Journal of Chemical Neuroanatomy 77 (2016) 176–186

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

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

Spred-2 expression is associated with neural repair of injured adult zebrafish brain

Fei Tieng Lim[1], Satoshi Ogawa, Ishwar S. Parhar*

Brain Research Institute, School of Medicine and Health Sciences, Monash University Malaysia, 47500 Bandar Sunway, Selangor, Malaysia

ARTICLE INFO

Article history:
Received 7 May 2016
Received in revised form 13 July 2016
Accepted 13 July 2016
Available online 15 July 2016

Keywords:
Neuroregeneration
BrdU
ERK1/2
GFAP
in situ hybridization

ABSTRACT

Sprouty-related protein-2 (Spred-2) is a negative regulator of extracellular signal-regulated kinases (ERK) pathway, which is important for cell proliferation, neuronal differentiation, plasticity and survival. Nevertheless, its general molecular characteristics such as gene expression patterns and potential role in neural repair in the brain remain unknown. Thus, this study aimed to characterise the expression of *spred-2* in the zebrafish brain. Digoxigenin-*in situ* hybridization showed *spred-2* mRNA-expressing cells were mainly seen in the proliferative zones such as the olfactory bulb, telencephalon, optic tectum, cerebellum, and the dorsal and ventral hypothalamus, and most of which were neuronal cells. To evaluate the potential role of *spred-2* in neuro-regeneration, *spred-2* gene expression was examined in the dorsal telencephalon followed by mechanical-lesion. Real-time PCR showed a significant reduction of *spred-2* mRNA levels in the telencephalon on 1-day till 2-days post-lesion and gradually increased to normal levels as compared with intact. Furthermore, to confirm involvement of Spred-2 signalling in the cell proliferation after brain injury, double-labelling of *spred-2 in-situ* hybridization with immunofluorescence of BrdU and phosphorylated-ERK1/2 (p-ERK1/2), a downstream of Spred-2 was performed. Increase of BrdU and p-ERK1/2 immunoreactive cells suggest that a decrease in *spred-2* after injury might associated with activation of the ERK pathway to stimulate cell proliferation in the adult zebrafish brain. The present study demonstrates the possible role of Spred-2 signalling in cell proliferative phase during the neural repair in the injured zebrafish brain.

1. Introduction

Spred (Sprouty-related EVH1 domain containing) family is a member of Sprouty-like gene family that downregulates the receptor tyrosine kinases (RTKs) pathway by controlling Ras and Raf (Wakioka et al., 2001; King et al., 2005; Kim and Bar-Sagi, 2004; Li et al., 2010). The RTKs pathway is known to be involved in the regulation of cell proliferation, differentiation and survival (Wakioka et al., 2001).

Proteins of the Spred family are known to function in the down-regulation of rat sarcoma/extracellular signal-regulated kinase (ERK) in the mitogen-activated protein kinase (MAPK) signalling pathway (Bundschu, 2005). This pathway can be activated by a wide range of growth factors (epidermal growth factors, fibroblast growth factors and vascular epidermal growth factors) and cytokines (interleukin-3 and interleukin-5) (Bundschu et al., 2007; Kato et al., 2003). The binding of Spred to Ras suppresses its phosphorylation and thus deactivate Raf, and also downstream MAPK kinase (MEK) and ERK cascade (Tuduce et al., 2010). In the mammalian CNS, the activation of ERK1 and 2 are important for cell proliferation, neuronal differentiation, plasticity and survival through the activation of Elk-1 transcription (Zhang and Liu, 2002; Hetman and Gozdz, 2004)

In mammals, there are three types of Spred family (Spred-1, 2 and 3), all of which are expressed in the brain and spinal cord (Bundschu et al., 2007). All three members of the Spred family have been reported to be involved in the Ras-ERK pathway in mammals (Bundschu et al., 2007). Out of these three types, Spred-2 (Sprouty-related EVH1 domain-containing 2) protein has been reported to modulate cell proliferation and differentiation in cancer cells (Kachroo et al., 2013; Yang et al., 2015). Spred-2 protein is a signal transduction regulator, which consists of three domains: an N-terminal EVH1 domain, a central c-Kit-binding domain and a

* Corresponding author at: Brain Research Institute, School of Medicine and Health Sciences, Monash University Malaysia, 47500 Bandar Sunway, Selangor, Malaysia.
E-mail address: ishwar@monash.edu (I.S. Parhar).
[1] Faculty of Pharmacy, University Teknologi MARA (UiTM), 42300 Bandar Puncak Alam, Selangor Darul Ehsan, Malaysia.

http://dx.doi.org/10.1016/j.jchemneu.2016.07.005
0891-0618/

conserved, cysteine-rich Sprouty related (SPR) domain at the C terminus (Wakioka et al., 2001; King et al., 2005). However, the association between Spred2 and neuroregeneration remains poorly understood.

In this study, zebrafish was used as the model organism. Similarly, in zebrafish, three types of Spred-homologous genes: Spred-1, 2 and 3 have been identified by the NIH – Zebrafish Gene Collection (ZGC) project. We investigated the role of Spred2 in adult neural repair after brain injury. The distribution of *spred-2* mRNA-expressing cells in the zebrafish brain was visualised by *in-situ* hybridisation and characterised by double labelling with markers for neuronal (Hu C/D) and astrocytic [Glial fibrillary acidic protein (GFAP)] cells. To evaluate the role of Spred-2 signalling in the neural repair process, we analysed *spred-2* mRNA expression in the brain using real-time polymerase chain reaction (PCR) after the mechanical creation of lesions. In addition, we examined the association between *spred-2* expression patterns in the brain and cell proliferation [as indicated by bromodeoxyuridine (BrdU)] or activation of the ERK signalling pathway [as indicated by phosphorylated ERK1/2 (p-ERK1/2)] during neural repair.

2. Materials and methods

2.1. Animals

Sexually mature male zebrafish (*Danio rerio*) with standard length of 2.8–3.0 cm were used for the study. The fish were maintained in freshwater aquaria at 27 ± 0.5 °C with a controlled natural photoregimen (14/10 h, light/dark). This study was carried out in strict accordance with the recommendations in the Guidelines to promote the wellbeing of animals used for scientific purposes: The assessment and alleviation of pain and distress in research animals (2008) by the National Health and Medical Research Council of Australia (https://www.nhmrc.gov.au/guidelines-publications/ea18). The protocol was approved by the Animal Ethics Committee of Monash University (Approval Number: SOBSB/MY/2008/42).

2.2. Phylogenetic tree and comparative genomics analysis of Spred2 gene

Sequences of vertebrate Spred-2 gene were retrieved from National Center for Biotechnology Information (NCBI) database. Multiple alignments of Spred-2 amino acid sequences were obtained using the program CLUSTAL O (1.2.1) of UniProt- Blast (http://www.uniprot.org/). The core sequences of three domains of Spred-2 protein of zebrafish were used to query several available protein sequences, from non-mammalian vertebrate (*Xenopus*) to mammalian vertebrates (mouse, rat and human), using UniProt-Blast. Phylogenetic tree was inferred by the Maximum Likehood method via MEGA 6.06.

2.3. In-situ *hybridization*

For *in-situ* hybridization, the plasmid containing coding regions of zebrafish *spred-2* (787 bp, GenBank accession No. BC066561; primer sequences are listed in Supplementary Table 1) was linearized; and digoxigenin (DIG)-labelled sense and antisense riboprobes were synthesized using SP6 and T7 RNA polymerase (Maxiscript kit, Ambion, TX, USA) with DIG RNA labelling mix (Roche Diagnostics, Mannheim, Germany).

Fish were anesthetized in a 0.025% of benzocaine (Sigma, MO, USA) solution and killed by decapitation (n = 6). The brains were fixed in buffered 4% paraformaldehyde (PFA) in phosphate buffer (PB) (pH 7.4) at 4 °C for 4 h. The brains were cryoprotected in 20% sucrose at 4 °C for overnight. The brains were embedded in optimal cutting temperature (OCT) compound (TissueTek O.C.T. compound, Sakura Fine Technical, Tokyo, Japan) and frozen with dry ice. The brains were sectioned coronally on a cryostat at 14 μm and thaw-mounted on 3-aminopropylsilane-coated glass slides.

In-situ hybridization was performed as described previously (Kitahashi et al., 2009). Briefly, sections were treated for 30 min in proteinase K (1 μg/ml) at 37 °C and hybridized with DIG-labelled riboprobes (50 ng/ml) at 55 °C overnight in a humidified chamber. The sections were then incubated with 1:500 anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics) for 2 h at room temperature and developed with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics).

2.4. Brain mechanical lesion

The dorsal part of the telencephalic region was lesioned to observe changes of *spred-2* mRNA expression. Fish were anesthetized by immersion in 0.025% of benzocaine solution for operation. The immobilized fish were placed on a water-soaked sponge for positioning and to avoid drying off of the fish body and gills. Guided by specific landmarks on zebrafish's head (4.0 mm from mouth, 2.0 mm from eye frontal; slightly above clover-liked structure located in between eyes), a small hole of $2 \times 2\,mm^2$ area was opened on the skull by means of a sterile surgical blade (Grade 11, Sheffield, England). An extra fine tweezer (EMS 78320-5A, Swiss) was inserted 1.0 mm depth into the left telencephalon lobe for 2.0 mm length cut mechanically (Fig. 3A), while the right lobe was kept intact. The opening was sealed by locating back the skull with water proof instant adhesive (LOCTITE 404, Sunnyvale, CA, USA). The fish were then returned to an isolated tank for recovery for various post-lesion survival times: 1, 2, 3 and 4-day (n = 6–8/group) for gene expression analysis, while 1-, 2- and 4-day (n = 4/group) for histological analysis. As a negative control, sham operated fish (open skull without ablation) were sampled at the same time points.

2.5. Real-time PCR

Gene expression of *spred-2* at different timepoints (intact, 1, 2, 3 and 4 days) after brain lesion was analysed by real-time PCR. The fish (n = 6–8/group) were anesthetized in a 0.025% of benzocaine solution and killed by decapitation at intact (initial control), 1, 2, 3 and 4-day post-lesion. Changes of spred-2 mRNA gene expression study were divided to two parts:-

To show region specific: The brains for intact and 1-day post-lesion only were divided into 6 regions using a fine tweezer: the olfactory bulb, telencephalon, optic tectum, hypothalamus, cerebellum and others (included hindbrain and spinal cord).

To investigate recovery point: Part A showed gene expression in lesioned region, telencephalon, had significantly changed. Therefore, only telencephalon was focused for all subsequent studies. Timepoints 2, 3 and 4-days post-lesion were added to study the recovery point of *spred-2* mRNA expression.

Total RNA from each region was isolated with TRIzol reagent (Invitrogen Corp., CA, USA) according to the manufacturer's instruction. One hundred nanograms of the total RNA were reverse-transcribed in 20 μl reaction volume containing 4 mM deoxynucleotide triphosphate (dNTP) mix, 1X random primers, 1X RT buffer, 20U of RNase inhibitor and 50U of RT enzyme (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems).

Real-time PCR was conducted using 7500 Real Time PCR System with software SDS version 1.3.1 (Applied Biosystems). The reactions of 10 μl contained 1X Power SYBR Green Master Mix (Applied Biosystems), 0.25 μM gene-specific forward and reverse primers (listed in Supplementary Table S1) and 1 μl cDNA were