Developmental Biology 321 (2008) 175–187

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Inhibition of protocadherin-α function results in neuronal death in the developing zebrafish

Michelle R. Emond, James D. Jontes *

Center for Molecular Neurobiology and Department of Neuroscience, 115 Rightmire Hall, 1060 Carmack Road, The Ohio State University Medical Center, Columbus, OH 43210, USA

ARTICLE INFO

Article history:
Received for publication 19 December 2007
Revised 5 June 2008
Accepted 5 June 2008
Available online 16 June 2008

Keywords:
Zebrafish
Protocadherin
Apoptosis
Imaging
In vivo

ABSTRACT

The *pcdhα/CNR* gene comprises a diverse array of neuronal cell-surface proteins of the cadherin superfamily, although very little is known about their role in neural development. Here we provide the first in-depth characterization of *pcdh1α* in zebrafish. Whole-mount immunocytochemistry demonstrates that a large proportion of endogenous cytoplasmic domain immunoreactivity is present in the nucleus, suggesting that endoproteolytic cleavage and nuclear translocation of the intracellular domain are important aspects of *pcdh1α* activity *in vivo*. Using whole-mount immunocytochemistry and BAC-based expression of Pcdh1α-GFP fusion proteins, we find that Pcdh1α does not appear to form stable, synaptic puncta at early stages of synaptogenesis. We also demonstrate that the presence of the Pcdh1α cytoplasmic domain is essential for normal function. Truncation of Pcdh1α proteins, using splice-blocking antisense morpholinos to prevent the addition of the common intracellular domain to the entire *pcdh1α* cluster, results in neuronal apoptosis throughout the developing brain and spinal cord, demonstrating an essential role for *pcdh1α* in early neural development. This cell death phenotype can be attenuated by the expression of a soluble Pcdh1α cytoplasmic domain.

Introduction

In mammals, the clustered protocadherins (pcdhs) comprise three tandemly-arrayed genes of cadherin-like cell surface receptors, *pcdhα*, *pcdhβ* and *pcdhγ*, encoding over 50 distinct proteins (Sugino et al., 2000; Wu and Maniatis, 1999; Wu et al., 2001; Zou et al., 2007). The *pcdhα* and *pcdhγ* genes are notable for their organization into sets of 5′ variable exons, each encoding the entire extracellular and transmembrane domains, and three 3′ constant exons that encode a common cytoplasmic domain (Wu and Maniatis, 1999). Each variable exon is expressed under the control of its own promoter, which is then spliced to the constant exons to generate a full-length protein (Tasic et al., 2002; Wang et al., 2002a). The clustered protocadherins are expressed predominantly in neurons (Kohmura et al., 1998; Wang et al., 2002b; Zou et al., 2007) and, within a brain region, individual neurons express only a small subset of protocadherin isoforms (Esumi et al., 2005; Kaneko et al., 2006; Kohmura et al., 1998; Wang et al., 2002b; Zou et al., 2007). Due to these observations, it has been widely hypothesized that the clustered protocadherins may contribute to a molecular code for synaptic connectivity (Hilschmann et al., 2001; Redies et al., 2003; Shapiro and Colman, 1999; Suzuki, 2000). However, there is little direct evidence to support this hypothesis, and the specific biological functions of these genes are not known.

The cell biology of Pcdhα and the role of Pcdhα in neural development are poorly understood. These proteins were first discovered on the basis of a yeast 2-hybrid screen for Fyn kinase-interacting proteins (Kohmura et al., 1998). Pcdhα protein has been shown to be present in developing axons (Blank et al., 2004; Morishita et al., 2004a; Morishita et al., 2004b). In contrast to expectations, Pcdhα isoforms exhibit little cell–cell adhesion (Morishita et al., 2006; Triana-Baltzer and Blank, 2006). Recently, Pcdhα has been shown to undergo sequential proteolytic processing by matrix metalloproteases (MMPs) and Presenilin-1 when expressed in heterologous cells (Bonn et al., 2007). The carboxy-terminal intracellular domain is released as a soluble fragment, which is capable of translocating to the nucleus. However, the fraction of Pcdhα undergoing proteolysis appears relatively low, and the *in vivo* significance of these observations is uncertain.

While no functional data for the *pcdhα* gene are presently available, some information is available for the *pcdhγ* gene, coming from studies of mice in which *pcdhγ* has been deleted (Wang et al., 2002b; Weiner et al., 2005). These mice die shortly after birth, exhibiting extensive apoptosis of spinal interneurons (Wang et al., 2002b). More detailed analysis has also revealed synaptic deficits, although neither the cell biological roles played by Pcdhγ, nor the relationship of the synaptic deficits to the observed cell death, have been determined (Weiner et al., 2005).

As the zebrafish has a highly stereotyped and relatively simple embryonic nervous system, it offers an excellent opportunity to investigate the role of protocadherins in neural development. Here we focus on *pcdh1α*, providing the first *in vivo* analysis of this gene in zebrafish. We characterize the expression of *pcdh1α*, as well as the

* Corresponding author. Fax: +1 614 292 5379.
E-mail address: jontes.1@osu.edu (J.D. Jontes).

0012-1606/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.06.011

subcellular localization and dynamics, using whole-mount immunocytochemistry and *in vivo* 2-photon time-lapse microscopy of Pcdhα-GFP fusion proteins. In addition, we demonstrate that loss of *pcdh1α* activity results in a dramatic increase in programmed cell death throughout the nervous system, which can be partially attenuated by expression of a soluble cytoplasmic domain fragment.

Materials and methods

Fish maintenance

Adult zebrafish (*Danio rerio*) and embryos of the Tübingen longfin and AB* strains were maintained at ~28.5 °C and staged according to Westerfield (1995). Embryos were raised in E3 embryo medium (Westerfield, 1995) with 0.003% phenylthiourea (Sigma-Aldrich) to inhibit pigment formation.

Whole-mount in situ hybridization

The constant cytoplasmic domains of *pcdh1α* and *pcdh2α* were cloned by RT-PCR and sequenced. A T7 RNA Polymerase binding site was added to the antisense strand by PCR and this PCR product was used as template for *in vitro* transcription reactions (Promega). Antisense riboprobe was labeled with DIG-dUTP (Roche Applied Science) and purified on ProbeQuant G-50 spin columns (Amersham Biosciences). Embryos were fixed at 4 °C overnight in 4% paraformaldehyde in PBS, permeabilized in methanol at −20°C overnight, rehydrated, treated with proteinase K (10 μg/ml, Roche Applied Science), and refixed in 4% paraformaldehyde prior to hybridization. Labeled riboprobe was added to a final concentration of 400 ng/ml and hybridization occurred overnight at 65 °C. AP-conjugated anti-DIG Fab fragments (Roche Applied Science) were used at 1:5000, and the *in situs* were developed with NBT/BCIP (Roche Applied Science).

BAC recombineering and DNA injections

BAC recombineering

The BAC clone CH211-150p9 (Accession # AC144823) was obtained from BACPAC Resources (http://bacpac.chori.org). BAC DNA was introduced into EL250 cells (obtained from N. Copeland, NIH), to generate EL150p9 cells. EL150p9 cells were grown to midlog phase at 32 °C, induced at 42 °C for 15 min, chilled rapidly, and washed twice with ice-cold sterile H_2O. Linear targeting contructs (200–300 ng) were electroporated into electrocompetent EL150p9 cells and plated after a 1 h recovery. Recombinants were grown on kanamycin selective plates, and colonies were screened by PCR. Recombinant BAC DNA was purified using Nucleobond AX-100 columns (Clontech Laboratories). Purified BAC DNA was diluted in 0.1 M KCl and injected at a concentration of 100–200 ng into 1-cell stage embryos.

Targeting construct

The constant cytoplasmic domain (CD) of the *pcdh1α* cluster was amplified from cDNA by PCR and subcloned into pEGFP-N1 (Clontech Laboratories) using EcoRI and AgeI sites (which were included in the PCR primers). The resulting Pcdh1αCD-GFP fusion was sequenced. PCR primers were made which amplified the Pcdh1αCD fusion protein and the downstream kanamycin selection marker, and contained 60 base pairs of homology to the targeted BAC. The 5'-primer was designed to place the Pcdh1αCD-GFP in register with the replaced constant exon 1, thus preserving the splice acceptor site and its relationship to the αCD.

Morpholino and mRNA injections

Antisense morpholino oligonucleotides were purchased from Gene-Tools LLC. The morpholinos were dissolved in dH_2O at ~8 ng/nl, aliquoted and stored at −80°C. For use, morpholinos were diluted to a working concentration of 2–4 ng/nl in dH_2O and 1 nl was injected into 1-cell stage embryos. For the mRNA experiments, we injected 200 pg of mRNA encoding a soluble myc-tagged Pcdh1α cytoplasmic domain, which was inserted into pCS2+ and synthesized with an SP6 mMessage mMachine kit (Ambion). The mRNA injection experiments were performed double-blind.

Morpholinos used in this study were the following:

MO1.1: 5′AAACTGCTTACCTCTGCACTCCTGC3′;
MO1.2 5′CTGCTTACCTCTGCACTCCTGCCCT3′;
MO1.1mis 5′AATCTCCTTAGCTCTGGACTCGTGC3′;
MO1.2mis 5′CTCCTTAGCTCTCCACTCGTGCCCT3′;
p53MO 5′GCGCCATTGCTTTGCAAGAATTG3′;
MO2.1 5′AACTATCTTACCAGGAGCGCTGGAA3′
MO2.2 5′AAGTCTTACCTTTGCATTCCTGCTC3′
MO2.2mis 5′AACTCTTAGCTTTCCATTCGTGGTC3′

Time-lapse imaging and analysis

Imaging

GFP-labeled embryos were mounted in 1.5% low melting point agarose and placed on a custom-built laser-scanning two-photon microscope. Excitation was provided by a Chameleon-XR Ti:Sapphire laser (Coherent, Inc.), tuned to 920 nm. We used a Zeiss 63× Achroplan-IR NA 0.9 objective (Carl Zeiss Microimaging) for imaging. The software for control of the microscope was very kindly provided by Dr. Noam Ziv (Technion, Haifa, Israel). Images were scanned with a pixel size of 0.22 μm. Time-lapse sequences consisted of image stacks of 15–25 optical sections at 1 μm spacing, collected at 2 min intervals for 2–24 h. Image processing was performed using ImageJ (http://rsb.info.nih.gov/ij). For presentation purposes, regions of interest were cropped and the image contrast was inverted and adjusted in Adobe Photoshop.

Generation of polyclonal Pcdh1αCD antibody

Antibodies were produced against the common cytoplasmic domain of the zebrafish Pcdh1α cluster (Pcdh1αCD). Rabbits were immunized against a GST-fusion protein (GST-Pcdh1αCD) and affinity purification was performed using an MBP-fusion (MBP-Pcdh1αCD; Covance Research Products).

Whole-mount immunocytochemistry

Embryos were fixed overnight in 4% paraformaldehyde, permeabilized in 1% TX-100 in PBS for 1 h and blocked for 1 h in PBS containing 1% dimethylsulfoxide, 2 mg/ml BSA, 0.2% Triton X-100, and 5% normal goat serum. Antibody was added at a dilution of 1:100 (Pcdh1α-CD), 1:1000 (acetylated tubulin; Sigma-Aldrich), 1:500 (SV2; DSHB) or 1:500 (myc; Sigma-Aldrich) and incubated overnight at 4 °C. Rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch) were used at a dilution of 1:500. In some experiments, DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich) was added with secondary antibody at 100 ng/mL to label nuclei. Embryos were embedded in 1.5% agarose and imaged on a two-photon microscope. A myc-epitope was added to the carboxy-terminus of the constant cytoplasmic domain and was subcloned into a UAS vector. The UAS:myc-pcdh1αCD was expressed by co-injection with a goldfish α1-tubulin promoter:Gal4VP16 driver plasmid, and the embryos were processed for myc immunoreactivity as described above. Statistical analysis was performed using a Student's *t*-test.

HEK293 cell culture, transfection, and immunostaining

Human embryonic kidney (HEK293) cells were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin at 37°C in 5% CO_2. One day before transfection, cells were split and plated