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

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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. Morphological 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.

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, \(\beta\)-endorphin, neurotensin and \(\alpha\)-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 is a negative regulator of inositol (1,4,5)-trisphosphate (IP₃) 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 IP₃ [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 neuronal 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-pyrroldinecarboxamide 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 µg/ml of streptomycin. Cells (0.5 × 10⁵) 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 EC₅₀) 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).

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**Lysis and Cell Fractionation for PREP Activity Assay**

The lysates 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 μl of 50 mM KH₂PO₄, 1.5 mM MgCl₂, 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 μl of cold hypotonic buffer (50 mM KH₂PO₄, 1.5 mM MgCl₂, 250 mM NaCl, 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 μM Suc-Gly-Pro-AMC as substrate in 0.1 M potassium phosphate, pH 7 (final volume 500 μl) 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. 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 x 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 x 10⁶ cells was plated into each well of 6-well plates containing 2 ml of medium. After 4 h, a PREP inhibitor (10 μM of JTP-4819, KYP-2047 or Z-Pro-Prolinal) was added into the corresponding wells. After 24 h, RA (10 μM) 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 x 10⁶) 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 IscriptTM cDNA Synthesis Kit (Biorad) on 10 μl of total RNA (around 60–50 ng). cDNA was amplified by PCR (forward primer 5’-ATG CTG TCC TTT CAG TAC C-3’; reverse primer 5’-GCT GAA CGC ATA ACC TC-G G-3’) using MiniOpticon cycler (BioRad) according to the manufacturer’s instructions. The size of the amplified fragment was 360 pb. The Ct values were compared with a standard curve of Prep cDNA plasmid fragment (BamHI/EcoRI fragment from pBADHumPREP plasmid as described in Venäläinen et al. [15]). The determinations were made in triplicate.

**IP₃ Determination**

The assay is based on competitive binding between an IP₃-[³H] tracer and unlabelled IP₃ from cell lysates or standards to an IP₃ receptor preparation (rat cerebellar membranes) according to an IP₃ radioreceptor assay kit (Perkin-Elmer). Supernatants containing cellular IP₃ were prepared extracting the cells for 20 min with 20% HClO₄ neutralizing (1.5 m KOH, 60 mM HEPES), and centrifuging (2,000 g). Binding was conducted in tubes containing 165 μl of incubation buffer (0.1 M Tris-HCl, pH 9.4 mM EDTA), 10 μl IP₃-[³H] (18 Ci/mmol) 200 μl (250 μg) of an IP₃ receptor preparation and 125 μl of each standard (6.25–100 pmol IP₃), 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 x 10⁶) were lysed...
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×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 × 10⁶) under standard conditions were incubated with 10 μM 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.
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 $\pm 1.3$-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 [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 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
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 μM JTP-4819, shown in fig. 1c; 10 μM KYP-2047 or 10 μM Z-Pro-Proline, 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 levels (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 Mipp1 and inositol monophosphatase [9]. We explored the steady-state levels of IP$_3$ 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 IP$_3$ 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 IP$_3$ 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 IP$_3$ 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 IP$_3$ levels was observed but
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 IP₃ levels reached their peak, cell growth had been completely arrested (fig. 1c) and the overall PREP activity was low. At DIV 11, when the IP₃ level was again low, increasing cytoplasmic PREP activity reached a plateau. PREP inhibition by JTP-4819 (10 \text{M}) from DIV 0 had no effect on the IP₃ levels in these experiments (fig. 3b).

Extra cellular 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 \text{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 \text{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
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).

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**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.
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 |
|------------|----------------------------------------------------------------------|------|
| **Upregulated genes** |                                                                      |      |
| Translation: p value = 1.8 • 10^{-12} Benjamini = 1.5 • 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 • 10^{-3} Benjamini = 2.5 • 10^{-1}

| Probe set  | Gene                                                                 | Fold |
|------------|----------------------------------------------------------------------|------|
| 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 |
| 213926_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  |

**Downregulated genes**

| Probe set  | Gene                                                                 | Fold |
|------------|----------------------------------------------------------------------|------|
| 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|
| 204336_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 and 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...
Inhibition can elevate the levels of IP$_3$. There is some evidence that PREP inhibition had no effect on the levels of IP$_3$ in cultured cells are inversely related to the levels of PREP expression and that inhibitors further increase the IP$_3$ 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 IP$_3$ 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 IP$_3$ 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 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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References

1 Walter R, Shlank H, Glass JD, Schwartz IL, Kerenyi TD: Leucylglycinamide released from oxytocin by human uterine enzyme. Science 1971;173:827–829.
2 García-Horsman JA, Männistö PT, Venäläinen JJ: On the role of prolyl oligopeptidase in health and disease. Neurotpetides 2007;41:1–24.
3 Männistö PT, Venäläinen J, Jalkanen A, García-Horsman JA: Prolyl oligopeptidase: A potential target for the treatment of cognitive disorders. Drug News Perspect 2007;20:293–305.
4 Cavasin MA, Liao TD, Yang XP, Yang JJ, Carretero OA: Decreased endogenous levels of Ac-SDKP promote organ fibrosis. Hypertension 2007;50:130–136.
5 Breen G, Harwood AJ, Gregory K, Sinclair M, Collier D, St Clair D, Williams RS: Two peptidase activities decrease in treated bipolar disorder not schizophrenic patients. Bipolar Disord 2004;6:156–161.
6 Williams RS, Cheng L, Mudge AW, Harwood AJ: A common mechanism of action for three mood-stabilizing drugs. Nature 2002;417:292–295.
7 Harwood AJ, Agam G: Search for a common mechanism of mood stabilizers. Biochem Pharmacol 2003;66:179–189.
8 Harwood AJ: Lithium and bipolar mood disorder: The inositol-depletion hypothesis revisited. Mol Psychiatry 2005;10:117–126.
9 King J, Keim M, Teo R, Weening KE, Kapur M, McQuillan K, Ryves J, Rogers B, Dalton E, Williams RS, Harwood AJ: Genetic control of lithium sensitivity and regulation of inositol biosynthetic genes. PloS One 2010;5:e1151.
10 King JS, Teo R, Ryves J, Reddy IV, Peters O, Orabi B, Hoeller O, Williams RS, Harwood AJ: The mood stabiliser lithium suppresses PI3 signalling in dictyostelium and human cells. Dis Model Mech 2009;2:306–312.
11 Moreno-Baylach MJ, Félix V, Männistö PT, García-Horsman JA: Expression and traffic of cellular prolyl oligopeptidase are regulated during cerebellar granule cell differentiation, maturation, and aging. Neuroscience 2008;156:580–585.
12 Puttonen KA, Lehtonen S, Raasmaja A, Männistö PT: A prolyl oligopeptidase inhibitor, Z-Pro-prolinal, inhibits glyceraldehyde-3-phosphate dehydrogenase translocation and production of reactive oxygen species in CV-1 p cells exposed to 6-hydroxydopamine. Toxicol In Vitro 2006;20:1446–1454.
13 Puttonen KA, Lehtonen S, Lampela P, Männistö PT, Raasmaja A: Different viabilities and toxicity types after 6-OHDA and Ara-C exposure evaluated by four assays in five cell lines. Toxicol In Vitro 2008;22:182–189.
14 Myöhänen TT, Venäläinen JJ, Tupalu E, García-Horsman JA, Miettinen R, Männistö PT: Distribution of immunoreactive prolyl oligopeptidase in human and rat brain. Neurochem Res 2007;32:1365–1374.
15 Venäläinen JJ, García-Horsman JA, Forsberg MM, Jalkanen A, Wallén EA, Jarho EM, Christiaans JA, Gynther J, Männistö PT: Binding kinetics and duration of in vivo action of novel prolyl oligopeptidase inhibitors. Biochem Pharmacol 2006;71:683–692.
16 Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA: DAVID: Database for annotation, visualization, and integrated discovery. Genome Biol 2003;4;F3.
17 Huang da W, Sherman BT, Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat Protoc 2009; 4: 44–57.

18 Al-Shahrour F, Carbonell I, Minguez P, Goetz S, Conesa A, Tàrraga J, Medina I, Alloza E, Montaner D, Dopazo J: Babelomics: Advanced functional profiling of transcriptomics, proteomics and genomics experiments. Nucleic Acids Res 2008; 36(Web Server issue): W341–W346.

19 Al-Shahrour F, Diaz-Uriarte R, Dopazo J: Fatigo: A web tool for finding significant associations of gene ontology terms with groups of genes. Bioinformatics 2004; 20: 578–580.

20 Al-Shahrour F, Minguez P, Tàrraga J, Medina I, Alloza E, Montaner D, Dopazo J: Fatigo+: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. Nucleic Acids Res 2007; 35(Web Server issue): W91–W96.

21 Al-Shahrour F, Minguez P, Vaquerizas JM, Conde I, Dopazo J: Babelomics: A suite of web tools for functional annotation and analysis of groups of genes in high-throughput experiments. Nucleic Acids Res 2005; 33(Web Server issue): W460–W464.

22 McCullagh P, Nelder JA: Generalized Linear Models, ed 2. London, Chapman & Hall, 1989.

23 Kyosseva SV: Mitogen-activated protein kinase signaling. Int Rev Neurobiol 2004; 59: 201–220.

24 Marcucci H, Paoletti L, Jackowski S, Banchio C: Phosphatidylycholine biosynthesis during neuronal differentiation and its role in cell fate determination. J Biol Chem 2010; 285: 25382–25393.

25 Xie HR, Hu LS, Li GY: SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson’s disease. Chin Med J (Engl) 2010; 123: 1086–1092.

26 Brandt I, Scharpé S, Lambeir AM: Suggested functions for prolyl oligopeptidase: a puzzling paradox. Clin Chim Acta 2007; 377: 50–61.

27 Rossner S, Hårtig W, Schliebs R, Brückner G, Brauer K, Perez-Polo JR, Wiley RG, Bigl V: 1921G-saporin immunotoxin-induced loss of cholinergic cells differentially activates microglia in rat basal forebrain nuclei. J Neurosci 1995; 41: 335–346.

28 Jiang CH, Tsien JZ, Schultz PG, Hu Y: The effects of aging on gene expression in the hypothalamus and cortex of mice. Proc Natl Acad Sci USA 2001; 98: 1930–1934.

29 Katsube N, Sunaga K, Aishita H, Chuang DM, Ishitani R: ONO-1603, a potential antidementia drug, delays age-induced apoptosis and suppresses overexpression of glycerolaldehyde-3-phosphate dehydrogenase in cultured central nervous system neurons. J Pharmacol Exp Ther 1999; 286: 6–13.

30 Katsube N, Sunaga K, Chuang DM, Ishitani R: ONO-1603, a potential antidementia drug, shows neuroprotective effects and increases m3-muscarinic receptor mRNA levels in differentiating rat cerebellar granule neurons. Neurosci Lett 1996; 214: 151–154.

31 Shishido Y, Furushiro M, Tanabe S, Nishiyama S, Hashimoto S, Ohno M, Yamamoto T, Watanabe S: ZTTA, a postproline cleaving enzyme inhibitor, improves cerebral ischemia-induced deficits in a three-panel runway task in rats. Pharmacol Biochem Behav 1996; 55: 333–338.

32 Odaka C, Mizuochi T, Shirasawa T, Morain P, Checler F: Murine T cells expressing high activity of prolyl endopeptidase are susceptible to activation-induced cell death. FERS Lett 2002; 512: 163–167.

33 Schulz I, Zeitschel U, Rudolph T, Ruiz-Carrillo D, Rahfeld JU, Gerhartz B, Bigl V, Demuth HU, Rossner S: Subcellular localization suggests novel functions for prolyl endopeptidase in protein secretion. J Neurochem 2005; 94: 970–979.

34 Williams RS, Eames M, Ryves WJ, Viggars J, Harwood AJ: Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate. EMBO J 1999; 18: 2734–2745.

35 Agirregoitia N, Casis L, Gil J, Ruiz F, Irazusta J: Ontogeny of prolyl endopeptidase and pyroglutamyl peptidase I in rat tissues. Regul Pept 2007; 139: 52–58.

36 Agirregoitia N, Gil J, Ruiz F, Irazusta J, Casis L: Effect of aging on rat tissue peptidase activities. J Gerontol A Biol Sci Med Sci 2003; 58:B792–B797.

37 Agirregoitia N, Irazusta A, Ruiz F, Irazusta J, Gil J: Ontogeny of soluble and particulate prolyl endopeptidase activity in several areas of the rat brain and in the pituitary gland. Dev Neurosci 2003; 25: 316–325.

38 Agirregoitia N, Laiz-Carrion R, Varona A, Rio MP, Mancera JM, Irazusta J: Distribution of peptidase activity in teleost and rat tissues. J Comp Physiol [B] 2005; 175: 433–444.

39 Bertagnolo V, Brugnoli F, Marchisio M, Capitanio S: Inositide-modifying enzymes: A cooperative role in regulating nuclear morphology during differentiation of myeloid cells. J Biol Regul Homeost Agents 2004; 18: 381–386.

40 Harwood AJ: Neurodevelopment and mood stabilizers. Curr Mol Med 2003; 3: 472–482.

41 Schulz I, Gerhartz B, Neubauer A, Holloschi A, Heiser U, Hafner M, Demuth HU: Modulation of inositol 1,4,5-trisphosphate concentration by prolyl endopeptidase inhibition. Eur J Biochem 2002; 269: 5813–5820.

42 Lu J, Tan L, Li P, Gao H, Fang B, Ye S, Geng Z, Zheng P, Song H: All-trans retinoic acid promotes neural lineage entry by pluripotent embryonic stem cells via multiple pathways. BMC Cell Biol 2009; 10: 57.