Developmental Biology 327 (2009) 497–507

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Involvement of an inner nuclear membrane protein, Nemp1, in *Xenopus* neural development through an interaction with the chromatin protein BAF

Hiroshi Mamada, Noriyuki Takahashi, Masanori Taira *

Department of Biological Sciences, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received for publication 8 July 2008
Revised 22 December 2008
Accepted 23 December 2008
Available online 8 January 2009

Keywords:
Nemp1
Barrier-to-autointegration factor (BAF)
Inner nuclear membrane protein
Eye development
Xenopus laevis

ABSTRACT

To clarify the molecular mechanisms of neural development in vertebrates, we analyzed a novel gene, termed *nemp1* (*n*uclear *e*nvelope integral *m*embrane *p*rotein *1*), which is expressed in the *Xenopus* anterior neuroectoderm at the neurula stage. Nemp1 has a putative signal peptide and five transmembrane domains, but does not have any other known domains. We show that Nemp1 is localized to the inner nuclear membrane (INM) with its evolutionarily conserved C-terminal region facing the nucleoplasm. Both overexpression and knockdown of Nemp1 in *Xenopus* embryos reduced the expression of early eye marker genes, *rax*, *tbx3*, and *pax6*, and later resulted mainly in severe eye defects at the tailbud stage. In contrast, the expression of a forebrain/midbrain marker, *otx2*, and a pan-neural marker, *sox2*, was largely unaffected. Deletion analysis of Nemp1 showed that nuclear envelope-localization of the C-terminal region is necessary for its eye-reducing activity. Furthermore, *nemp1* is coexpressed with *baf* (barrier-to-autointegration factor) in the eye anlagen, and that Nemp1 interacts with BAF through the BAF-binding site in the C-terminal region and this site is required for Nemp1 activity. These data suggest that Nemp1 is involved in the expression of eye marker genes by functioning at the INM at least partly through BAF.

Introduction

During early *Xenopus* development, the neuroectoderm is induced from the dorsal ectoderm of the gastrula embryo by signals from the Spemann organizer (De Robertis et al., 2000; Niehrs, 2004). The neuroectoderm then acquires regional specificity along the dorsal–ventral (DV) and anterior–posterior (AP) axes. The early neural patterning is visualized by the expression of various marker genes for the presumptive midbrain–hindbrain boundary region (Shinga et al., 2001; Takada et al., 2005), the neural crest (Steventon et al., 2005), and the eye field (Bailey et al., 2004; Esteve and Bovolenta, 2006; Lupo et al., 2000), among other regions. To clarify the molecular mechanisms of early neural patterning and brain development in vertebrates, we previously performed a systematic expression pattern screening with a *Xenopus* anterior neuroectoderm (ANE) cDNA library and obtained new candidate genes for developmental regulators (Takahashi et al., 2005). This paper reports that one of those clones encodes a novel protein localized to the inner nuclear membrane and is involved in early eye development.

The nuclear envelope (NE) consists of the outer (ONM) and inner (INM) nuclear membranes, nuclear pore complexes (NPCs), and nuclear lamina. The INM contains a distinctive set of integral membrane proteins, such as LAP2, MAN1, and Emerin, which interact with the nuclear lamina, a meshwork of intermediate filament proteins that underlies the INM (Burke and Stewart, 2002). These proteins play an essential role in maintaining the structural integrity of the NE (Gruenbaum et al., 2005; Hutchison, 2002). LAP2, MAN1, and Emerin interact with barrier-to-autointegration factor (BAF) (Furukawa, 1999; Lee et al., 2001; Mansharamani and Wilson, 2005; Shumaker et al., 2001), which binds to DNA as a homodimer to form a bridge between these INM proteins and DNA, and is thought to be involved in chromatin decondensation and nuclear assembly (Cai et al., 1998; Margalit et al., 2007; Segura-Totten et al., 2002). We have previously shown that a nuclear envelope protein, MAN1, acts as a Smad-interacting protein to antagonize BMP signaling during early *Xenopus* development (Osada et al., 2003) and to antagonize TGF-β signaling to regulate vascular remodeling in mice (Ishimura et al., 2006). Recently, Emerin has been shown to attenuate Wnt signaling by exporting β-catenin from the nucleus (Markiewicz et al., 2006). Thus, knowledge of the developmental roles of INM proteins is being accumulated, but identification and functional analysis of a novel INM protein are necessary for further understanding of their developmental roles.

Studies of vertebrate eye development have shown that the presumptive eye territories originate from a single bilaterally expanded field positioned within the ANE demarcated by *otx2* expression (Eagleson et al., 1995; Inoue et al., 2000; Li et al., 1997; Varga et al., 1999). To date, several transcription factors have been suggested as being involved in eye development. Those transcription factors include *rax* (representing alloalleles Rx1 and Rx2A), *pax6*, and *six3* (Andreazzoli et al., 1999; Chow et al., 1999; Chuang and Raymond, 2001; Loosli et al., 1999; Mathers et al., 1997; Oliver et al., 1996). In *Xenopus*, a gene cascade of transcription factors from *otx2* and *tbx3*/*ET* through *rax* to *pax6*, and a gene network containing *pax6*, *six3*,

* Corresponding author. Fax: +81 3 5841 4434.
E-mail address: m_taira@biol.s.u-tokyo.ac.jp (M. Taira).

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.12.038

and *lhx2* have been proposed for eye field specification (Zuber et al., 2003). Genetic analyses in the human and mouse have also shown the importance of *rax*, *pax6*, *six3*, and *lhx2* genes in eye development (Hill et al., 1991; Lagutin et al., 2003; Mathers et al., 1997; Porter et al., 1997; Tucker et al., 2001). Although eye development has thus been extensively studied, the mechanisms of gene regulation of these transcription factors have not been fully investigated.

We report here a novel INM protein, Nemp1 (for *n*uclear *e*nvelope integral *m*embrane *p*rotein *1*), which is expressed in the ANE and is involved mainly in eye development from the late gastrula to the neurula stage. Our functional and biochemical data suggest the involvement of Nemp1 protein in gene regulation for eye development through binding to BAF, shedding light on both the molecular mechanisms of early eye development and the role of an INM protein in gene regulation.

Materials and methods

cDNA cloning, sequence analysis, and constructs

cDNA library construction and screening were previously described (Osada et al., 2003; Takahashi et al., 2005). A full-length cDNA clone, pBluescript II-SK(−)*nemp1a* [SK(−)*nemp1a*; DDBJ accession no. AB474919] was isolated from an ANE library (Takahashi et al., 2005). An alloallele gene, *nemp1b* (clone XL436g07ex; DDBJ accession no. AB474920), and *baf* alloallele genes, *baf-a* (clone XL456h02ex) and *baf-b* (clone XL455o03ex), were identified in a *Xenopus* EST database of NBRP (http://www.nbrp.jp/index.jsp), and were isolated from an ANE expression cDNA library (Osada et al., 2003). Computational sequence analyses were done using SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004), SMART (http://smart.embl-heidelberg.de/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001), Pfam (http://www.sanger.ac.uk/Software/Pfam/), and the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Plasmid constructs were made with pCS107, pCS2+Myc, and pCS2+mcs4HAmcs as vectors at the BamHI site for full-length Nemp1 [amino acid numbers (aa) 1–434], ΔN (lacking aa 37–175), ΔA (lacking aa 176–287), ΔBt (aa 1–325), ΔSP (aa 35–434), ΔKR (lacking aa 317–325), ΔBBS (lacking aa 368–375), SPNA (aa 1–287), SP+TMs (aa 1–34 plus 149–317), ΔTMs (lacking aa 149–317), SP+A (aa 1–36 plus 176–287), SP+Bt (aa 1–35 plus 326–434), Ct (aa 318–434), KR (aa 317–325), and Bt (aa 326–434). All Nemp1 constructs were C-terminally tagged with five-repeat Myc or four-repeat HA, designated as Nemp1-Myc or Nemp1-HA, respectively, and so on, using pCS2+MT or pCS2+mcs4HAmcs as a vector. N- and C-terminally HA-tagged human Emerin (HAn-Emerin and Emerin-HAc, respectively) and internally HA-tagged *Xenopus* MAN1 (MAN1-HAi), in which four-repeat HA was inserted after aa 421 between the two transmembrane domains, were made with pCS2+ MT-Emerin and pCS2+MT-MAN1 as PCR templates (Osada et al., 2003) and pCS2 +mcs4HAmcs as a vector. N-terminally two-repeat FLAG-tagged BAF was made with *baf-b* and pCS2+2FTn2.

Xenopus embryo manipulation and mRNA microinjection

Xenopus embryos were obtained by artificial fertilization, dejellied, and incubated in 0.1× Steinberg's solution (Peng, 1991). Embryos were staged according to the normal table of Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Microinjection of synthetic mRNA was done with a fine glass capillary and the pneumatic pressure injector IM300 (Narishige) in 3% Ficoll in 1× Modified Bath's solution (MBS) (Peng, 1991). Injected embryos were kept in 3% Ficoll in 1× MBS for 2–3 h, transferred into 0.1× Steinberg's solution containing 50 μg/ml gentamicin sulfate, and incubated until embryos reached the appropriate stages. For mRNA synthesis, plasmids were linearized with AscI or NotI and transcribed with SP6 polymerase (MEGAscript, Ambion) as described (Suga et al., 2006). mRNAs were injected into the dorsal blastomere of four cell-stage embryos. Nuclear β-*galactosidase* (nβ-*gal*) mRNA (60 pg/embryo) was coinjected for lineage tracing.

Whole-mount in situ hybridization and RT-PCR analysis

Whole-mount in situ hybridization (WISH) was performed according to Harland (1991) using an automated system (AIH-201, Aloka). Antisense *nemp1a* and *baf-b* RNA probes were transcribed with T7 RNA polymerase from BamHI-linearized SK(−)*nemp1* and a *baf-b* PCR product, which was amplified from pCS105-*baf-b* with the SP6 primer and a T7 promoter containing primer (5′-GTAATACGACTCACTATAGGGCGAGAGGCTC-3′). Other RNA probes were synthesized according to plasmid providers. RT-PCR was performed as described (Suga et al., 2006), with primer sets of *nemp1a* (forward, 5′-GAGGAGGCTGTAGAGTTAGT-3′; reverse, 5′-GGACCACTTTACCTTCATAG-3′; 29 cycles) and *histone H4* (Niehrs et al., 1994) (25 cycles).

Immunofluorescence microscopy

Preparation of COS7 cells transfected with pCS2 expression constructs and confocal microscopic analysis with LSM Pascal (Zeiss) were performed as described (Osada et al., 2003) using mouse anti-Myc 9E10 (BIOMOL), mouse anti-FLAG M2 (Sigma), and rabbit anti-HA Y-11 (Santa Cruz) antibodies as primary antibody and Alexa Fluor 488-, Alexa Fluor 555-, and Alexa 546-conjugated antibodies (Molecular Probes) as secondary antibody. Nuclei were stained with SytoxGreen (Molecular Probes). Digitonin extraction of fixed cells was done with 40 μg/ml digitonin (Wako) in PBS for 3 min on ice, and digitonin-treated cells were blocked in 0.5% gelatin in PBS for 15 min (Brachner et al., 2005) and in 10% lamb serum in PBS for 1 h at room temperature. Fixed *Xenopus* embryos were permeabilized and blocked with 0.1% Triton X-100, 0.2% BSA, 10% lamb serum in Tris-buffered saline (pH. 7.5) at room temperature.

Knockdown experiments

Antisense morpholino oligos, MOa and MOb, against exon 1–intron 1 boundary sequences of *nemp1* alloalleles, *nemp1a* and *nemp1b*, were obtained from Gene Tools, LLC (MOa, 5′-TTTAAATTACCTGAGGCCAATGTAC-3′; MOb, 5′-ACACTTTATGTTATATTACGTGTCC-3′). Five mismatched MO (5mmMO 5′-ACAgTTTATcTTAaATTACgTGTgC-3′; lower cases are mismatched with MOb) was used as negative control. Prior to designing MOa and MOb, RNA splice junctions for *nemp1a* and *nemp1b* were identified by alignment of cDNA sequences with the *Xenopus/Silurana tropicalis* genome sequence (http://genome.jgi-psf.org/Xentr3/Xentr3.home.html). *nemp1a* and *nemp1b* genomic DNA fragments were PCR-amplified with *Xenopus laevis* genomic DNA and primers specific to exons 1 and 2 (5′-GAGGAGGCTGTAGAGTTAGT-3′ and 5′-CGGACCACTTTACCTTCATAG-3′ from the *nemp1a* gene, respectively) and sequenced. MOa and MOb were dissolved in water and injected into the dorsal blastomere of four cell-stage embryos.

Glutathione S-transferase (GST) pull-down assay and western blotting

The C-terminal region of Nemp1 (Bt; aa 325–434) and the Bt lacking the BBS (BtΔBBS; aa 325–434 lacking aa 368–375) were subcloned into the pGEX-6P-1 vector to produce the GST-Bt and GST-BtΔBBS fusion proteins. Generation of GST fusion proteins was carried out as described previously (Hiratani et al., 2003; Osada et al., 2003). mRNA for FLAG-BAF was injected into the animal pole region of two-cell stage embryos. Injected embryos were cultured until the gastrula (stage 10.5), homogenized in 1× lysis buffer (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 8 mM DTT, 40 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM PMSF) containing 0.1% NP-40. Ten μg of purified GST, GST-MAN1-Ct, GST-Bt, or GST-BtΔBBS proteins bound to glutathione