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Critical Role of Cys168 in Noggin Protein's Biological Function

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Abstract Previous studies have indicated that noggin exerts its neural inducing effect by binding and antagonizing bone morphogenetic protein 4 (BMP4). In order to further clarify the relationship between the structure and the function of noggin, and elucidate the possible mechanism responsible for noggin-BMP4 interaction, we generated three noggin mutants, C168S, C174S and C197S, by using a site-directed mutagenesis method. Ectopic expression of wild-type (WT) noggin, C174S or C197S, in *Xenopus* animal caps (ACs) by mRNA injection converted the explants (prospective ectoderm) into neural tissue, as indicated by the neural-like morphology and expression of the neural cell adhesion molecule (*NCAM*) in the ACs. In contrast, ACs expressing C168S suffered an epidermal fate similar to the control caps. Similarly, among the three mutants, only C168S lost the dorsalizing function. These studies highlight the critical role played by Cys168 in noggin's biological activities. It probably participates in the formation of an intermolecular disulfide bridge.

Key words noggin; site-directed mutagenesis; neural induction; dorsalizing

The amphibian nervous system is derived from a portion of the ectoderm by its interaction with the dorsal mesoderm, which is called Spemann organizer. When transplanted from one blastula stage embryo to the ventral side of another, the dorsal lip of the blastopore, the first site of invagination at gastrulation, induces the ectoderm (presumptive epidermis region) to become neural tissue [1]. It is known that this Spemann organizer signaling center secretes several polypeptides that induce neurogenesis [2].

Noggin, which was identified in *Xenopus* embryos, is the first molecule shown to have the neural inducing effect of the organizer center [3]. It is expressed in the organizer at the gastrula stage and encodes a novel 32 kDa glycoprotein that is secreted and functions as a homodimer. Soluble noggin protein induces anterior neural markers in animal cap (AC) ectoderm that would otherwise develop epidermis [4]. Xu *et al.* [5] and other researchers have shown that inhibition of the BMP signal by a dominant negative BMP receptor (DN-BR) elicits neuralization in the AC tissue, whereas noggin exerts its neuralizing effect by binding and inactivating BMP4 [5–10]. We have previously shown that C145S or 3CS mutation leads to loss of noggin's inducing effect [11].

The present study attempted to further dissect the functionally related sites in the noggin molecule. Our results suggest that Cys168 is required for the neuralizing and dorsalizing effects of noggin.

Materials and Methods

Site-directed mutagenesis of noggin

The noggin cDNA in plasmid pGEM5zf(–) was kindly provided by Dr. R. M. HARLAND (University of California, CA, USA). From the wild-type (WT) clone, we generated three mutated forms of noggin, C168S, C174S and C197S, by replacing cysteine residues at these sites with serine residues using the QuikChange site-directed mutagenesis kit (Clontech, Palo Alto, USA). The following primers were used for these mutations: (1) C168S: 5′-GTGAAAGTAGGGAGCTCCTACAGTAAGAGGTC-3′ (forward), 5′-GACCTCTTACTGTAGGAGCT-

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CCCTACTTTCAC-3′ (reverse); (2) C174S: 5′-CTACAGTAAGAGGTCTTCTTCTGTGCCAG AGGGC-3′ (forward), 5′-GCCCTCTGGCACAGA-AGAAGACCTCTTACTGTAG-3′ (reverse); and (3) C197S: 5′-CATCTTAAGGTGGAGATCTCAAC-GCAGGGTTCAGC-3′ (forward), 5′-GCTGAACCC-TGCGTTGAGATCTCCACCTTAAGATG-3′ (reverse). Mutagenesis PCR reactions were carried out in accordance with the manufacturer's instructions.

In vitro RNA synthesis

The WT and the mutated noggin plasmids were linearized by *Not*I and subjected to synthesis of capped mRNA using an *in vitro* transcription kit (Ambion, Austin, TX, USA). The synthetic RNA was quantified by ethydium bromide staining with a standard RNA ladder as the control.

Xenopus embryo

Xenopus laevis adults were purchased from the Beijing Institute of Developmental Biology, and the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. *Xenopus* embryos were obtained by *in vitro* fertilization after induction of the females with 500 units of human chorionic gonadotropin. Developmental stages were designated according to the descriptions provided by Nieuwkoop and Faber [12].

Embryo injection and AC explant culture

At the two-cell stage, each blastomere of the embryos was injected with 1–2 ng of the synthesized mRNA. The ACs were dissected at stage 8.5 to 9, cultured at 22 °C in 0.3×modified Marc's Ringer's (MMR) solution (0.1 M NaCl, 2.0 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8, 0.1 mM EDTA) until stage 24, and then harvested for morphological observation and reverse transcription-PCR (RT-PCR) assays.

In some experiments,0.1–1 ng of the synthetic mRNAs were injected into the ventral marginal zone of the four-cell stage embryos. The injected embryos were allowed to develop until the tadpole stage and the dorsoanterior index (DAI) was calculated in accordance with the criteria described by Kao and Elinson [13].

RT-PCR

Total RNAs were extracted from cultured ACs with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and subsequently digested with DNase I to remove residual genomic DNA. RT-PCR was carried out using the Superscript preamplification system (Invitrogen, Carlsbad, CA, USA) to detect the expression of the *NCAM* gene as a pan-neural tissue marker [14] and elongation factor-1 alpha (EF-1α) [15] was used as an internal loading control. Primers for *NCAM* and EF-1α were designed according to the descriptions provided by Hawley *et al*. [6] and Hemmati-Brivanlou and Melton [16], respectively. The PCR conditions were as follows: 4 min at 94 °C, 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C for 30 cycles, and 10 min extension at 72 °C.

Results

Mutagenesis of noggin

Noggin contains a conservative spacing of seven cysteines, a motif characteristic of Kunitz-class protease inhibitors (KPIs) [17]. The most critical function of the cysteine-rich Kunitz domain is to mediate specific protein-protein interaction by forming bisulfide bonds between each protein. We artificially created cysteine to serine mutations at Cys168, Cys174 and Cys197 sites [**Fig. 1(A)**], and obtained three mutants, namely, C168S, C174S and C197S, respectively. Mutagenesis PCR reactions were carried out as described in "Materials and Methods". After digested with *Dpn*I, each of the PCR products showed a clear and single band of the same size as WT noggin when electrophoresized on agarose gel, whereas the negative control products did not show any bands. An optimal amount of digested PCR products was transformed into the competent *E. coli* strain XL1-Blue for multiplication. The purified plasmids were subjected to DNA sequencing to confirm the mutations [**Fig. 1(B)**]. The WT and mu-

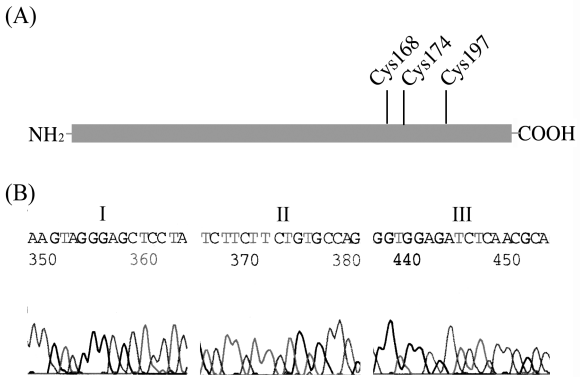

Fig. 1 The mutated sites in noggin protein (A) and the DNA sequencing of C168S, C174S and C197S (B)

I, II and III represent C168S, C174S and C197S mutations, respectively.

tated noggin mRNAs were synthesized by *in vitro* transcription, with the linearized WT and mutated noggin plasmids as templates. Each mRNA showed a single band at 1.32 kb. These mRNAs were quantified in comparison with an RNA ladder.

Analysis of the neuralizing effect of noggin mutants

Approximately 2 ng of the WT mRNA or each of the mutated noggin mRNAs was injected into each of the two blastomeres of the two-cell stage *Xenopus* embryos. The ACs were microdissected at stage 8.5–9 and cultured until the equivalent of stage 24. As shown in **Fig. 2**, WT mRNA-, C174S mRNA- or C197S mRNA-injected ACs exhibited a neural-like phenotype: slightly prolonged, partially white, and accompanied by a black spot (cement gland). In contrast, C168S mRNA-injected ACs, like the uninjected control ACs, sustained an epidermal phenotype: brownish and round, and without white/black delineation. To further confirm the neural differentiation, we examined the expression of the pan-neural marker *NCAM* using RT-PCR. The *NCAM* gene was expressed in the WT mRNA-, C174S mRNA- and C197S mRNA-injected ACs, but not in C168S mRNA-injected ACs (**Fig. 3**). These results suggest that Cys168 plays a critical role in the neuralizing effect of noggin.

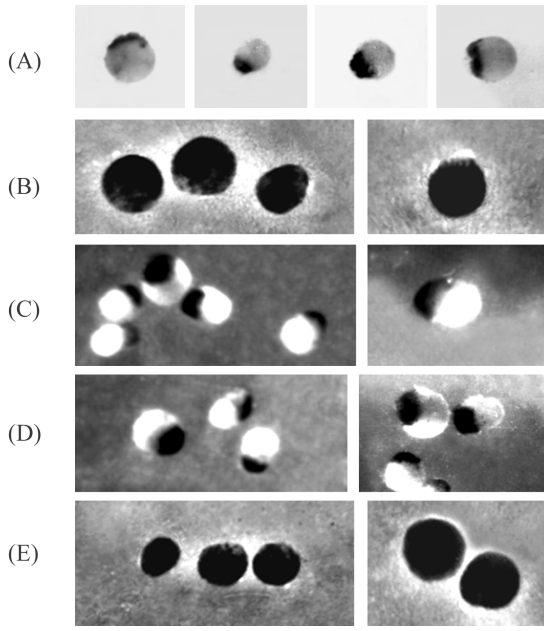

Fig. 2 Morphological observation of animal caps injected with WT and mutated noggin mRNAs

1–2 ng of mRNA was injected into the animal hemispheres of the two blastomeres at two-cell stage. Animal caps were dissected at stage 8.5–9 followed by culturing. At the equivalent of stage 24, the ACs injected with WT, C174S and C197S noggin mRNAs developed neural tissue and cement glands (A, C, D), while the ACs injected with C168S noggin mRNA and the negative control ACs showed epidermal morphology (B, E).

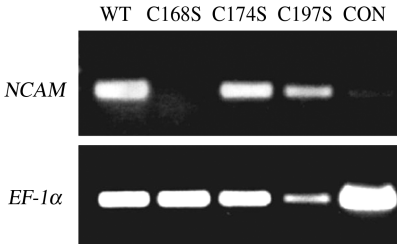

Fig. 3 RT-PCR analysis of *NCAM* gene expression in ACs

WT, C174S and C197S noggin mRNA-treated ACs expressed *NCAM*, whereas C168S noggin mRNA-treated ACs did not. EF-1α gene was used as an internal control.

Analysis of the dorsalizing effect of noggin mutants

The neural inducer noggin also possesses a dorsalizing effect in the mesoderm [3], another feature of an organizer factor. To investigate the functional changes of the noggin mutants in the mesoderm, we injected each of their mRNAs into the ventral marginal zone of the four-cell stage embryos. The injected embryos were allowed to develop to the tadpole stage and the DAI was calculated. As shown in **Table 1**, the WT mRNA-, C174S mRNA- and C197S mRNA-injected embryos were dorsalized with DAI scores

Table 1 Comparison of the dorsalizing effect of noggin and its mutants

No.	Dorsoanterior index (DAI)				
	WT noggin	C168S	C174S	C197S	Con
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
4	–	–	–	–	–
5	–	13.0	–	2.0	20.0
6	2.0	–	–	3.0	–
7	12.0	–	4.0	4.0	–
8	3.0	–	2.0	1.0	–
9	5.0	–	2.0	2.0	–
10	4.0	–	1.0	2.0	–
Mean	7.9	5.0	8.0	7.3	5.0

0.1–1 ng of WT noggin mRNA, C168S mRNA, C174S mRNA and C197S mRNA were injected into the ventral marginal zone of four-cell-stage embryos. The dead embryos were not counted. The numbers in this table indicate the frequency of each DAI score. Con, control.

of 7.9, 8.0 and 7.3, respectively, whereas the C168S mRNA-injected embryos, like the water-injected control embryos, remained normal with a DAI score of 5.0 for all of them. These results suggest that Cys168 is also crucial for noggin to exercise its dorsalizing effect in the mesoderm as well as its neuralizing effect in the ectoderm.

Discussion

In amphibia, neural induction occurs in the dorsal ectoderm during gastrulation when the dorsal mesoderm involutes beneath the ectoderm. Initially, the anterior mesoderm induces the overlying ectoderm to become anterior neural tissue (e.g. forebrain). Then a transformation posteriorizes the ectoderm to form more posterior structures such as the hindbrain and spinal cord [18]. Three molecules, noggin, chordin and follistatin, have been shown to exhibit anterior neural inducing effects *in vitro*, and are expressed in the right place (dorsal mesoderm) and at the right time (during gastrulation) to exert neurogenic effects [3,4,7,16,19]. Noggin is a secreted molecule that can induce anterior neural markers directly in naive gastrula ectoderm without inducing the mesoderm. It has been shown that BMP4 inhibits neuralization of the ectoderm and noggin physically binds the BMP4 ligand to block BMP4 signaling [9]. Neuralization is a default process of the ectodermal cells that occurs when the BMP4 signaling is inhibited by "neural inducers".

There are nine cysteine residues in noggin protein, among which seven match the pattern found in the KPI superfamily [17]. We believe that some of the cysteine residues in noggin are responsible for its biological activities during early *Xenopus* development. In the present study, we have made further investigations to analyze the molecular mechanism involved in noggin activity during neuralization. The three conservative cysteines were mutated to serines using the site-directed mutagenesis technique. The results showed that C174S or C197S mutation had no effect on the neuralizing and dorsalizing activities of noggin, while the C168S mutation abolished these biological effects.

The Kunitz domain is crucial for the inhibitory effect of KPI proteins. For example, the hepatocyte growth factor activator inhibitor-1 (HAI-1) contains two extracellular Kunitz domains. In tumorigenesis and tissue regeneration, HAI-1 inhibits the activity of the HGF activator (HGFA) and matriptase. A soluble form of HAI-1B comprising the entire extracellular domain has been found to display remarkable enzyme specificity by potently inhibiting HGFA, matriptase and trypsin [20]. The tissue factor pathway inhibitor-alpha (TFPI-α) contains three tandem Kunitz domains. Functionally, the first two Kunitz domains of the TFPI-α bind and inhibit the activity of factors IIa and Xa, respectively. The third Kunitz domain is involved in the cell surface localization of TFPI-α [21]. The inhibitory effect of the KPI is highly dependent on its cysteine residues. For instance, serine proteinase inhibitors have a modest effect on the processing of the potential beta-amyloid precursor protein from the brain, while cysteine modification completely inhibits them [22]. By replacing Cys168 of noggin protein with a serine residue, we observe a similar phenomenon: the cysteine residue is indispensable for the biological activities of noggin. Noggin fails to function biologically when this cysteine residue is mutated. How the cysteine residue affects the biological activities of noggin remains to be clarified. There are several possible ways by which Cys168 plays its role. First, it may form intramolecular disulfide bonds. Second, it may form an intermolecular disulfide bridge. Third, there is the possibility that it forms hydrogen bonds with other amino acid residues. It most likely participates in the formation of a disulfide bond between noggin and BMP4, or behaves within the noggin dimer as cysteine residues behave in other KPI factors, which can be investigated by further studies.

Recent studies have revealed that noggin has multiple functions *in vivo*. In cloned mouse embryonic stem (ES) cells, noggin can coordinate with BMP4 to regulate the progression of chondrogenic differentiation [23]. Mouse ES cells transfected with pCS2/noggin expression plasmids or cultured in noggin-conditioned medium can be directed to differentiate into neurons [24]. BMPs, BMP receptors and noggin are expressed in developing cerebral cortex, and *in vitro* experiments have shown that BMP and noggin co-regulate the differentiation and morphogenesis of cortical cells [25]. Various noggin mutations have been detected in patients with symphalangism, multiple synostosis syndrome, autosomal dominant stapes ankylosis and neural tube defects [26–30]. Taken together, an understanding of the structure-function relationship of noggin will be helpful in therapeutical cloning studies and will significantly contribute to fundamental and clinical research on some neural and boned diseases.

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