, the activity of nuclear PREP decreased sharply, whereas the cytoplasmic activity remained unchanged until the end of the experiment (DIV 20–30). On the other hand, soon after RA addition, PREP activity was observed to be substantially decreased in the nucleus, with a concomitant increase occurring in the cytoplasm, as differentiation and morphological maturation proceeded (fig. 1). Furthermore, when the cells were undergoing differentiation, we detected an increase in activity with ageing, i.e. present several days after neurons had matured. These results are evidence for regulation of the levels and location of PREP activity during growth and in response to induction of differentiation in SH-SY5Y cells.

The increase in cytoplasmic activity observed with maturation, evoked by RA addition, was, at least in part, due to the considerable increase in PREP mRNA levels as

compared to the control condition (fig. 2). Moreover, this increase was reversed by the removal of RA, which restored proliferative growth (fig. 1c). The changes in PREP expression were also validated by Western blotting. These results clearly indicate that the expression of *Prep* is controlled by RA signalling. Variations in *Prep* expression have been studied in rodents from their embryonic stage until the animals are aged [35–38]; however, the mechanisms involved in the control of this phenomenon have not been elucidated. This is the first result indicating that a specific signalling system, such as RA signalling, modulates PREP expression.

We did not observe any effect on cell growth curves after PREP inhibition in any of the conditions tested (fig. 1c). However, a more detailed inspection of the differentiating cells by light microscopy suggested that addition of the PREP inhibitor had retarded differentiation (fig. 5a–c). Under the conditions described here, RA is able to arrest proliferation (fig. 1c) and triggers morphological changes with the formation of neurites, neurite trees and spikes. In the presence of JTP-4819, fewer fully differentiated cells were observed as well as a lower number of neurites (and of shorter length), and a lower frequency of single or double contacts between two neurons. However, there was a significantly higher frequency of multiple contacts (more than 2) between more than two neurons in the presence of the inhibitor (fig. 6). PREP activity seems to be important for the onset of neurite biogenesis and for cell-to-cell communication.

We also studied the changes in the intermediates of the cellular signalling system during cell culture under proliferative and differentiation conditions and the effect of PREP inhibitors. We measured intermediates which have been reported as being modified by PREP activity or by RA. It was found that the levels of IP₃ were higher in the presence of RA as compared to control cells (fig. 3). We also observed that this increase in the IP₃ level was more prominent at DIV7, a time at which cell growth has been totally arrested by RA (fig. 1c). Cell differentiation requires cytoskeleton remodelling in a dynamic system that involves subcellular organization and regional signalling, and inositol metabolism is known to be involved in governing the architectural transitions of the cell via modulation of enzymes that control the amount and phosphorylation levels of inositol intermediates [39], where PREP might be involved. Contrary to expectations, PREP inhibition had no effect on the levels of IP₃ both in control and during differentiation conditions. Our results do not agree with previous claims that PREP inhibition can elevate the levels of IP₃. There is some ex-

perimental evidence that PREP has an inhibitory action on the enzymes which feed IP₃ into the cycle [40]. Furthermore, it has been reported that the levels of IP₃ in cultured cells are inversely related to the levels of PREP expression and that inhibitors further increase the IP₃ pool [41]. The difference with previous studies might be attributed to the different cell culture conditions. Furthermore, there is a recent report indicating that the effects of PREP activity on IP₃ are transient and not direct; they are, instead, being mediated through the control of the expression of inositol cycle related genes, e.g. the inositol monophosphatase gene via multiple inositol polyphosphate phosphatase [9]. Accordingly, the relative levels of IP₃ reported here may be close to the equilibrium of cell signalling, and they might also be modulated by putative compensatory mechanisms. It is important to note that when JTP-4819 was present, PREP was continuously inhibited throughout the culture period (online suppl. fig. S4).

It has been reported that the effects of RA are dependent on crosstalk with the ERK pathway [42]. We explored the changes of phosphorylated levels of ERK1/2 during proliferative or differentiating cell cultures and the effect of PREP inhibition. We found that although PREP inhibition modified the levels of p-ERK1/2 during the early stages of differentiation, these conditions did not have any effect on the RA-induced increase of ERK1/2 during later stages. This indicates that the site of action of PREP in mediating RA modulation of cell signalling might be upstream of ERK1/2 activation at early stages, and could well be linked to the nuclear localization of PREP, but downstream to these kinases during the later phase when PREP is mainly found in the cytoplasm. However, this theory will need to be clarified by further experiments before reaching any conclusion.

It is clear that PREP transcription is controlled in RA-induced signalling, and this is in some way involved in the onset of neurite biogenesis. Indeed, we have found that the expression of a significant number of genes was modified in the presence of the PREP inhibitor. Most of the modified genes encoded for cytoplasmic proteins or proteins contained in organelles other than mitochondria. An important effect was observed also in the expression of nuclear proteins (fig. 7). Furthermore, functional analysis revealed that the upregulated genes were those coding for proteins involved in nuclear machinery and protein translation, but genes coding for proteins involved in intracellular signalling were upregulated too (table 1). On the other hand, downregulated genes belonged to the protein kinase cascade family (table 1).

These results indicate that PREP seems to be involved in intracellular signalling as well as possibly controlling protein synthesis. Further studies are needed to confirm the role of PREP in these processes.

In summary, we have demonstrated that the level of PREP and its cellular location correlate with cellular life stage, and that PREP is genetically regulated through RA cell signalling processes. This is of particular relevance since RA modulates neurogenesis, neuronal survival and synaptic plasticity. In adulthood, it is important in the continued formation, differentiation and maintenance of neuronal phenotypes. Retinoids are known to affect hippocampal long-term depression and potentiation. This might be the mechanism through which PREP inhibition can affect memory and learning and perhaps also combat against neurodegeneration. Furthermore, it was observed that PREP was able to influence neurite biogenesis. Genetic profiling revealed the importance of PREP in intracellular signalling through modulation of intracellular transport, kinase cascades and protein biosynthesis. In turn, these processes have important roles in neuronal differentiation and biogenesis. More studies are ongoing to unravel the molecular links between these processes and PREP.

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