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Prolyl oligopeptidase is a glyceraldehyde-3-phosphate dehydrogenase-binding protein that regulates genotoxic stress-induced cell death

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ABSTRACT

Prolyl oligopeptidase is a serine protease that cleaves peptides shorter 30-mer at carboxyl side of an internal proline. This enzyme has been proposed to be involved in the maturation and degradation of peptide hormones and neuropeptides. However, conclusive results have not yet been reported, and the primary physiological role remains to be elucidated. Here, we describe the identification of a novel protein that interacts with prolyl oligopeptidase in a human neuroblastoma cell line NB-1. Using an affinity column with immobilized recombinant human prolyl oligopeptidase as ligand, we identified glyceraldehyde-3-phosphate dehydrogenase as a novel prolyl oligopeptidase binding protein in NB-1 cell extracts. The interaction between prolyl oligopeptidase and glyceraldehyde-3-phosphate dehydrogenase was confirmed by immunoprecipitation both *in vitro* and *in vivo*. To study the functional relevance of prolyl oligopeptidase–glyceraldehyde-3-phosphate dehydrogenase interactions, we investigated whether this interaction was involved in cytosine arabinoside-induced glyceraldehyde-3-phosphate dehydrogenase nuclear translocation and cell death. Prolyl oligopeptidase inhibitor, SUAM-14746, and prolyl oligopeptidase knockdown successfully inhibited glyceraldehyde-3-phosphate dehydrogenase translocation and promoted the survival of cytosine arabinoside-treated NB-1 cells. We also found that the interactions between prolyl oligopeptidase and glyceraldehyde-3-phosphate dehydrogenase in the cytoplasm but not in nuclei of NB-1 cell treated with cytosine arabinoside using an *in situ* proximity ligation assay. These results indicate that the interaction between prolyl oligopeptidase and glyceraldehyde-3-phosphate dehydrogenase is required for cytosine arabinoside-induced glyceraldehyde-3-phosphate dehydrogenase nuclear translocation and cell death. Therefore, the results of the present study demonstrate a novel function for prolyl oligopeptidase in cell death.

1. Introduction

Prolyl oligopeptidase (POP) is a post-proline cleaving serine peptidase that is conserved with high similarities in several species (Venäläinen et al., 2004; Garcia-Horsman et al., 2007). This enzyme preferentially hydrolyzes peptides up to around 30 residues long (Polgár, 2002). Its structure includes a β-propeller domain, which excludes access of large peptides or proteins to the catalytic site (Fülöp et al., 1998, 2001; Fuxreiter et al., 2005). This enzyme has been proposed to be involved in the maturation and degradation of peptide hormones and neuropeptides (Mentlein, 1988). POP is ubiquitously distributed, with high activities in the brain (Goossens et al., 1996). This has led to links with roles in learning and memory (Yoshimoto et al., 1987; Cunningham and O'Connor, 1997). Administration of the POP inhibitor to rats increases brain levels of the proline-containing peptides, such as substance P, α-melanocyte-stimulating hormone, thyrotrophin-releasing hormone, and arginine-vasopressin (Bellemère et al., 2003, 2005). Changes in POP expression or activity have been observed in neurodegenerative disorders including Alzheimer's and Parkinson's (Männisto et al., 2007). However, it has been difficult to determine the physiological relevance of cytosolic POP and the role of this peptidase in extracellular neuropeptide metabolism.

Recently, POP was reported to have roles associated with cell cycle regulation (Sakaguchi et al., 2011), angiogenesis (Myöhänen et al., 2011) and cellular signaling (Moreno-Baylach et al., 2011). In addition, there appears to be some consensus that POP possesses a non-hydrolase function. Schulz et al. (2005) demonstrated that POP is located in close association with the cytoskeletal component tubulin. POP was also found to physically interact with the

Abbreviations: POP, prolyl oligopeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ara-C, cytosine arabinoside; rhPOP, recombinant human POP; PLA, proximity ligation assay.

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C-terminus of α-tubulin in a yeast two-hybrid screen. The authors suggest that POP might be involved in microtubule-associated processes, such as intracellular trafficking and vesicle sorting. Additionally, in mouse brain POP was detected immunohistochemically in axonal and dendritic processes, substantiating a function of POP in cellular transport processes in neurons *in vivo* (Roßner et al., 2005). POP has been also reported to bind with the growth-associated protein 43 (GAP-43) and regulate synaptic plasticity without its peptidase activity (Di Daniel et al., 2009). Moreover, the aggregation of α-synuclein is connected to the pathology of Parkinson's disease and POP accelerates the aggregation of α-synuclein *via* a protein–protein interaction (Brandt et al., 2008; Lambeir, 2011; Myöhänen et al., 2012). These reports suggest that identifying novel POP interaction partners should provide additional insights into the cellular roles of POP.

In the present study, to investigate the possible roles of POP, we constructed a high expression system for human POP in *Escherichia coli* cells and generated an affinity chromatography column with immobilized human recombinant POP (hrPOP) as ligand to search for molecules that interacted with POP. Using this technique, we identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a novel POP-binding protein. We confirmed the interaction between POP and GAPDH by immunoprecipitation both *in vitro* and *in vivo*.

GAPDH is widely regarded as a ubiquitous glycolytic enzyme and is considered to be a simple 'housekeeping' protein. However, this enzyme has been shown to be involved in many cellular processes, including DNA repair (Meyer-Siegler et al., 1991), tRNA export (Singh and Green, 1991), membrane fusion and transport (Tisdale, 2001), cytoskeletal dynamics (Kumagai and Sakai, 1983). Moreover, GAPDH participates in cell death. A number of reports reveal that nuclear accumulation of GAPDH precedes apoptosis (Colell et al., 2009). Evidence for the potential apoptotic role of GAPDH first came from studies on cultured cerebellar neurons, in which an increase in GAPDH levels and its subsequent translocation to the nucleus preceded neuronal death induced by culture aging or cytosine arabinoside (Ara-C) exposure (Saunders et al., 1997; Ishitani et al., 1998). Antisense oligonucleotides directed against GAPDH mRNA or depletion of GAPDH with RNA interference exerted a protective effect to cytotoxicity of Ara-C (Ishitani et al., 1998; Phadke et al., 2009). However, the mechanisms involved in the nuclear translocation of GAPDH are still a controversial matter. Interestingly, it was previously reported that a POP inhibitor, ONO-1603, suppressed the overexpression of GAPDH mRNA in cultured central nervous system neurons that were undergoing age-induced apoptosis and prevented GAPDH nuclear translocation (Katsube et al., 1999). Puttonen et al. (2006) also reported that another POP inhibitor, Z-Pro-Prolinal, inhibited 6-hydroxydopamine-induced GAPDH nuclear translocation in monkey fibroblast CV1-P cells. Based on these reports, we investigated whether the POP–GAPDH interaction was involved in Ara-C-induced GAPDH nuclear translocation and cell death. Our results show that the interaction between POP and GAPDH is required for Ara-C-induced GAPDH nuclear translocation and cell death.

2. Materials and methods

2.1. Cell culture and reagents

NB-1 (IFO 50295) human neuroblastoma cells were obtained from the Health Science Research Resources Bank (HSRRB) and maintained in RPMI-1640 (Sigma) containing 10% fetal calf serum (FCS) and 50 μg/mL kanamycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air. 3-({4-[2-(E)-Styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) was obtained from the Peptide Institute. Cytosine arabinoside (Ara-C) and doxorubicin (DOX) was obtained from Sigma. Antibodies were purchased from Santa Cruz Biotechnology (GAPDH and β-actin) and Clontech (AcGFP).

2.2. Preparation of recombinant human POP (rhPOP) and POP antibody

Human POP cDNA was amplified by RT-PCR using total RNA extracted from NB-1 cells. The PCR products were cloned into pET-41 Ek/LIC vectors (Novagen) according to the manufacturer's protocol. The encoded glutathione S-transferase (GST)–POP fusion proteins were expressed in *E. coli* strain BL21-AI (Invitrogen) and purified using a Gluthathione Sepharose 4B column (GE Healthcare). After the GST moiety of the purified fusion protein was removed with thrombin, rhPOP was purified using a Mono Q 5/50 GL column (GE Healthcare). Human POP antiserum was obtained from rabbits that had been immunized with the purified rhPOP at Sigma Genosys Custom Services (Sigma Genosys). This antiserum was purified with a packed Protein A column (Applied Biosystems). This antibody was used in Western blot and immunocytochemical analyses to detect POP protein and in co-immunoprecipitation experiments to confirm the interaction between POP and GAPDH.

2.3. POP affinity chromatography and analysis of unknown proteins

A POP affinity column was prepared by coupling 3 mg of purified rhPOP to a 1-mL NHS-activated HiTrap column (GE Healthcare) according to the manufacturer's protocol. NB-1 cells were lysed in a RIPA buffer (Nacalai Tesque), after which cell extracts (3.5 mg protein) were loaded onto the column that had been equilibrated with a buffer (50 mM Tris–HCl, pH 7.4). After washing with 10 volumes of the same buffer, the bound proteins were then eluted with 2 M NaCl in the same buffer. These fractions were then analyzed by SDS-polyacryl amide gel electrophoresis (SDS-PAGE). For N-terminal amino acid sequence analysis, the proteins were blotted onto PVDF membranes (Bio-Rad) and analyzed with a protein sequencer (Procise 491HT, Applied Biosystems).

2.4. Construction of AcGFP-fused POP expression vector

The POP gene was first cloned into a pET-41 Ek/LIC vector and then inserted into a pAcGFP1-C1 vector (Clontech) along with SacI and EcoRI digestion to generate the pAcGFP1-C1-POP construct, in which the target gene was placed under the control of the cytomegalovirus (CMV) promoter. In addition, the Kozak consensus sequence was added upstream of the start codon to improve translational performance.

2.5. Cell proliferation and viability assays

Cell proliferation was determined using WST-1 reagent (Dojindo). Cells were seeded onto 96-well plates at a density of 3×10^3 cells/well and incubated for 24 h. The cells were then treated with varying doses of Ara-C or DOX with or without 40 μM of SUAM-14746. After different treatment periods, cells were treated with 10 μL of 5 mM WST-1 reagent for 3.5 h at 37 °C. The quantity of formazan product was determined spectrophotometrically with a microplate reader (Bio-Rad) at 450 nm. Cell viability was determined using a propidium iodide exclusion assay.

2.6. Western blot analysis of fractionated GAPDH

Cells were seeded at a density of 5×10^5 cells in 60 mm culture dishes and incubated for 24 h, followed by adding 40 μM of