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Characterization of the Functionally Related Sites in the Neural Inducing Gene Noggin

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Previously we have shown that blocking bone morphogenetic protein (BMP) receptor signaling by a dominant negative BMP receptor causes neurogenesis in *Xenopus* animal caps (ACs), whereas the physiological neural inducer noggin acts as a homodimer physically binding to BMP-4 and disrupting its signaling at the ligand level. The present study attempted to elucidate the relationship between the structure and function of noggin. By replacing some cysteine residues with serine residues through a site-directed mutagenesis strategy, we generated three noggin mutants, C145S, C205S, and C(218, 220, 222)S (3CS). Although mRNAs encoded by these mutants were translated as efficiently as wild-type (WT) noggin mRNA, they behaved differently when expressed *in vivo*. Expression of WT noggin or C205S in *Xenopus* ACs converted the explants (prospective ectoderm) into neural tissue, indicated by the neural-like morphology and expression of the pan neural marker NCAM in the ACs. In contrast, ACs expressing C145S or 3CS sustained an epidermal fate like the control caps. Similar results were observed in the mesoderm where C205S (but not C145S and 3CS) displayed dorsalizing activity as well as WT noggin. Altogether, our results suggest that Cys145 alone or Cys(218, 220, 222) as a whole in noggin protein is required for the biological activities of noggin, probably participating in the dimerization of noggin with BMP-4 or itself. © 2000 Academic Press

The vertebrate nervous system arises in embryonic development from a portion of the ectoderm that is induced to form neural tissue through an interaction with another region of the embryo called the Spemann organizer (dorsal mesoderm). The role of the organizer in neural induction was first demonstrated in amphibian embryos when the dorsal lip of the blastopore, the first site of invagination at gastrulation, was transplanted from one blastula stage embryo onto the ventral side of another. The grafted organizer induced the ventral ectoderm that would normally have developed epidermis, to form neural tissue (1). More recent work suggests that this Spemann organizer signaling center secretes a cocktail of polypeptides that induce neural ectoderm (2).

The first molecule shown to have the property expected as an organizer signal, noggin, was identified in *Xenopus* embryos (3). Noggin is expressed in the organizer at the gastrula stage. It is a novel 32-kDa glycoprotein that is secreted as a homodimer. Soluble noggin protein induces anterior neural markers in animal cap (AC) ectoderm that would otherwise become epidermis (4). We, and other investigators, have demonstrated that the neuralizing activity of noggin is antagonized by bone morphogenetic protein 4 (BMP-4), whereas inhibiting the BMP signaling with a dominant negative BMP receptor (DN-BR) elicits neuralization in the AC tissue (5–8). Later on, an array of biochemical evidence proved that noggin binds BMP-4/DPP (the *Drosophila* homolog of BMP-4) with high affinity and inactivates BMP-4/DPP by blocking their binding to cognate cell-surface receptors (9, 10). The present studies were attempted to dissect the functionally related sites in the noggin molecule. Our results suggest that Cys145 alone or Cys(218, 220, and 222) as a whole in noggin protein is required for the neuralizing and dorsalizing activities of noggin.

MATERIALS AND METHODS

Site-directed mutagenesis of noggin. The noggin cDNA in pGEM5zf(−) was kindly provided by Dr. R. M. Harland (3). From the

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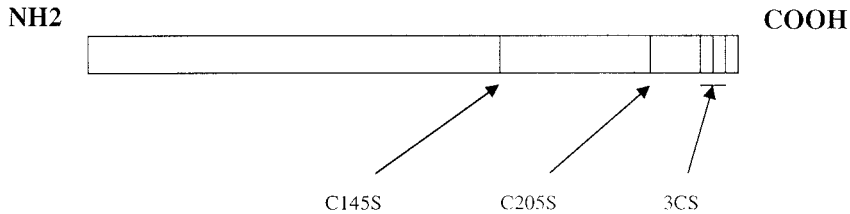

FIG. 1. Scheme for the mutated sites in noggin protein.

wild-type (WT) clone, we generated three mutated forms of noggin, C145S, C205S, and C(218, 220, and 222)S (3CS), by replacing cysteine residues at these sites with serine residues using the QuikChange Site-directed Mutagenesis kit (Clontech, Palo Alto, CA). Following primers were used for these mutations:

1. C145S: forward, 5′-CTGGTCCCAGACCTTCTCTCCTGTCCTT-TACACATG3-′; reverse: 5′-CATGTGTAAAGGACAGGAGAGAA-GGTCTGGGACCAG-3′.
2. C205S: forward, 5′-CAGGGTTCAGCAGAAGTCTGCGTGGAT-AACCATTC-3′; reverse, 5′-GAATGGTTATCCACGCAGACTTCT-GCT GAACCCTG-3′;
3. 3CS: forward, 5′-CATTTCCGAGTCCAAATCCTCATCCTGAG-ACTCTTG-3′; reverse, 5′-CAAGAGTCTCAGGATGAGGATTTG-GACTCGGAAATG-3′.

Mutagenesis PCRs were done in accordance with the manufacturer's instructions. Briefly, the PCR conditions were as follows: 30 s at 95°C, 1 min at 55°C, and 12 min at 68°C for 15 cycles. Part of the PCR products was digested with *Dpn*I at 37°C for 1 h followed by electrophoresis on 1% agarose gel to check the sizes. Non-digested PCR products were transformed into the competent *E. coli* strain XL1-Blue. Positive (mutated) clones were selected and confirmed by DNA sequencing.

In vitro RNA synthesis. The WT and mutated noggin plasmids were linearized by *Not*I and subjected to synthesis of capped mRNA using an *in vitro* transcription kit (Ambion, Austin, TX). The synthetic RNAs were quantified by ethidium bromide staining in comparison with a standard RNA.

In vitro translation. Synthetic RNAs were translated in a reticulocyte lysate system using the *In Vitro* Express translation kit (Stratagene, La Jolla, CA) in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The products of the translation reaction were subjected to SDS–PAGE. Dried gels were analyzed by autoradiography.

Xenopus embryo. Xenopus laevis adults were purchased from Beijing Institute of Developmental Biology, Beijing, and Shanghai Institute of Cell Biology, Academia Sinica, Shanghai, China. *Xenopus* embryos were obtained by *in vitro* fertilization after induction of females with 500 units of human chorionic gonadotropin. Developmental stages were designated according to Nieuwkoop and Faber (11).

Embryo injection, animal cap (AC) explant culture, and morphological observation. At the two-cell stage each blastomere of the embryos was injected with the synthesized mRNA. ACs were dissected at stages 8.5 to 9, cultured at 22°C in 0.3× MMR (NaCl 33 mM, KCl 0.67 mM, $MgSO_4$ 0.33 mM, $CaCl_2$ 0.67 mM, EDTA 0.03 mM, Hepes 1.67 mM, pH 7.8) until stage 24, and then harvested for morphological observation and reverse transcription-PCR (RT-PCR) assays. In some experiments, the mRNAs were each injected into the ventral marginal zone of the four-cell stage embryos. The injected embryos were allowed to develop until tadpole stages and the dorsoanterior index (DAI) was scored in accordance with the criteria as described by Kao and Elinson (12).

RT-PCR. Total RNA was extracted from cultured ACs with TRIzol reagent (GIBCO BRL, Bethesda, MD) according to the manufacturer's instructions, and subsequently digested with DNase I to remove genomic DNA. RT-PCR was carried out using the Superscript preamplification system (GIBCO BRL, Bethesda, MD) to detect the expression of NCAM as a pan-neural tissue marker (13) and elongation factor-1α (EF-1α) (14) as an internal loading control. Primers for NCAM and EF-1α were designed as described by Hawley *et al.* (6) and Hammati-Brivanlou and Melton (15), respectively. 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. Although data from individual experiments are shown, in all cases the results were confirmed independently.

RESULTS

Mutagenesis of noggin. Noggin has been reported to contain a conserved region of seven cysteines characteristic of a motif found in Kunitz class protease inhibitors (16). The cysteine-rich Kunitz domain can mediate specific interaction with another protein by forming bisulfide bonds between each other. We artificially created cysteine-to-serine mutations at Cys145, Cys205 and Cys(218, 220, and 222) (Fig. 1), and obtained three mutants C145S, C205S, and 3CS, respectively. The mutations were confirmed by DNA sequencing. mRNA was synthesized from each of the mutants as well as WT noggin. Translation efficiency of the mRNAs was tested (see Materials and Methods). As shown in Fig. 2, mRNAs encoded by all these mutants were translated at similar efficiency as WT noggin mRNA, suggesting that these mutations did not affect the translatability. Thus, we used these mRNAs for the following biological studies.

Analysis of neuralizing activity of the noggin mutants. At the two-cell stage, 0.2 ng of the WT or each of the mutated noggin mRNAs was injected into each of the two blastomeres of *Xenopus* embryos. ACs were

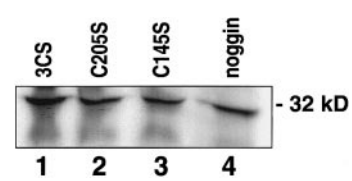

FIG. 2. Translation of WT and mutated noggin. Synthetic WT and mutated noggin mRNAs were translated in a reticulocyte lysate translation system in the presence of [^{35}S]methionine; products were analyzed by autoradiography on an SDS Tris–HCl Ready Gel (Bio-Rad, Hercules, CA). A 32-kDa band appeared at similar intensity in all the lanes loaded with the translation products.

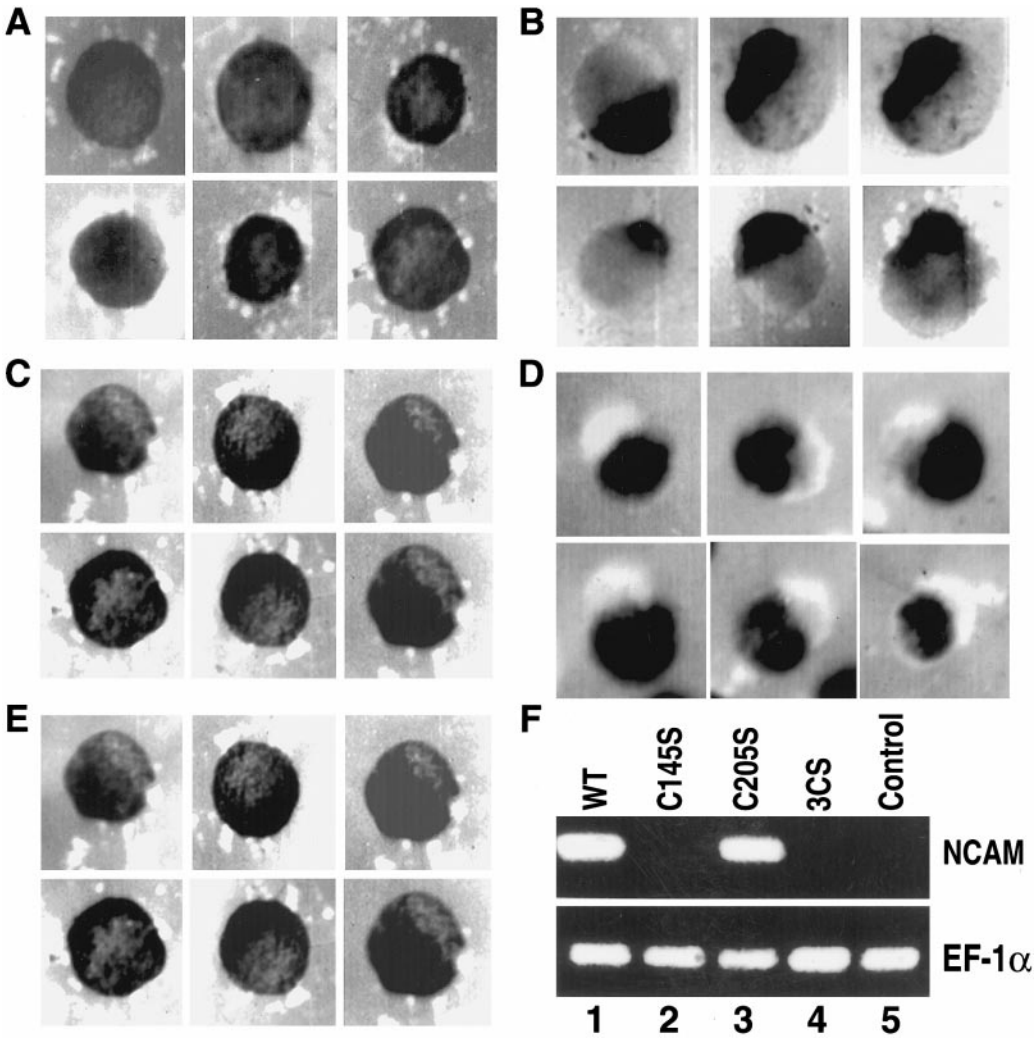

FIG. 3. Morphological observation and RT-PCR analysis of ACs expressing WT or mutated noggin. Water or 0.2 ng mRNA encoding WT noggin or each of the noggin mutants was injected into the animal hemispheres of the two blastomeres at the two-cell stage. ACs were dissected at stages 8.5–9 followed by culture. At equivalent of stage 24, ACs were harvested for photography (A to E) or RT-PCR analysis for expression of NCAM (a pan neural marker) and EF-1α (internal control) (F).

dissected at stages 8.5–9 and cultured until the equivalent of stage 24. As shown in Fig. 3, WT (B) or C205S (D) mRNA-injected ACs developed a neural-like phenotype: slightly swollen with one side being white while the other side black (cement gland). On the other hand, C145S (C) or 3CS (E) mRNA-injected ACs, like the water-injected control ACs (A), sustained an epidermal phenotype: basically round without clear white/black delineation. To confirm the neural differentiation, we examined the expression of the pan-neural marker NCAM using RT-PCR assay. NCAM was expressed only in the WT and C205S (but not C145S and 3CS) mRNA-injected ACs (F). These results suggest that Cys145 and Cys(218, 220, and 222) are uncompromising elements for noggin to exert its neuralizing activity.

Analysis of dorsalizing activity of the noggin mutants. The neural inducer noggin is known to also possess the dorsalizing effect in the mesoderm (3), another feature for an organizer factor. To investigate the functional consistency of the noggin mutants in the mesoderm as well as the ectoderm, we injected each of their mRNAs into the ventral marginal zone of the four-cell stage embryos. The injected embryos were allowed to develop until tadpole stages and the DAI was scored. As shown in Table 1, the WT or C205S mRNA-injected embryos were dorsalized with DAI 7.9 and 6.4, respectively. In contrast, the C145S or 3CS mRNA-injected embryos, like the water-injected control embryos, remained normal with DAI 5.0 for all. These results suggest that Cys145 and Cys(218, 220, and 222) are also crucial for noggin to execute dorsal-

TABLE 1

Comparison of Dorsalizing Activity of Noggin and Its Mutants

RNA injected	DAI
WT noggin	7.9
C145S	5.0
C205S	6.4
3CS	5.0
Water	5.0

Note. The four-cell-stage embryos were injected ventrally with water or 0.2 ng mRNA for WT noggin or each of the noggin mutants. The embryos were allowed to develop to tailbud stage and scored for DAI (12). Briefly, a normal embryo was scored 5, a completely ventralized embryo was scored 0, while a completely dorsalized embryo was scored 10. Other intermediate phenotypes were scored between 0 and 10 according to their degree in ventralization or dorsalization.

izing activity in the mesoderm as well as neuralizing activity in the ectoderm.

DISCUSSION

Previously, we have demonstrated that BMP-4 antagonizes the neuralizing effect of noggin in *Xenopus* AC, and blocking BMP activity with DN-BR brings about a neural fate in the ectodermal tissue (5). The anti-neurogenic action of BMP-4 later proved to be mediated by some components downstream of the BMP-4 signaling. We found that the erythroid transcription factor GATA-1b, but not its homologous gene GATA-1a or GATA-2, is able to negate the neuralizing activity of the DN-BR (17). We further proved that the homeobox-containing gene PV.1 (18) also specifies the ectoderm by repressing another neuralizing factor chordin that, like noggin, physically binds to BMP-4 (18, 19). GATA-1a, -1b, and -2 as well as PV.1 are inducible in response to BMP-4 (17, 18). Similar effect for msx.1, another BMP-4-inducible transcription factor, was reported by other investigators (20). In addition, other BMP members such as BMP-2 and -7 were also found to induce epidermis and inhibit neural tissue in the ectoderm (21). These findings suggest that neurogenic (noggin, chordin, etc.) versus anti-neurogenic (BMPs) signals co-specify the ectoderm to commit a neural versus epidermal fate. The neurogenic signals act via interruption of the BMP signaling either at its ligand level (by noggin or chordin) or receptor level (by the artificially made DN-BR).

In the present study, we furthered our investigation to analyze the molecular mechanism involved in the noggin actions during neuralization. We propose that some of the cysteine residues in noggin are responsible for its biological activities during early *Xenopus* development. Some cysteine-to-serine mutations were created by using the site-directed mutagenesis strategy. In comparison with WT noggin, while mutation at Cys205 had no effect on the neuralizing activity and only slight inhibition on the dorsalizing activity (DAI down from 7.9 to 6.4), mutations at Cys145 and Cys(218, 220, and 222) abrogated these biological activities (Fig. 3 and Table 1).

Noggin protein contains nine cystein residues, seven of which are within the carboxyl terminal region matching the pattern found in the Kunitz-type protease inhibitor (KPI) superfamily (16). The KPI domain is crucial for the inhibitory activity. For example, the tissue factor pathway inhibitor-1 (TFPI-1) contains three tandem KPI domains and has been well characterized for its role as a natural anticoagulant in the extrinsic coagulation pathway. Functionally, the first two KPI domains of the TFPI-1 bind and inhibit the activity of factor Xa and VIIa, respectively (22). The inhibitory effect of KPI is highly dependent on its cysteine residues. For instance, serine proteinase inhibitors had only modest effect in the processing activities of the potential β-amyloid precursor protein from brain, whereas cysteine modification completely inhibited them (23). By replacing Cys145 and Cys (218, 220, and 222) in noggin protein with serine residues, we observed a similar phenomenon: these cysteine residues are uncompromising for the biological activities of noggin. Noggin failed to function when these sites were mutated. How these cysteine residues contribute to the biological activities awaits to be defined. Most likely, they may participate in the formation of disulfide bonds in the noggin–noggin homeodimers and/or noggin–BMP-4 heterodimers as they behave in other KPI factors. Further studies are underway to address this possibility.

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