Developmental Biology 421 (2017) 161–170

Contents lists available at ScienceDirect

Developmental Biology

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

Calreticulin is a secreted BMP antagonist, expressed in Hensen's node during neural induction

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ARTICLE INFO

Keywords:
Neural plate
Gastrulation
Neurulation
Chick embryo
Phospho-Smad
Calnexin
Calcium-binding proteins

ABSTRACT

Hensen's node is the "organizer" of the avian and mammalian early embryo. It has many functions, including neural induction and patterning of the ectoderm and mesoderm. Some of the signals responsible for these activities are known but these do not explain the full complexity of organizer activity. Here we undertake a functional screen to discover new secreted factors expressed by the node at this time of development. Using a Signal Sequence Trap in yeast, we identify several candidates. Here we focus on Calreticulin. We show that in addition to its known functions in intracellular Calcium regulation and protein folding, Calreticulin is secreted, it can bind to BMP4 and act as a BMP antagonist in vivo and in vitro. Calreticulin is not sufficient to account for all organizer functions but may contribute to the complexity of its activity.

1. Introduction

Neural induction is the process by which signals secreted by the organizer (Hensen's node in amniotes, a structure at the tip of the primitive streak) can instruct cells in the epiblast to change their fate from non-neural (eg. epidermis) to neural plate. In chick, a graft of Hensen's node to the proximal anterolateral extraembryonic region (area opaca) can elicit the formation of a complete, patterned nervous system in less than 24 h (for review see Stern, 2005). Although BMP inhibition is absolutely required for neural induction to take place (De Robertis and Kuroda, 2004; Harland, 2000; Hemmati-Brivanlou and Melton, 1997; Linker and Stern, 2004), it is increasingly clear that other signals are also required (Stern, 2005). Known additional factors include FGFs, IGFs and Wnt inhibition, but even a combination of all of these factors is insufficient to mimic the effect of a node graft to the area opaca in the chick (de Almeida et al., 2008; Linker and Stern, 2004), suggesting that other factors are also involved.

To identify new secreted factors expressed in Hensen's node, we took advantage of a Signal Sequence Trap, a rapid strategy to isolate large numbers of cDNAs encoding putative secreted proteins by genetic selection in yeast. A strain of *Saccharomyces cerevisiae* with a genomic deletion at the SUC2 locus (Klein et al., 1996a) is unable to secrete invertase and is therefore unable to grow on sucrose or raffinose as the sole carbon source. A vector with the SUC2 gene lacking the signal sequence and the start codon is then used to construct a library of cDNAs from the tissue of interest. If the cDNA clone provides the elements required for secretion, the fusion protein is translocated to the secretion pathway, allowing the transformant to grow on sucrose or raffinose as their only source of carbon (Jacobs et al., 1997).

Here we use this functional genetic screen to seek new secreted factors from the chick organizer, Hensen's node. Out of 137 putative secreted factors identified, 16 have appropriate expression patterns in the node. These include Calnexin (CANX) and Calreticulin (CALR), molecules previously well studied in connection with intracellular Calcium regulation and glycoprotein folding in the endoplasmic reticulum (Bedard et al., 2005). Misexpression of Calreticulin, but not Calnexin, at the neural plate border can expand the domain of expression of neural plate markers, similar to the effect of BMP antagonists in the same assay. We further show that Calreticulin can be secreted by cells, that it can inhibit BMP, and that soluble Calreticulin can bind to BMP4.

2. Materials and methods

2.1. Eggs, embryo manipulations and electroporation

Fertilized hens' eggs (Brown Bovan Gold; Henry Stewart and Company) were incubated at 38 °C to the desired stages, following

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http://dx.doi.org/10.1016/j.ydbio.2016.12.001
Received 28 October 2016; Received in revised form 29 November 2016; Accepted 1 December 2016
Available online 02 December 2016
0012-1606/

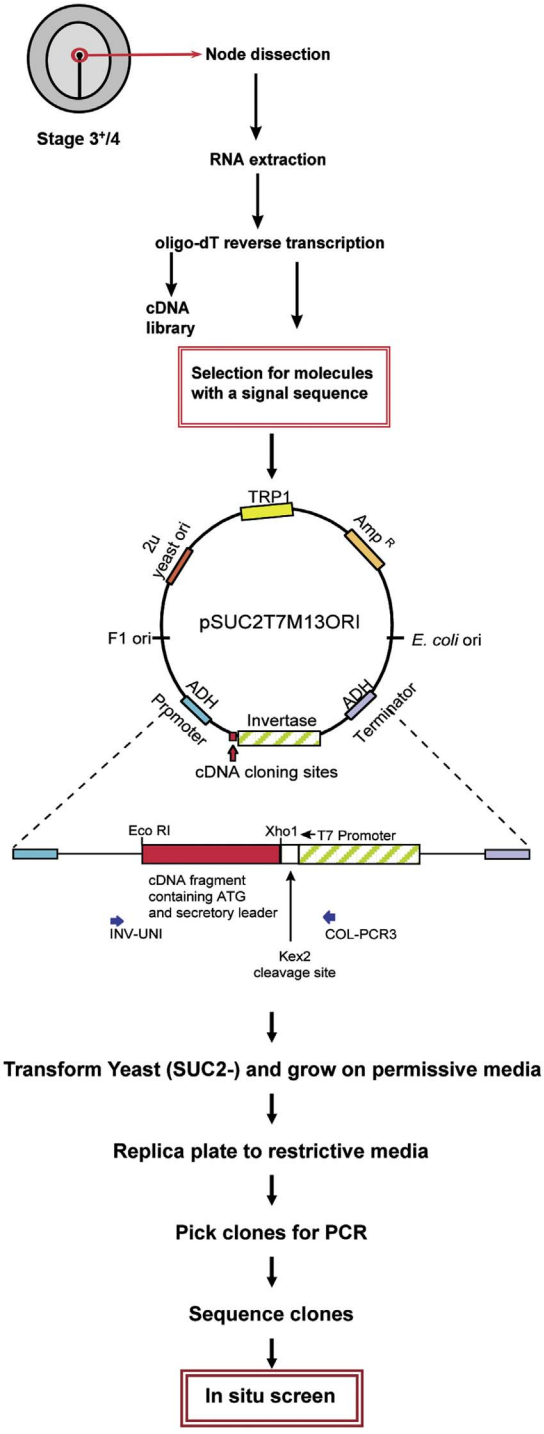

Fig. 1. Identification of secreted molecules using the Signal Sequence Trap strategy. Diagram showing the screen methodology: Hensen's nodes were dissected from Stage 3+-4 chick embryos; after RNA extraction and reverse transcription the clones were put through the secretion selection and the resulting sequences further screened by in situ hybridization.

the Hamburger and Hamilton system (Hamburger and Hamilton, 1951). Electroporation, whole-mount in situ hybridization and whole-mount immunostaining were performed using standard methods as previously described (Sheng et al., 2003; Stern, 1993; Streit and Stern, 2001; Voiculescu et al., 2008). All DNA solutions for electroporation were used at 1.5 μg/μl. FGF8 (50 μg/ml) and Calreticulin (50 μg/ml) proteins were delivered on heparin beads (Sigma; prepared as described by Streit et al., 2000).

2.2. Signal Sequence Trap screen and cloning of Calreticulin

A Signal Sequence Trap screen to identify putative secreted factors was performed in yeast as described by Jacobs et al., 1997) (Fig. 1) using a cDNA library constructed by Oligo-dT-primed reverse transcription from mRNA purified from Hensen's nodes of embryos at stage HH3+-4. All inserts that passed the selection step (see Fig. 1 and Results) were sequenced and identified initially by BLAST homology searches querying public sequence databases.

Full length Calreticulin was obtained from a stage 2–4 cDNA library as previously described (Streit et al., 2000). The coding regions of chick Calreticulin (CALR), zebrafish Calreticulin (calr) (Rubinstein et al., 2000), human Calnexin (CANX) (kind gift from Marek Michalak (Vassilakos et al., 1998), Xenopus truncated BMP receptor (Suzuki et al., 1994), cSmad6 (a kind gift from P Szendro and G Eichele) (de Almeida et al., 2008; Yamada et al., 1999), cChordin (Streit et al., 1998) and xSmad7 (Casellas and Brivanlou, 1998; de Almeida et al., 2008) were each cloned into pCAβ-IRES-GFP.

The coding region of Calreticulin was also cloned in the pCDNA 3.1/Myc-His (Invitrogen) expression vector using the NotI and BamHI cloning sites. Inserts were generated by PCR using the primers GATCGCGGCCGCATGAGCCGCCTCTGCCTCCCG (adds a NotI restriction site prior to the start codon) and GATCGGATCCTCTTCCTCTCA GCCTCC (removes the stop codon from Calreticulin and adds a BamHI restriction site) and pfuTaq polymerase (Promega) (94 °C, 2 min; 42 °C, 2 min; 72 °C, 2 min; 30 cycles). After digestion of both the PCR fragment and the pCDNA vector with NotI and BamHI, the DNAs were gel purified using a gel extraction kit (Promega) and ligated with T4 ligase (Promega). The resulting plasmid (CALR-Myc) was verified by sequencing.

2.3. Cell culture and co-immunoprecipitation

Cell culture and treatments were performed as previously described (Howell et al., 2002) with a few modifications: HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and transfected using Lipofectamine™ 2000 in combination with Plus Reagent (Invitrogen) according to the manufacturer's instructions. Cells were seeded at 10^5 cells per well in a 6-well plate. The next day, each well was transfected with 1 μg DNA (either control vector [no insert], dominant-negative BMP receptor (dnBMPr), CANX or CALR, all in pCAβ-[insert]-IRES-GFP). To study BMP inhibition, transfected cells were grown for 72 h and then treated with human BMP4 protein (R & D Systems) at 20 ng/ml for 1 h prior to preparation of whole cell extracts. Cell lyses and Western blots were performed as previously described (Howell et al., 2002).

COS cells were transfected with CALR-Myc, Chordin-Myc (Streit et al., 1998) or control, empty Vector by a similar method. Pellets of transfected COS cells for grafting into embryos were generated from hanging drops as previously described (Streit et al., 1998; Streit and Stern, 1999).

For co-immunoprecipitations (Co-IP), 500 μl of lysates from transfected cells were clarified by centrifugation for 10 min at 13,200 r.p.m. at 4 °C and diluted (1:2) in Co-IP wash buffer (0.025 M Tris pH7.4, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40 and 5% glycerol). The sample was first immunoprecipitated with 1–2 μg of the protein-specific antibody (either mouse anti-BMP4 IgG, Enzo Life Sciences or rabbit anti-Calreticulin, Cell Signalling Technology or control mouse or rabbit Ig [mouse IgG from Santa Cruz, Rb IgG from BD Pharmigen]) overnight at 4 °C on a rocking platform. As additional negative controls, we used cell lysates of non-transfected HEK-293T cells. Recombinant BMP4 (Biotechne) and Calreticulin (Abcam) were used as positive controls, diluted in Co-IP wash buffer (up to 1 μg/ml). The precipitated supernatants (1 ml) were complexed with 20 μl prewashed Sepharose L (Santa Cruz) and incubated for 3 h at 4 °C on a