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Expression of Reg/PAP family members during motor nerve regeneration in rat

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Abstract

In this study, we examined the expression of mRNAs for *Regenerating gene* (*Reg*)/*pancreatitis-associated protein* (*PAP*) family members following hypoglossal nerve injury in rats. In addition to four rat family members (*RegI*, *Reg-2*/*PAP I*, *PAP II*, and *PAP III*) that had been identified, we newly cloned and sequenced a type-IV *Reg* gene in rats. Among these five family members, the expression of *Reg-2*/*PAP I* mRNA was predominantly enhanced in injured motor neurons after axotomy. Furthermore, a marked induction of *PAP III* mRNA was observed in the distal part of the injured nerve. A polyclonal antibody was raised against PAP III, and a Western blotting analysis using this antibody confirmed an increased level of PAP III protein in the injured nerve. These results suggest that *Reg* family members would be new mediators among injured neurons and glial cells, and may play pivotal roles during nerve regeneration.

Keywords: Reg family; PAP; HIP; Motor neuron; Schwann cell; Inflammation; Nerve regeneration

Following peripheral nerve injury, glial cells surrounding axotomized neurons secrete a number of growth factors and/or cytokines, including glial cell-line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF), etc., many of which can prevent injured neurons from undergoing cell death as well as promote axonal regeneration [1–3]. To elicit these responses, well-organized interactions between injured neurons and their surrounding glial cells are essential [4,5]. Among these glial cells, Schwann cells may play a major role in peripheral nerve regeneration, and the existence of Schwann cells may be the major reason why nerve regeneration occurs in the peripheral nervous system, but not in the central nervous system [6]. In addition to their role as producers of neurotrophic factors, Schwann cells provide a suitable molecular pavement for regenerating axons by releasing several cell adhesion molecules or materials into the extracellular matrix [4,7]. Therefore, a variety of molecular signals from Schwann cells to injured neurons that promote regeneration have been identified and characterized, whereas the reverse signals from injured neurons to Schwann cells are not fully understood. One molecule that is released from injured neurons towards Schwann cells is Reg-2 [8], which is also known as pancreatitis-associated protein I (PAP I) or Peptide 23 in rats (referred to as PAP I below) [9]. PAP I is a small secretory protein (relative molecular mass, 16 kDa) that belongs to the Reg family and is structurally classified as the

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calcium-dependent type (c-type) lectins [10]. PAP I expression is substantially induced in spinal motor neurons as well as subsets of the dorsal root ganglion (DRG) neurons after sciatic nerve injury [8,11]. In vitro, PAP I has a mitogenic effect on Schwann cells [8], and inhibition of PAP I by application of a neutralizing antibody against PAP I retards peripheral nerve regeneration in vivo [8], strongly suggesting that injury-induced PAP I is a crucial mediator from neurons to Schwann cells during nerve regeneration. To date, the members of this family can be grouped into four subclasses, type-I, -II, -III, and -IV, according to their structural similarities [12]. One type-I gene (*RegI*) and three type-III genes (*PAP I*, *PAP II*, and *PAP III*) have been cloned in rats. Of the family members, only *PAP I* gene expression has been reported following nerve injury [8,11], although the other remaining members have not yet been examined. Therefore, in this study, we examined the expression of *Reg* family members, including the newly identified rat *RegIV*, in response to peripheral nerve injury, and revealed that the expressions were differentially regulated among the family members, not only in injured motor neurons, but also in injured nerve.

Materials and methods

Animals and surgery. Adult male Wistar rats weighing 150–200 g (6–8 weeks of age) were used throughout this study after anesthesia with pentobarbital. They were placed in a supine position and their right hypoglossal nerves were then cut with a pair of scissors just proximal to the bifurcation at the hyoid bone. Experiments were carried out in accordance with the Guideline laid down by Osaka City University Medical School regarding the care and use of animals for experimental procedures.

In situ hybridization. The techniques used were described in detail in our previous manuscript [13]. For mRNA detection in injured hypoglossal motor neurons, rats were decapitated 3 days after surgery. The cDNA fragments comprising the entire coding regions for each rat *Reg* family member (shown in Table 1) were cloned into pBluescript KS (Stratagene) and used as probes. α-^{35}S-labeled cRNA probes (5×10^5 cpm/μl) were hybridized to 18-μm thick sections at 55 °C, subjected to two high stringency washes (in 50% deionized formamide, 2× SSC, and 10% 2-mercaptoethanol) at 65 °C, and then dipped in photographic emulsion (Kodak NTB2). The sections were then exposed for 3–4 weeks at 4 °C, developed in Kodak D-19 developer, and examined using a microscope.

RT-PCR. Total RNA was extracted from each tissue using the acid guanidinium–phenol–chloroform method [14]. In order to isolate rat *RegIV*, the following primers, which amplify the mouse *RegIV* coding sequence, were used for reverse transcription-polymerase chain reaction (RT-PCR): forward primer: 5′-TCGAAAGAGGAAGATGGCTT-3′; reverse primer: 5′-GCTTGATTTTTGCTCTATG-3′. cDNA prepared from rat ileum was used as the PCR template. For mRNA detection in an injured hypoglossal nucleus or injured nerve, operations were performed on 10 rats and these were sacrificed at 12 h, 1, 3, 7, 14 or 28 days after axotomy. Total RNA was also prepared from control and injured hypoglossal nuclei, control nerves, and distal segments of injured nerves (0–15 mm from the site of transection). Aliquots from the RT reaction were used for PCR amplification. The primers used were as follows: *Reg* family: see Table 1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): nucleotides 836–855 and 1149–1168 of NM017008 (GenBank Accession No.); activating transcription factor-3 (ATF-3): nucleotides 165–187 and 688–708 of M63282; p75: nucleotides 1005–1026 and 1326–1347 of NM012610; and P0: nucleotides 82–106 and 660–681 of MN017207. The numbers of amplifying cycles were: 30 for *Reg* family members; 28 for *ATF-3* and *p75*; and 18 for *P0* and *GAPDH*. The reaction conditions consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s. The reaction products were separated electrophoretically in 6–8% polyacrylamide gels and visualized using a Fluoroimager (Amersham Pharmacia Biotech) after staining with CYBR Green II (Molecular Probes) for 15 min. The visualized bands were analyzed quantitatively using NIH Image (National Institute of Health, USA). The intensities of the PCR products of *PAP I*, *PAP III*, *p75*, and *P0* were compared with that of *GAPDH* as an internal control.

Preparation of recombinant PAP III and generation of an anti-PAP III antibody. Recombinant PAP III protein was produced by the methylotrophic yeast *Pichia pastoris* as described previously [15,16]. The rat PAP III-encoding yeast expression plasmid, pPIC9 PAP III, was kindly provided by Dr. R. Graf (University Hospital, Zürich). The linearized plasmid was transformed into *Pichia pastoris* GS115 using a Pichia EasyComp kit (Invitrogen), and the yeast transformants were selected on histidine-deficient RDB agar plates prepared according to the manufacturer's protocol (Invitrogen). Individual colonies on the plates were screened for insert integration by PCR, using the factor primer and the 3′AOX (alcohol oxidase) primer. Positive clones were selected for further analysis to check the level of PAP III production in the culture medium. Colonies were grown at 30 °C in 2 ml glass test-tubes, each containing 1 ml of buffered minimal glycerol (BMG) medium (100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen

Table 1
Probes for the in situ hybridization study and primer pairs for the RT-PCR analysis in detecting rat Reg family members

Rat Reg family member (other names)	Accession No.	PCR primers used in this study	References
Reg I	M18962	Sense 5′-GCATCCTAAGCAGAAGACAGT-3′ Antisense 5′-TTCTTGTCTGGCTCTGTATGAC-3′	Terazono et al. (1988) [19]
PAP I (Reg-2, Peptide 23)	M55149	Sense 5′-AAGATGTTGCATCGCTTGGCC-3′ Antisense 5′-CTAAAGCTGTTTGCTGTCTGG-3′	Iovanna et al. (1991) [58] Kamimura et al. (1992) [59] Katsumata et al. (1995) [27]
PAP II (Reg III)	D23676	Sense 5′-ATCCCAGATCACTGCAAGGC-3′ Antisense 5′-AAGGTCTCTTCTGGCAGGCC-3′	Frigerio et al. (1993) [60] Suzuki et al. (1994) [61]
PAP III	L20869	Sense 5′-GGAAGATGTGCCCACTTCACG-3′ Antisense 5′-GTTGTTGATCTTCCCATTGGG-3′	Frigerio et al. (1993) [62]
RegIV	AB164049	Sense 5′-CTGCTGAGCTGGGTAGCTGGCCC-3′ Antisense 5′-TTTATCCTTGGGGTTCATCTCAG-3′	This paper

base, 0.000004% biotin, and 1% glycerol). After 2 days of cultivation, when the A_{600} was between 2 and 6, the cells were harvested by centrifugation and resuspended in 0.5 ml BMM (BMG containing 0.5% methanol instead of glycerol) for induction of PAP III protein expression. After a further 4 days of cultivation, 10 ml of the supernatant of each clone was subjected to SDS–PAGE analysis, and the resulting gel was stained with SYPRO Ruby (Amersham Pharmacia Biotech) to visualize the PAP III band. The most productive clone was selected and cultured in 1 L start volumes for large scale PAP III preparation. High yield media were collected by centrifugation at 1500*g* for 5 min at 4 °C, and the supernatants were then centrifuged again at 5000*g* for 15 min. The supernatant was diluted 1:3 with water, filtered, and adjusted to pH 3.6 with HCl. The diluted supernatant was applied, at a rate of 5 ml/min, to a cation exchange column (Hi-prep 16/10 SP XL; Amersham Pharmacia Biotech) with a bed volume of 40 ml. The column was washed with 2 volumes of starting buffer (50 mM Mes, 10 mM LiCl, pH 5.3), and the proteins were eluted with a stepwise gradient (0–35%) of elution buffer (50 mM Mes, 2 M LiCl, pH 6.3), generated by an AKTAprime system (Amersham Pharmacia Biotech). The purified protein was subjected to SDS–PAGE analysis along with concentrated culture medium samples derived from 293T cells which were mock-transfected or transfected with a PAP III-expressing pcDNA3 vector (Invitrogen) as described below, followed by SYPRO Ruby protein staining and visualization using a Fluoroimager. A rabbit polyclonal anti-PAP III antiserum was raised against the recombinant PAP III described above and the antiserum obtained was concentrated by ammonium sulfate precipitation. The anti-PAP III antiserum was further purified by chromatography through an affinity column in which the recombinant PAP III was coupled to HiTrap NHS-activated HP (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Western blotting. Each Reg family member was cloned into pcDNA3 and transfected into 293T cells. Thirty-six hours after transfection, the cells were lysed in buffer containing 50 mM Tris–HCl at pH 6.8, 2% SDS, 6% β-mercaptoethanol, and 10% glycerol. Samples of hypoglossal nerves (uninjured site and distal segment of the injured site) 7 days after axotomy were also used. Equal amounts of protein (20 μg) from each sample were subjected to SDS–PAGE and the resulting gels were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The following primary antibodies were used as probes: anti-PAP III antibody (1:1000), mouse anti-p75 monoclonal antibody (1:50; cell supernatant of 192 hybridoma; kindly provided by Dr. C.E. Henderson, Developmental Biology Institute, Marseille), and anti-GAPDH monoclonal antibody (1:10000; Ambion). The membranes were then probed with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Amersham Pharmacia Biotech) as appropriate and visualized using a chemiluminescence system (Amersham Pharmacia Biotech).

Immunocytochemistry. The PAP III coding sequence was cloned to the pcDNA3, expression vector and transfected into COS7 cells using Lipofectamine 2000 (Invitrogen). Thirty-six hours later, immunocytochemistry was performed using the anti-PAP III antibody (1:500). An Alexa 488-conjugated antibody (Molecular Probes) was used as the secondary antibody. Subsequently, Hoechst staining was performed (Hoechst 33258, 2.5 μg/ml; Wako, Tokyo, Japan) to visualize the cell nuclei.

Results

mRNA expressions of Reg family in injured motor neurons

To date, four isoforms of *Reg* family member genes, *RegI*, *PAP I*, *PAP II*, and *PAP III*, have been cloned in rats (Table 1). To examine the expression of the complete set of *Reg* family mRNAs in rats, we attempted to clone the unidentified rat *RegIV* gene. Eventually we obtained and sequenced the rat ortholog of human or mouse *RegIV*/*RELP* (regenerating protein-like protein) [17,18] and designated it as rat *RegIV* (GenBank Accession No.: AB164049 in Table 1). First, we examined the mRNA expressions of these five *Reg* family members in injured motor neurons after rat hypoglossal nerve transection. In situ hybridization histochemistry using probes for the five family members revealed that the mRNA expression of *PAP I* was markedly induced in injured motor neurons 3 days after axotomy (Fig. 1A), which is consistent with previous data that *PAP I* expression is induced in injured spinal motor neurons after sciatic nerve axotomy [8]. In addition, weak *PAP II* mRNA expression was observed in the injured motor neurons (Fig. 1A). No expression of the other three *Reg* family members, *RegI*, *PAP III*, and *RegIV*, was detected in either the normal or nerve-injured sides of hypoglossal nuclei. RT-PCR analysis using cDNAs prepared from injured hypoglossal nuclei was performed during the time-course after axotomy (Fig. 1B). Consistent with the results for the in situ hybridization analysis, the RT-PCR analysis confirmed that there was a marked increase in *PAP I* mRNA and a faint increase in *PAP II* mRNA. A slight increase in the amplified band for the *PAP I* gene was detected initially 12 h after nerve transection and the intensity of the band markedly increased to a peak level at 3 days after the nerve injury. Thereafter, the expressions were maintained for the following 2 weeks. This analysis also revealed that there were no detectable mRNA expressions of the other three *Reg* family members in either normal or injured hypoglossal nuclei.

mRNA expressions of Reg family in injured nerve

We next examined changes in the *Reg* family member mRNA expressions in injured hypoglossal nerve after axotomy. In the intact hypoglossal nerve, no expression of the mRNAs for *RegI* and *PAP II* was detected by RT-PCR, whereas faint expressions of *PAP I* and *PAP III* mRNAs were observed (Fig. 2A). After nerve injury, *PAP III* mRNA was dramatically induced within 12 h in the injured nerve to reach a peak level at 1 day after the lesion, and the induced expression was maintained for the following 4 weeks (Fig. 2A). In addition, a slight increase in *PAP I* mRNA expression was also detected in the injured nerve from 12 h to 1 day after axotomy. On the other hand, such alterations in *RegI*, *PAP II*, and *RegIV* mRNA expressions were not observed in the injured nerves. The rapid increase in *PAP III* mRNA expression was significantly faster than the alterations in *p75* and *P0* expressions (Figs. 2A and B).

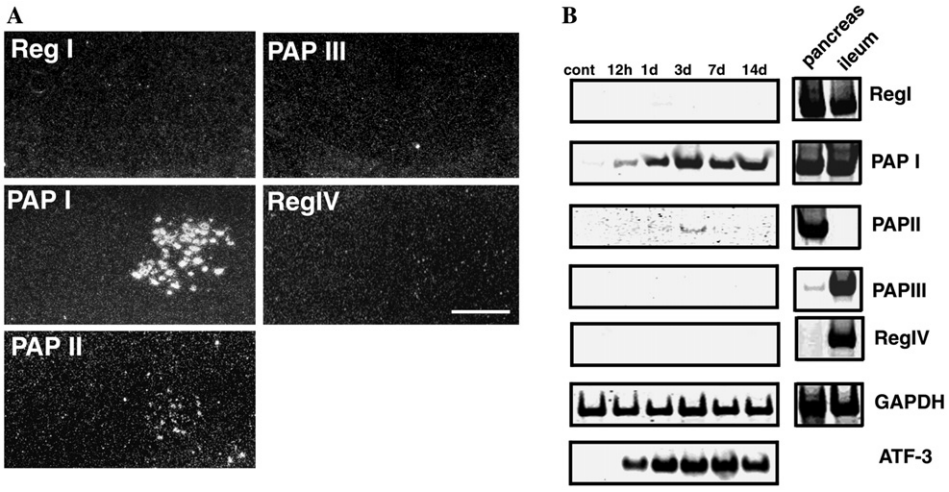

Fig. 1. mRNA expressions of *Reg* family members in hypoglossal motor neurons. (A) Emulsion autoradiography of tissue sections (3 days after nerve injury) hybridized with probes for the *Reg* family members. In each panel, the right side is the injured side, and the left side is the uninjured one. When the *PAP I* or *PAP II* probes are used, an up-regulated hybridization signal (accumulation of silver grains) is observed for the injured motor neuron. Bar = 0.5 mm. (B) During the course of nerve regeneration (control (cont), 12 h, 1, 3, 7, and 14 days after axotomy), mRNA expressions for the *Reg* family members were determined in the nerve-injured hypoglossal nuclei by RT-PCR. Other RT-PCRs using cDNA prepared from rat pancreas or ileum, which abundantly express *Reg* family members, were performed to confirm that the primer pairs used in this study were suitable for PCR amplification. *GAPDH* mRNA expression was used as an internal control, and *ATF-3* mRNA expression was used as a positive control [63].

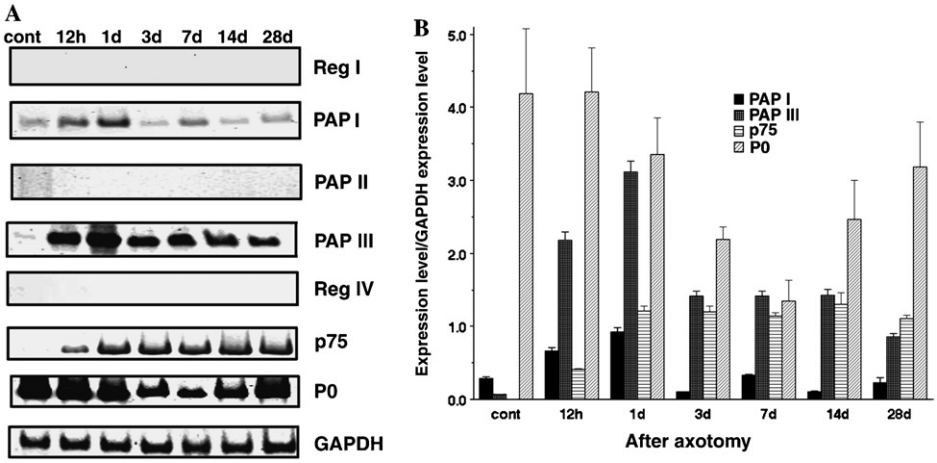

Fig. 2. (A) Expression of mRNAs for *Reg* family members in the distal part of injured hypoglossal nerves during the course of nerve regeneration examined by RT-PCR. *p75* and *P0* mRNA expressions, which are up-regulated and down-regulated, respectively, in injured nerves, were used as positive controls. The expression of GAPDH mRNA was used as an internal control. (B) Graph showing the temporal mRNA expression patterns for *PAP I*, *PAP III*, *p75*, and *P0*. At least three individual RT-PCR experiments were performed, and each time point shows the average intensity of the amplified band as a ratio to that of GAPDH, and the standard deviation.

Preparation of an antibody against PAP III and the PAP III protein induction in injured nerve

Since we had identified *PAP III* mRNA as a novel lesion-induced mRNA in injured peripheral nerves, the generation of a rabbit antibody against rat PAP III was attempted. For use as a PAP III immunogen, recombinant rat PAP III was prepared using the methylotrophic yeast *Pichia pastoris*, which has been shown to be able to effectively produce Reg proteins in its culture supernatant [15,16]. After one step of cation exchange column chromatography, purified recombinant PAP III was obtained and visualized by SDS–PAGE as a single band that was the predicted size of 16 kDa, and exactly the same size as the secreted PAP III in the culture medium from *PAP III*-transfected 293T cells (Fig. 3A). Using this recombinant protein as an immunogen, we successfully obtained a rabbit antibody against PAP III. In order to examine the specificity of the generated antibody among the family members, we first performed Western blotting using cell lysates prepared from 293T cells transfected with each *Reg* family member. This

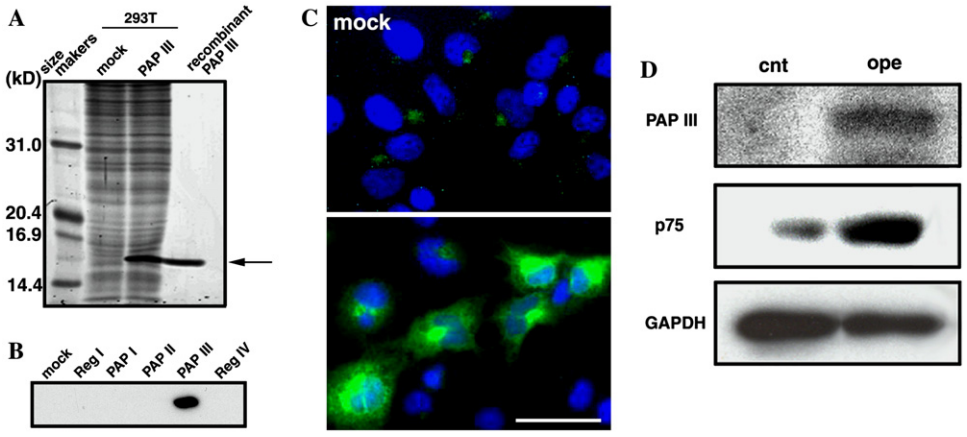

Fig. 3. Generation of an anti-PAP III antibody and induction of PAP III protein expression in injured nerves. (A) SDS–PAGE analysis and subsequent protein staining demonstrating that the purified recombinant PAP III is exactly the same size as the secreted PAP III protein (arrow) in the supernatant of 293T cells transfected with a PAP III-expressing plasmid. (B) Plasmids expressing each rat Reg family member were separately transfected into 293T cells, and Western blotting analysis of their cell lysates revealed that the generated antibody specifically recognizes PAP III. (C) Immunocytochemistry using mock-transfected COS7 cells (upper) or COS7 cells transfected with a PAP III-expressing plasmid (lower). The anti-PAP III antibody specifically recognizes PAP III (green), while Hoechst staining (blue) reveals the nuclei of the cells. Bar = 40 μm. (D) Western blotting analysis using the anti-PAP III antibody reveals the induction of PAP III protein expression in the distal segment of the injured hypoglossal nerve at 7 days after axotomy. The same membrane was re-probed with an anti-p75 antibody. Equal amounts of protein loading are confirmed by the Western blot using the anti-GAPDH antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

analysis revealed that only PAP III was strongly recognized by the antibody, while the other members were not (Fig. 3B). In addition, to examine the specificity of the immunocytochemical detection of PAP III by the generated antibody, immunocytochemical staining was performed on COS7 cells that were transfected with an empty vector or a PAP III expression vector. Even in the mock-transfected cells, faint immunopositive reactions were detected in the perinuclear region of the cytoplasm, possibly corresponding to the Golgi apparatus (Fig. 3C, upper). On the other hand, PAP III-transfected cells were strongly stained by the antibody (Fig. 3C, lower). Collectively, these results indicate that the generated antibody is useful for the specific detection of PAP III. Finally, we performed Western blotting analysis to detect PAP III protein induction in the distal portion of injured nerve using the generated antibody. Similar to its transcriptional regulation, the PAP III protein expression was also up-regulated in the injured hypoglossal nerve after axotomy (Fig. 3D).

Discussion

In this study, we examined the alterations in rat *Reg* family member expressions after peripheral motor nerve injury, and revealed the cell type-specific induction of *Reg* family members in response to nerve injury. PAP I expression is predominantly induced in injured motor neurons, whereas PAP III expression is preferentially enhanced in injured nerve. These specific expressions of PAP I and PAP III in injured motor neurons and nerves, respectively, suggest that these Reg family proteins are possible signal mediators from injured motor neurons and Schwann cells during the process of nerve regeneration.

The initially identified *Reg* gene (now called *RegI*) was originally isolated from a regenerating islet-derived cDNA library [19]. Subsequently Reg family members have been identified by other laboratories using different approaches, and shown to have functional diversity, especially in the cells of the digestive organs [9,20]. One of the family members, RegI protein, has been identified as a predominant pancreatic β-cell replication factor and also as an islet regeneration inducer in vivo [21,22]. In addition, much attention has recently been focused on their significant expressions in human cancer cells residing in the liver [23], colon [24] or stomach [25], although some normal tissues such as the small intestine or pituitary express some of the *Reg* family members [26,27]. Moreover, they are dramatically induced as acute phase reactants after tissue injuries, such as pancreatitis [26], colonic inflammation [28] or gastric ulcers [29], and these functions may be associated with cell protection from inflammatory responses [30,31] or cell proliferation during the wound healing stages [21,32,33]. In neural injury, as revealed in the present study and previous reports [8,11], *Reg* family members are significantly induced in both neurons and nerves after peripheral nerve transection. Of the rat *Reg* family members, three *PAP* family members (*PAP I*, *PAP II*, and *PAP III*), which are structurally similar and constitute a subfamily of type III *Reg*, are all up-regulated, although the predominance of their expressions varies among cell types.

As shown in this study, injured hypoglossal motor neurons markedly express *PAP I*. The *PAP I* mRNA up-regulation was detected within 12 h and peaked 3 days after the nerve injury. This relatively quicker expression profile supports the idea that PAP I released from injured neurons stimulates the growth of Schwann cells [8]. Indeed, we observed that ERK activation, which would act as a critical intracellular growth signal in various types of cells, is induced in the injured hypoglossal nerve within 24 h after axotomy [34], and subsequently the injured Schwann cells begin to proliferate 1–2 days later. Like nerve-injured mature neurons, subsets of normal developing motor or sensory neurons specifically and abundantly express mRNA for *PAP I* [8,35] or its mouse homolog *RegIIIβ* [36], and these expressions seem to function as a trophic factor for neurons by being released in an autocrine and/or paracrine manner during different developmental periods. Furthermore, it has been proved that PAP I possesses vital survival activity for cultured motor neurons [35]. Previously, it has been demonstrated that the induction of *PAP I* mRNA after nerve injury is mainly regulated by the signal transducer and activator of transcription (STAT)-3 transcription factor, which has been separately revealed to function in protection against axotomy-induced motor neuron death. In fact, a functional IL-6-responsive element, to which STAT-3 can bind, exists in the promoter region of the *PAP I* gene [37,38]. STAT-3 deficiency accelerated axotomy-induced motor neuron death, and a remarkable reduction in *PAP I* mRNA was observed in axotomized motor neurons [39]. It is well known that CNTF-related cytokines, including CNTF and LIF, are released from injured Schwann cells [40,41], and have a potent motor neuron survival activity [42,43] after nerve injury. These members can enhance STAT-3 transcriptional activity via gp130 receptors in various types of cells [44]. In fact, a previous report demonstrated that PAP I, which is induced by CNTF, is a signaling intermediate in the CNTF-elicited survival pathway in cultured motor neurons, since PAP I inhibition using an antisense strategy induced marked motor neuron death even in the presence of CNTF [35]. Therefore, PAP I induction by the CNTF family would be a key mechanism for promoting the survival of motor neurons in culture. Furthermore, in vivo, it is likely that the CNTF family is required for *PAP I* mRNA expression, since there is no PAP I expression in developing motor and sensory neurons deficient in the gene for the LIF receptor β chain which is a component of CNTF and/or LIF receptor complexes [8,35]. Further experiments examining whether direct application of PAP I protein or overexpression of the *PAP I* gene in injured motor neurons are capable of rescuing axotomy-induced cell death are needed to clarify a more precise role for PAP I in vivo. Regarding *PAP I* expression regulation in this study, the enriched CNTF in mature Schwann cells may be released by passive lysis of the injured nerve immediately after injury [40,41], and at this immediate early phase other family members have not yet been synthesized in response to the nerve injury. Thus, CNTF would be recognized as an early lesion-induced retrograde signal from Schwann cells to axotomized motor neurons [45]. We observed that the higher level of *PAP I* mRNA expression was maintained for at least 2 weeks after axotomy. Since the *CNTF* mRNA level reduces quickly after nerve injury [40,46], subsequently induced and released LIF from Schwann cells [47,48], for instance, might maintain the long-lasting PAP I expression during nerve regeneration.

In this study, for the first time, we found that PAP III is prominently induced in injured nerve. Although the type of cells that express PAP III in injured nerve should be precisely determined in further experiments, Schwann cells seem to be the major source of PAP III because of its rapid and significant phenotypic responses to nerve injury [4]. *PAP III* mRNA is induced in the pancreas by acute pancreatitis [49] together with *PAP I* and *PAP II* mRNAs, suggesting that the expressions of these three PAPs may be regulated in a similar manner. However, PAP family members are differentially regulated in a cell type-specific manner in the nervous system after nerve injury. The reason why such selective regulation of PAP family members occurs in the nervous system remains unclear. The immediate increase in *PAP III* expression within 12 h following injury suggests that PAP III is an acute reactant derived from injured nerves as well as PAP I from injured motor neurons. Since the promoter region of the *PAP III* gene has one IL-6-responsive element [49] that is also located in the *PAP I* gene promoter, and since STAT-3 is immediately activated following axotomy at the site of injured sciatic nerves [50], involvement of the JAK-STAT pathway in the transcriptional regulation of *PAP III* is likely and should be examined in further studies.

Previous reports demonstrated that several secreted molecules are rapidly induced in injured Schwann cells [4], and some of these appeared to function on infiltrating macrophages that scavenge the debris of degenerated axons and myelin in injured nerves, leading to the rapid Wallerian degeneration required for effective nerve regeneration [51]. For instance, pro-inflammatory cytokines, including tumor necrosis factor-α (TNFα) and interleukin-1α (IL-1α) that are the most rapidly up-regulated molecules (within 5 h after nerve injury) and immediately released from injured Schwann cells, are likely to be involved in stimulating macrophage phagocytosis for the removal of debris [52]. In addition, other lesion factors such as monocyte chemoattractant protein-1 (MCP-1), IL-6 or LIF, which are immediately induced in injured Schwann cells, would act as direct or indirect chemotactic factors for macrophages after nerve injury [53,54]. Indeed, a previous report showed that

PAP I has the ability to enhance the motility of human melanocytes and melanoma cells [55]. Therefore, PAP III may be another acute reactant in injured nerves, although it remains unclear whether PAP III has the same role as PAP I which acts as a Schwann cell mitogen or motor neuronal survival factor [8,35].

There are other possible roles for the injury-induced PAP I and PAP III. Vasseur et al. [56] recently revealed a novel function of PAP I. Interestingly, PAP I could prevent the release of pro-inflammatory cytokines such as TNFα and IL-6 from activated macrophages in vitro [56], and administration of an anti-PAP I antibody to rats increased pancreatic inflammation during pancreatitis in vivo [56], suggesting that PAP I acts as an anti-inflammatory factor during acute pancreatitis. Since the nerve injury-induced pro-inflammatory cytokines that are released from both Schwann cells and infiltrating macrophages may have cytotoxic effects on the regenerating neurons and/or Schwann cells, it may be preferable that they are suppressed immediately after finishing their role related to rapid Wallerian degeneration as mentioned above. Since the PAP I produced in the injured neuron was transported to the injured axon tip [8,11], and may be released from the regenerating axons, it may have the ability to suppress such inflammatory responses elicited by nerve injury. In addition, it is also possible that injury-induced PAP I has a similar anti-inflammatory activity against microglial cells surrounding injured motor neurons, since these cells are possibly supportive for injured motor neurons without expressing cytotoxic cytokines in spite of their presentation of activated status markers after axotomy [5,57]. Although it remains to be elucidated whether the PAP III produced in injured nerves acts as a similar anti-inflammatory factor to PAP I, injury-induced Reg proteins seem to play a crucial role in suppressing such axotomy-induced inflammatory responses in both the axotomized motor nucleus and the site of injured nerves.

In conclusion, we have shown that the *Reg*/*PAP* family members PAP I and PAP III are induced in injured motor neurons and nerves in response to axotomy. Since the induction and release of PAP I and PAP III appeared to readily respond to nerve injury, PAP I and PAP III may function as mediators of the injury signal among the neuron and glial cells, and possibly macrophages. The proper expression of these molecules in the proper cells may be crucial for the promotion of nerve regeneration.

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References

[1] R.M. Lindsay, Neuron saving schemes, Nature 373 (1995) 289–290.
[2] G. Terenghi, Peripheral nerve regeneration and neurotrophic factors, J. Anat. 194 (Pt. 1) (1999) 1–14.
[3] J.G. Boyd, T. Gordon, Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury, Mol. Neurobiol. 27 (2003) 277–324.
[4] S.S. Scherer, J.L. Salzer, Axon-Schwann cell interactions during peripheral nerve regeneration, in: K.R. Jessen, W.D. Richardson (Eds.), Glial Cell Development, Oxford University Press, London, 2001, pp. 299–317.
[5] L.B. Moran, M.B. Graeber, The facial nerve axotomy model, Brain Res. Brain Res. Rev. 44 (2004) 154–178.
[6] R.Y. Cajal, Degeneration and Regeneration of the Nervous System, Oxford University Press, London, 1928.
[7] S.Y. Fu, T. Gordon, The cellular and molecular basis of peripheral nerve regeneration, Mol. Neurobiol. 14 (1997) 67–116.
[8] F.J. Livesey, J.A. O'Brien, M. Li, A.G. Smith, L.J. Murphy, S.P. Hunt, A Schwann cell mitogen accompanying regeneration of motor neurons, Nature 390 (1997) 614–618.
[9] H. Okamoto, The Reg gene family and Reg proteins: with special attention to the regeneration of pancreatic beta-cells, J. Hepatobiliary Pancreat. Surg. 6 (1999) 254–262.
[10] K. Drickamer, Increasing diversity of animal lectin structures, Curr. Opin. Struct. Biol. 5 (1995) 612–616.
[11] S. Averill, D.R. Davis, P.J. Shortland, J.V. Priestley, S.P. Hunt, Dynamic pattern of reg-2 expression in rat sensory neurons after peripheral nerve injury, J. Neurosci. 22 (2002) 7493–7501.
[12] H. Okamoto, S. Takasawa, Recent advances in the Okamoto model: the CD38-cyclic ADP-ribose signal system and the regenerating gene protein (Reg)-Reg receptor system in beta-cells, Diabetes 51 (Suppl. 3) (2002) S462–S473.
[13] M. Honma, K. Namikawa, K. Mansur, T. Iwata, N. Mori, H. Iizuka, H. Kiyama, Developmental alteration of nerve injury induced glial cell line-derived neurotrophic factor (GDNF) receptor expression is crucial for the determination of injured motoneuron fate, J. Neurochem. 82 (2002) 961–975.
[14] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[15] S. Kobayashi, T. Akiyama, K. Nata, M. Abe, M. Tajima, N.J. Shervani, M. Unno, S. Matsuno, H. Sasaki, S. Takasawa, H. Okamoto, Identification of a receptor for reg (regenerating gene) protein, a pancreatic beta-cell regeneration factor, J. Biol. Chem. 275 (2000) 10723–10726.
[16] R. Graf, M. Schiesser, G.A. Scheele, K. Marquardt, T.W. Frick, R.W. Ammann, D. Bimmler, A family of 16-kDa pancreatic secretory stress proteins form highly organized fibrillar structures upon tryptic activation, J. Biol. Chem. 276 (2001) 21028–21038.
[17] J.C. Hartupee, H. Zhang, M.F. Bonaldo, M.B. Soares, B.K. Dieckgraefe, Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV, Biochim. Biophys. Acta 1518 (2001) 287–293.
[18] M. Kämäräinen, K. Heiskala, S. Knuutila, M. Heiskala, O. Winqvist, L.C. Andersson, RELP, a novel human REG-like

protein with up-regulated expression in inflammatory and metaplastic gastrointestinal mucosa, Am. J. Pathol. 163 (2003) 11–20.
[19] K. Terazono, H. Yamamoto, S. Takasawa, K. Shiga, Y. Yonemura, Y. Tochino, H. Okamoto, A novel gene activated in regenerating islets, J. Biol. Chem. 263 (1988) 2111–2114.
[20] Y.W. Zhang, L.S. Ding, M.D. Lai, Reg gene family and human diseases, World J. Gastroenterol. 9 (2003) 2635–2641.
[21] T. Watanabe, Y. Yonemura, H. Yonekura, Y. Suzuki, H. Miyashita, K. Sugiyama, S. Moriizumi, M. Unno, O. Tanaka, H. Kondo, et al., Pancreatic beta-cell replication and amelioration of surgical diabetes by Reg protein, Proc. Natl. Acad. Sci. USA 91 (1994) 3589–3592.
[22] M. Unno, K. Nata, N. Noguchi, Y. Narushima, T. Akiyama, T. Ikeda, K. Nakagawa, S. Takasawa, H. Okamoto, Production and characterization of Reg knockout mice: reduced proliferation of pancreatic beta-cells in Reg knockout mice, Diabetes 51 (Suppl. 3) (2002) S478–S483.
[23] C. Lasserre, L. Christa, M.T. Simon, P. Vernier, C. Brechot, A novel gene (HIP) activated in human primary liver cancer, Cancer Res. 52 (1992) 5089–5095.
[24] H. Rechreche, G. Montalto, G.V. Mallo, S. Vasseur, L. Marasa, P. Soubeyran, J.C. Dagorn, J.L. Iovanna, pap, reg Ialpha and reg Ibeta mRNAs are concomitantly up-regulated during human colorectal carcinogenesis, Int. J. Cancer 81 (1999) 688–694.
[25] Y. Kadowaki, S. Ishihara, Y. Miyaoka, M.A. Rumi, H. Sato, H. Kazumori, K. Adachi, S. Takasawa, H. Okamoto, T. Chiba, Y. Kinoshita, Reg protein is overexpressed in gastric cancer cells, where it activates a signal transduction pathway that converges on ERK1/2 to stimulate growth, FEBS Lett. 530 (2002) 59–64.
[26] J.L. Iovanna, V. Keim, A. Bosshard, B. Orelle, J.M. Frigerio, N. Dusetti, J.C. Dagorn, PAP, a pancreatic secretory protein induced during acute pancreatitis, is expressed in rat intestine, Am. J. Physiol. 265 (1993) G611–G618.
[27] N. Katsumata, C. Chakraborty, Y. Myal, I.C. Schroedter, L.J. Murphy, R.P. Shiu, H.G. Friesen, Molecular cloning and expression of peptide 23, a growth hormone-releasing hormone-inducible pituitary protein, Endocrinology 136 (1995) 1332–1339.
[28] H. Ogawa, K. Fukushima, H. Naito, Y. Funayama, M. Unno, K. Takahashi, T. Kitayama, S. Matsuno, H. Ohtani, S. Takasawa, H. Okamoto, I. Sasaki, Increased expression of HIP/PAP and regenerating gene III in human inflammatory bowel disease and a murine bacterial reconstitution model, Inflamm. Bowel Dis. 9 (2003) 162–170.
[29] M. Asahara, S. Mushiake, S. Shimada, H. Fukui, Y. Kinoshita, C. Kawanami, T. Watanabe, S. Tanaka, A. Ichikawa, Y. Uchiyama, Y. Narushima, S. Takasawa, H. Okamoto, M. Tohyama, T. Chiba, Reg gene expression is increased in rat gastric enterochromaffin-like cells following water immersion stress, Gastroenterology 111 (1996) 45–55.
[30] E.M. Ortiz, N.J. Dusetti, S. Vasseur, D. Malka, H. Bodeker, J.C. Dagorn, J.L. Iovanna, The pancreatitis-associated protein is induced by free radicals in AR4-2J cells and confers cell resistance to apoptosis, Gastroenterology 114 (1998) 808–816.
[31] D. Malka, S. Vasseur, H. Bodeker, E.M. Ortiz, N.J. Dusetti, P. Verrando, J.C. Dagorn, J.L. Iovanna, Tumor necrosis factor alpha triggers antiapoptotic mechanisms in rat pancreatic cells through pancreatitis-associated protein I activation, Gastroenterology 119 (2000) 816–828.
[32] M.T. Simon, A. Pauloin, G. Normand, H.T. Lieu, H. Mouly, G. Pivert, F. Carnot, J.G. Tralhao, C. Brechot, L. Christa, HIP/PAP stimulates liver regeneration after partial hepatectomy and combines mitogenic and anti-apoptotic functions through the PKA signaling pathway, FASEB J. 17 (2003) 1441–1450.
[33] Y. Miyaoka, Y. Kadowaki, S. Ishihara, T. Ose, H. Fukuhara, H. Kazumori, S. Takasawa, H. Okamoto, T. Chiba, Y. Kinoshita, Transgenic overexpression of Reg protein caused gastric cell proliferation and differentiation along parietal cell and chief cell lineages, Oncogene 23 (2004) 3572–3579.
[34] K. Abe, K. Namikawa, M. Honma, T. Iwata, I. Matsuoka, K. Watabe, H. Kiyama, Inhibition of Ras extracellular-signal-regulated kinase (ERK) mediated signaling promotes ciliary neurotrophic factor (CNTF) expression in Schwann cells, J. Neurochem. 77 (2001) 700–703.
[35] H. Nishimune, S. Vasseur, S. Wiese, M.C. Birling, B. Holtmann, M. Sendtner, J.L. Iovanna, C.E. Henderson, Reg-2 is a motoneuron neurotrophic factor and a signalling intermediate in the CNTF survival pathway, Nat. Cell Biol. 2 (2000) 906–914.
[36] C. Lasserre, C. Colnot, C. Brechot, F. Poirier, HIP/PAP gene, encoding a C-type lectin overexpressed in primary liver cancer, is expressed in nervous system as well as in intestine and pancreas of the postimplantation mouse embryo, Am. J. Pathol. 154 (1999) 1601–1610.
[37] N.J. Dusetti, E.M. Ortiz, G.V. Mallo, J.C. Dagorn, J.L. Iovanna, Pancreatitis-associated protein I (PAP I), an acute phase protein induced by cytokines. Identification of two functional interleukin-6 response elements in the rat PAP I promoter region, J. Biol. Chem. 270 (1995) 22417–22421.
[38] D. Broekaert, S. Eyckerman, D. Lavens, A. Verhee, W. Waelput, J. Vandekerckhove, J. Tavernier, Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals, Eur. Cytokine Netw. 13 (2002) 78–85.
[39] U. Schweizer, J. Gunnersen, C. Karch, S. Wiese, B. Holtmann, K. Takeda, S. Akira, M. Sendtner, Conditional gene ablation of Stat3 reveals differential signaling requirements for survival of motoneurons during development and after nerve injury in the adult, J. Cell Biol. 156 (2002) 287–297.
[40] M. Sendtner, K.A. Stockli, H. Thoenen, Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration, J. Cell Biol. 118 (1992) 139–148.
[41] R. Curtis, K.M. Adryan, Y. Zhu, P.J. Harkness, R.M. Lindsay, P.S. DiStefano, Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury, Nature 365 (1993) 253–255.
[42] M. Sendtner, G.W. Kreutzberg, H. Thoenen, Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy, Nature 345 (1990) 440–441.
[43] S.S. Cheema, L.J. Richards, M. Murphy, P.F. Bartlett, Leukaemia inhibitory factor rescues motoneurones from axotomy-induced cell death, Neuroreport 5 (1994) 989–992.
[44] P.C. Heinrich, I. Behrmann, S. Haan, H.M. Hermanns, G. Muller-Newen, F. Schaper, Principles of interleukin (IL)-6-type cytokine signalling and its regulation, Biochem. J. 374 (2003) 1–20.
[45] M. Kirsch, U. Terheggen, H.D. Hofmann, Ciliary neurotrophic factor is an early lesion-induced retrograde signal for axotomized facial motoneurons, Mol. Cell. Neurosci. 24 (2003) 130–138.
[46] B. Friedman, S.S. Scherer, J.S. Rudge, M. Helgren, D. Morrisey, J. McClain, D.Y. Wang, S.J. Wiegand, M.E. Furth, R.M. Lindsay, et al., Regulation of ciliary neurotrophic factor expression in myelin-related Schwann cells in vivo, Neuron 9 (1992) 295–305.
[47] L.R. Banner, P.H. Patterson, Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia, Proc. Natl. Acad. Sci. USA 91 (1994) 7109–7113.
[48] R. Curtis, S.S. Scherer, R. Somogyi, K.M. Adryan, N.Y. Ip, Y. Zhu, R.M. Lindsay, P.S. DiStefano, Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve, Neuron 12 (1994) 191–204.
[49] N.J. Dusetti, J.M. Frigerio, C. Szpirer, J.C. Dagorn, J.L. Iovanna, Cloning, expression and chromosomal localization of the rat

pancreatitis-associated protein III gene, Biochem. J. 307 (Pt. 1) (1995) 9–16.
[50] J.Y. Sheu, D.J. Kulhanek, F.P. Eckenstein, Differential patterns of ERK and STAT3 phosphorylation after sciatic nerve transection in the rat, Exp. Neurol. 166 (2000) 392–402.
[51] P.M. Hughes, V.H. Perry, The role of macrophages in degeneration and regeneration in the peripheral nervous system, in: N.R. Saunders, K.M. Dziegielewska (Eds.), Degeneration and Regeneration in the Nervous System, Harwood Academic Publishers, Chichester, 2000, pp. 263–283.
[52] S. Shamash, F. Reichert, S. Rotshenker, The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta, J. Neurosci. 22 (2002) 3052–3060.
[53] S. Sugiura, R. Lahav, J. Han, S.Y. Kou, L.R. Banner, F. de Pablo, P.H. Patterson, Leukaemia inhibitory factor is required for normal inflammatory responses to injury in the peripheral and central nervous systems in vivo and is chemotactic for macrophages in vitro, Eur. J. Neurosci. 12 (2000) 457–466.
[54] G.K. Tofaris, P.H. Patterson, K.R. Jessen, R. Mirsky, Denervated Schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF, J. Neurosci. 22 (2002) 6696–6703.
[55] C. Valery, S. Vasseur, F. Sabatier, J.L. Iovanna, J.C. Dagorn, J.J. Grob, P. Verrando, Pancreatitis associated protein I (PAP-I) alters adhesion and motility of human melanocytes and melanoma cells, J. Invest. Dermatol. 116 (2001) 426–433.
[56] S. Vasseur, E. Folch-Puy, V. Hlouschek, S. Garcia, F. Fiedler, M.M. Lerch, J.C. Dagorn, D. Closa, J.L. Iovanna, p8 improves pancreatic response to acute pancreatitis by enhancing the expression of the anti-inflammatory protein pancreatitis-associated protein I, J. Biol. Chem. 279 (2004) 7199–7207.
[57] G.W. Kreutzberg, M.B. Graeber, W.J. Streit, Neuron-glial relationship during regeneration of motorneurons, Metab. Brain Dis. 4 (1989) 81–85.
[58] J.L. Iovanna, B. Orelle, V. Keim, J.C. Dagorn, Messenger RNA sequence and expression of rat pancreatitis-associated protein, a lectin-related protein overexpressed during acute experimental pancreatitis, J. Biol. Chem. 266 (1991) 24664–24669.
[59] T. Kamimura, C. West, E. Beutler, Sequence of a cDNA clone encoding a rat Reg-2 protein, Gene 118 (1992) 299–300.
[60] J.M. Frigerio, N.J. Dusetti, V. Keim, J.C. Dagorn, J.L. Iovanna, Identification of a second rat pancreatitis-associated protein. Messenger RNA cloning, gene structure, and expression during acute pancreatitis, Biochemistry 32 (1993) 9236–9241.
[61] Y. Suzuki, H. Yonekura, T. Watanabe, M. Unno, S. Moriizumi, H. Miyashita, H. Okamoto, Structure and expression of a novel rat RegIII gene, Gene 144 (1994) 315–316.
[62] J.M. Frigerio, N.J. Dusetti, P. Garrido, J.C. Dagorn, J.L. Iovanna, The pancreatitis associated protein III (PAP III), a new member of the PAP gene family, Biochim. Biophys. Acta 1216 (1993) 329–331.
[63] S. Nakagomi, Y. Suzuki, K. Namikawa, S. Kiryu-Seo, H. Kiyama, Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation, J. Neurosci. 23 (2003) 5187–5196.