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Short Communication

Cysteine-rich region of X-Serrate-1 is required for activation of Notch signaling in *Xenopus* primary neurogenesis

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ABSTRACT The *Notch* family genes encode single-pass transmembrane proteins which function in a variety of cell fate specifications in invertebrates and vertebrates. In *Xenopus* primary neurogenesis, the Notch ligands, X-Delta-1 and X-Serrate-1, mediate Notch signaling and regulate cell differentiation. In the present study, we examined the role of the Serrate-specific cysteine-rich (CR) region in the primary neurogenesis of *Xenopus* embryos. The ligand constructs containing the DSL (Delta/ Serrate/ Lag-2) domain in the extracellular region caused a reduction in primary neurons, whereas the DSL-deleted form of X-Delta-1 resulted in the overproduction of primary neurons. However, the DSL-deleted form of X-Serrate-1 or the construct containing only the CR region in the extracellular domain (SerCR) reduced the number of primary neurons. In contrast, the CR-deleted form of X-Serrate-1 (SerΔCR) lost activity as a Notch ligand, regardless of the presence of the DSL domain within the extracellular domain. Overexpression of *X-Delta-1* and *X-Serrate-1* strongly induced the expression of *Xenopus ESR-1 (XESR-1)*, a gene related to *Drosophila Enhancer of split*. SerCR alone also moderately induced the expression of *XESR-1*, but the SerΔCR form did not induce this expression. Co-injection of *X-Notch-1ΔICD,* which deletes the intracellular domain (ICD), with *SerCR* suppressed a neurogenic phenotype, although co-injection of *X-Su(H)1^{DBM}* with *SerCR* did not, indicating that SerCR affects primary neurogenesis through the Notch/Su(H) pathway. These results suggest that the CR region of *Xenopus* Serrate is required for the activation of Notch signaling and cell fate specification in primary neurogenesis.

KEY WORDS: *X-Serrate-1, cysteine-rich region, Notch signaling, XESR-1, X-Delta-1*

The interactions of LIN12/ Notch family proteins play essential roles in cellular differentiation in a number of organs and tissues in most multicellular organisms (Greenwald, 1998; Artavanis-Tsakonas *et al.*, 1999). The Notch signaling pathway appears to be substantially conserved in vertebrates and invertebrates (Weinmaster, 1997). The core Notch signaling pathway members include DSL (Delta/ Serrate/ LAG-2) ligands, LIN12/Notch receptors, CSL (CBF1/ Suppressor of Hairless (Su(H))/ Lag-1) transcriptional cofactors and target genes such as the HES (Hairy/ Enhancer of Split) family of basic helix-loop-helix transcriptional regulators (Munn and Kopan, 2000). In a current model, the Notch signaling converts CSL from a transcriptional repressor to a transcriptional activator by a forming complex with the Notch intracellular domain in the nucleus (Weinmaster, 2000). During primary neurogenesis of *Xenopus laevis*, overexpression of one of the Notch ligands, X-Delta-1, inhibits neurogenesis, although it has not been established whether Notch receptors are activated in the process (Chitnis *et al.*, 1995). Another Notch ligand, X-Serrate-1, also has an analogous effect (Kiyota *et al.*, 2001). Here, we show a difference in the functional interactions with Notch receptors between two ligands in the regulation of primary neurogenesis. A variety of truncated forms were constructed from full-length cDNAs of X-Delta-1 and X-Serrate-1, and RNAs prepared by transcription of these constructs were injected into one of the blastomeres of two-cell stage embryos. Effect of the truncated form of X-Delta-1/ X-Serrate-1 on the primary neurogenesis was assessed by examination of the expression pattern of *N-tubulin*, a neuron-specific type-II ß-tubulin gene (Oschwald *et al.*, 1991), as a marker gene of

Abbreviations used in this paper: β-gal, β-galactosidase; C. elegans, Caenorhabditis elegans; CR, cysteine-rich; CSL, CBF1/ Suppressor of Hairless/ Lag-1; Dl, X-Delta-1; DBM, DNA binding mutant; DSL, Delta/ Serrate/ LAG-2; EGF, epidermal growth factor; ELR, epidermal growth factor-like repeats; GFP, green fluorescent protein; HES, Hairy/ Enhancer of Split; N-tubulin, a neuron-specific type-II β-tubulin gene; ICD, intracellular domain; NΔICD, X-Notch-1ΔICD; RT-PCR, reverse transcript-polymerase chain reaction; Ser, X-Serrate-1; SP, signal peptide; Su(H), Suppressor of Hairless; XESR-1, a Xenopus gene related to Drosophila Enhancer of split.

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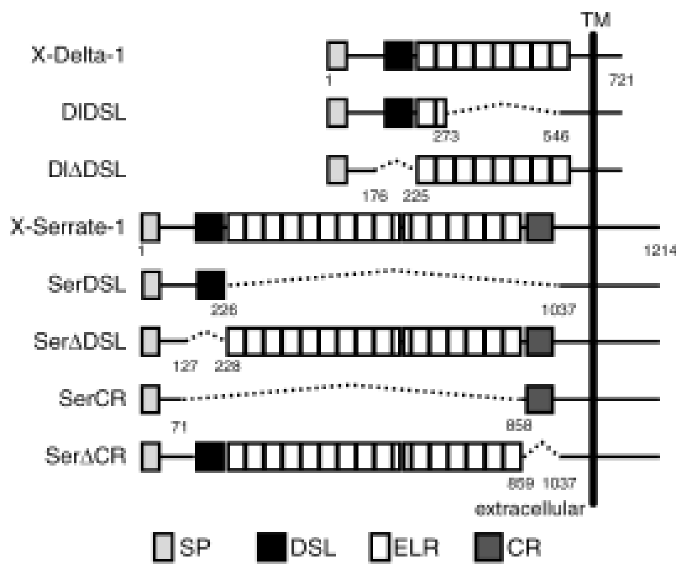

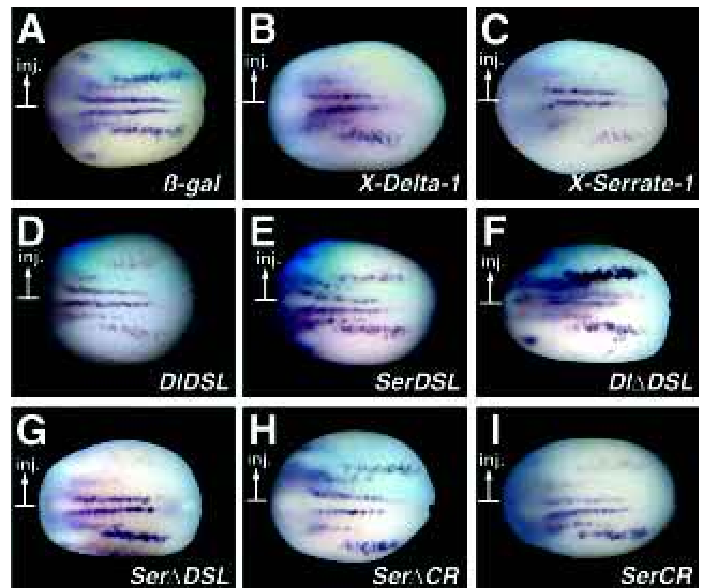

Fig. 1. (Left) Truncated forms of X-Delta-1 and X-Serrate-1. *Various truncation forms lacking portions of the extracellular domain of Notch ligands were produced. Numbers indicate the amino acid residue numbers. CR, cysteine-rich region; DSL, DSL domain; ELR, epidermal growth factor-like repeats; SP, signal peptide; TM, transmembrane.*

Fig. 2. (Right) Effect of Truncated-Notch ligands on primary neurogenesis. *Whole-mount* in situ *hybridization of* N-tubulin *(purplish blue) shows primary neurons (neurula stage, dorsal view, anterior to the left). The injected side labeled by X-gal staining (light-blue) for β-gal faces the top.* **(A)** *Injection of 1.0 ng* β-gal *RNA alone was used as a control.* **(B,C)** *Injection of 1.0 ng* X-Delta-1 *(B) or* X-Serrate-1 *(C) RNA. In both cases, a significant reduction of* N-tubulin *expression occurred in the injected side.* **(D,E)** Injection of *1.0 ng* DlDSL *(D) or* SerDSL *(E) RNA. In both cases, a moderate reduction of* N-tubulin *expression occurred in the injected side.* **(F,G)** *Injection of 1.0 ng* DlΔDSL *(F) or* SerΔDSL *(G) RNA. DlΔDSL caused overexpression of* N-tubulin, *whereas SerΔDSL showed a suppression of* N-tubulin *expression.* **(H)** *Injection of 1.0 ng* SerΔCR *RNA showed no effect on* N-tubulin *expression.* **(I)** *Injection of 1.0 ng* SerCR *RNA caused a reduction of* N-tubulin *expression.*

primary neurons. In addition, the expression of *XESR-1*, a target gene of Notch signaling (Wettstein *et al.*, 1997; Wittenberger *et al.*, 1999), was analyzed using an animal cap assay.

The CR Region is required for the X-Serrate-1-Mediated Regulation of Primary Neurogenesis

To investigate the functional domains of X-Delta-1 and X-Serrate-1 in *Xenopus* primary neurogenesis, various truncated forms were constructed from full-length cDNAs (Fig. 1). RNA transcripts of each construct were injected into one blastomere of a two-cell stage embryo, and primary neurogenesis was analyzed using *N-tubulin* at the neurula stage (Fig. 2 B-G, Table 1). Overexpression of DlDSL or SerDSL forms having a DSL motif in the extracellular domain caused a reduction of *N-tubulin*-expressing cells on the injected side, although this was moderate compared with the case of full-length X-Delta-1 or X-Serrate-1 (Fig. 2 B-E). The effect of deletion of the DSL motif was different between X-Delta-1 and X-Serrate-1. Injection of the form of X-Delta-1 lacking the DSL motif (DlΔDSL) caused the overproduction of *N-tubulin*-expressing cells on the injected side of the embryo (Fig. 2F), whereas the DSL-lacking form of X-Serrate-1 (SerΔDSL) reduced *N-tubulin*-expressing cells (Fig. 2G). A remarkable difference between X-Delta-1 and X-Serrate-1 is that X-Serrate-1 has a Serrate-specific cysteine-rich (CR) region between the epidermal growth factor (EGF)-like repeats and the transmembrane domain. To study the role of the CR region, we investigated the contribution of the CR region in the regulation of primary neurogenesis (Fig. 2 H,I, Table 1). Deletion of the CR region from X-Serrate-1 (SerΔCR) caused a loss of the suppressive effect on *N-tubulin*-expression (Fig. 2H), which is normally observed with full-length X-Serrate-1 (Fig. 2C). On the other hand, the form containing only the CR region of X-Serrate-1 in the extracellular region (SerCR) caused a signifi-

TABLE 1

EFFECT OF VARIOUS NOTCH LIGANDS ON PRIMARY NEUROGENESIS

Injected RNA	Amount (ng)	Total number of embryos	*N-tubulin* expression (%)		
			More	Unaffected	Fewer
ß-gal	(1.0)	21	0	100	0
X-Delta-1	(1.0)	34	0	12	88
X-Serrate-1	(1.0)	59	0	17	83
DlDSL	(1.0)	30	13	23	64
SerDSL	(1.0)	36	14	31	55
DlΔDSL	(1.0)	21	76	24	0
SerΔDSL	(1.0)	15	7	33	60
SerCR	(1.0)	30	0	33	67
	(0.3)	27	4	29	67
SerΔCR	(1.0)	20	10	80	10
NΔICD	(3.0)	14	64	18	18
NΔICD	(3.0)				
+ *SerCR*	(1.0)	20	20	45	35
NΔICD	(3.0)				
+ *X-Serrate-1*	(1.0)	13	15	31	54
X-Su(H)1^{DBM}	(1.0)	32	88	12	0
X-Su(H)1^{DBM}	(1.0)				
+ *SerCR*	(1.0)	22	82	13	5

The indicated amount of each RNA was injected into one blastomere of a 2-cell stage embryo and *N-tubulin* expression was examined at the neurula stage.

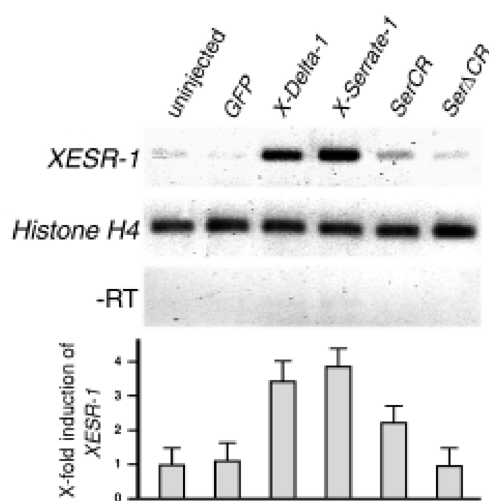

Fig. 3. *XESR-1* expression induced by injecting with *Notch* ligand RNA. (Upper panel) *Indicated RNAs (1ng) along with* Green fluorescent protein (GFP) *RNA (0.5ng) were injected into two-cell stage embryos. Animal cap was excised from the blastula stage (stage 8), cultured until the normal embryo reached stage 9.5, then assayed by quantitative RT-PCR for expression of* XESR-1 *and* Histone H4 *(internal marker). Injection of* X-Serrate-1 *or* X-Delta-1 *led to strong induction of* XESR-1 *expression. Moderate induction of* XESR-1 *was observed in the* SerCR*-injected sample. However, SerΔCR showed a significant reduction in the inductive capacity of* XESR-1 *expression, which was the same as the negative control (no injection or* GFP *RNA-injection).* **(Lower panel)** *Average levels of* XESR-1 *expression in triplicate samples. The level of uninjected sample was taken as 1.*

cant reduction of *N-tubulin*-expressing cells in the lateral stripe on the injected side (Fig. 2I). These results suggest that the Serrate-specific CR region plays a major role in X-Serrate-1-mediated regulation of primary neurogenesis.

The CR Domain of X-Serrate-1 is required for the Activation of XESR-1

The results described above showed that SerCR causes an inhibitory effect on primary neurogenesis, but SerΔCR does not. To observe if the effect depends on the canonical Notch-mediated signaling pathway, activation of the Notch target gene, *XESR-1*, was examined. We utilized the animal cap assay and quantitative reverse transcript-polymerase chain reaction (RT-PCR) for this analysis. As shown in Fig. 3, both wild-type X-Delta-1 and X-Serrate-1 caused strong induction of *XESR-1* expression. SerCR moderately induced the expression of *XESR-1*. However, SerΔCR showed as low an inductive capacity as the background level, regardless of the presence of the DSL domain within the extracellular region. These results indicate that the CR region of X-Serrate-1 plays a major role in the activation of *XESR-1* expression.

The CR Region of X-Serrate-1 controls Neurogenesis via the Notch/ Su(H) Pathway

To confirm that the activation of *XESR-1* expression by SerCR ligand is dependent on the Notch receptors, we examined the effect of a dominant-negative form of *Xenopus* Notch-1 (X-Notch-1ΔICD (NΔICD)), lacking the intracellular domain (ICD) (Greenwald, 1994). Injection of *NΔICD*-synthetic RNA caused the overproduction of primary neurons (Fig. 4A, Table 1), confirming the dominant-negative effect of the NΔICD form. Co-injection of *SerCR* RNA suppressed the *NΔICD*-induced *N-tubulin* expression, analogous to wild-type *X-Serrate-1* (Fig. 4 B,C, Table 1). Thus, the interaction of X-Serrate-1/ SerCR with their receptors, likely Notch receptors, can overcome the negative effect of NΔICD.

X-Su(H)1^{DBM} (DNA binding mutant (DBM) of X-Su(H)1), also has a dominant-negative effect downstream of the Notch receptor and caused the overproduction of primary neurons (Fig. 4D; Wettstein *et al.*, 1997). Co-expression of *SerCR* with *X-Su(H)1^{DBM}* did not overcome the dominant-negative effect of X-Su(H)1^{DBM} (Fig. 4E), a result expected if X-Su(H)1 is downstream of the SerCR receptor. These results support the idea that the inhibitory effect of the CR region of X-Serrate-1 on primary neurogenesis is mediated by the Notch/ Su(H) pathway.

From the data presented in this paper, we propose that the CR region of X-Serrate-1 has an important function in Notch signaling regulating *Xenopus* primary neurogenesis. In the *Caenorhabditis (C.) elegans* embryo, the *lag-2 (q411)* allele carries a nonsense mutation in the codon for the 79 amino acid between the NH_2-terminal and DSL domain, and results in the most severe Lag loss-of-function phenotype (Henderson *et al.*, 1994). In contrast, LAG-2(ΔEGF), a mutant LAG-2 protein lacking the EGF-like repeats, retains the full *lag-2* activity and rescues *lag-2(q411)*, indicating the dispensability of EGF-like repeats (Henderson *et al.*, 1997). In the present study, the DSL domain in both X-Delta-1 and X-Serrate-1 showed an inhibitory effect, although these activities were slightly weaker than those of full-length X-Delta-1/ X-Serrate-1 in primary neurogenesis (Kiyota *et al.*, 2001). These results suggest that the DSL domain of X-Delta-1 or X-Serrate-1 is functional without the other extracellular region of the ligand in the same way as *C. elegans* LAG-2.

The DSL domain of Notch ligands can interact with the EGF motif of Notch receptors in both *Drosophila* and vertebrates (Rebay *et al.*, 1991; Shimizu *et al.*, 2000). In the present study, DlΔDSL enhanced neurogenesis, indicating the loss of normal activity as a Notch ligand, and suggesting its interference with the interaction of endogenous X-Delta-1 and Notch receptors. This is consistent with the previous report of mouse embryo lacking the DSL domain of

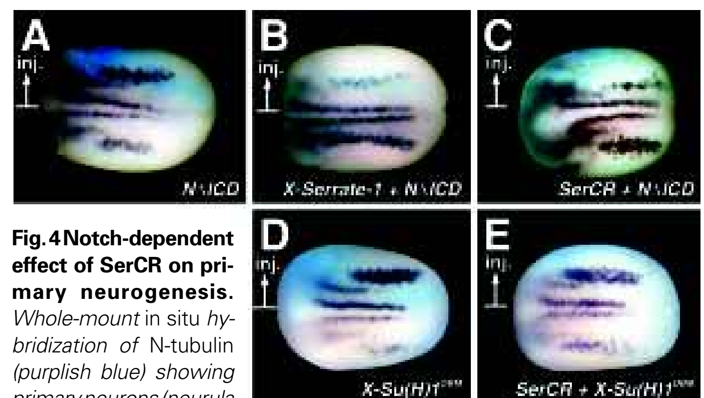

Fig. 4 Notch-dependent effect of SerCR on primary neurogenesis. *Whole-mount* in situ *hybridization of* N-tubulin *(purplish blue) showing primary neurons (neurula stage, dorsal view, anterior to the left). The injected side is top on each panel, confirmed by X-gal staining (light-blue) for β-gal.* **(A)** *Injection of 3.0 ng* NΔICD *RNA led to the overexpression of* N-tubulin. **(B,C)** *Co-injection of 3.0 ng* NΔICD *with 1.0 ng* X-Serrate-1 *(B) or 1.0 ng* SerCR *(C) RNA rescued the overexpression of* N-tubulin. **(D)** *Injection of 1.0 ng* X-Su(H)1^{DBM} *RNA produced a neurogenic phenotype.* **(E)** *The neurogenic phenotype caused by 1.0 ng* X-Su(H)1^{DBM} *was not rescued by co-injection of 1.0 ng* SerCR *RNA.*

Jagged2, a member of the mammalian Serrate family. In the homozygous mutant, histological defects occur in limb, craniofacial, and thymic development (Jiang *et al.*, 1998). In the present work, however, we demonstrated that the CR region has analogous activities since SerΔDSL or SerCR exerts an inhibitory effect and this is dependent on the canonical Notch signaling pathway on primary neurogenesis as if Notch signaling is activated. Furthermore, SerΔCR lacking the CR region lost the suppressive effect on *N-tubulin* expression and showed a significant reduction of inductive capacity for *XESR-1* expression, despite the existence of the DSL domain in the extracellular region. SerCR also moderately induced the expression of *XESR-1*. These results suggest that the CR region plays a major role in Serrate-mediating Notch signaling. Although SerDSL had a moderate inhibitory effect on *N-tubulin* expression, the activity was apparently lower than full-length X-Serrate-1 (Kiyota *et al.*, 2001). The lower capacity of SerDSL is probably the result of the structure lacking the CR region in the extracellular region. Overall, we demonstrated that the CR region of X-Serrate-1 is a second domain in addition to the DSL motif which is important for Notch signaling.

Experimental Procedures

The *X-Delta-1* clone inserted in pCS2+ (Turner and Weintraub, 1994) was digested with *Nco* I, and religated to produce *DlDSL*. To produce the *DlΔDSL* construct, the *Sca* I / *Sal* I fragment of *X-Delta-1* was replaced with the PCR fragment synthesized using primers:

5'-GGAATTCGCAGTACTCCACAGAACCAATTT-3' (upstream) and

5'-CAGCTATGACCTTGATTACGCCAAGCTCGA-3' (T3 primer) (downstream). To produce the *SerΔDSL* construct, the *Bgl* II fragment of *X-Serrate-1* inserted in pCS2+ was replaced with the PCR fragment synthesized using primers:

5'-GGAATTCAGATCTGCAATATGTCGTCAAGGC-3' (upstream) and

5'-TAAGTTGGGTAACGCCAGGGTTTTCCCAGT-3' (T7 primer) (downstream). To produce the *SerCR* construct, the *Bam* HI / *Stu* I fragment of *X-Serrate-1* was replaced with the *Bam* HI / *Dra* I fragment containing the 5' end of the *X-Serrate-1*. To produce the *SerΔCR* construct, the *Stu* I / *Bst* EII fragment of *X-Serrate-1* was replaced with the PCR fragment synthesized using primers:

5'-GGAATTCAGGCCTTGTAAGCGAGATGCCAA-3' (upstream) and T3 primer (downstream). To produce the *SerDSL* construct, the *Bam* HI / *Stu* I fragment of *SerΔCR* was replaced with the PCR fragment synthesized using primers: T3 primer (upstream) and 5'-GCTCTAGATCAAGTACTACATTCAGGCCCC-3' (downstream). The full-length X-Notch-1 gene (a gift from C. Kintner; Coffman *et al.*, 1990) was cloned into pCS+ vector at *Eco* RI site, and to produce the *NΔICD* construct, the *Sac* I / *Xho* I fragment of *X-Notch-1* was replaced with the PCR fragment synthesized using primers:

5'-GGAATTCACCGAGAGCTCGTGCTTTAATGG-3' (upstream) and

5'-GGAATTCCTCGAGGCTAGTTAACGATGACC-3' (downstream). Embryo manipulations, capped mRNA production, microinjection, *β–gal* staining, whole-mount *in situ* hybridization and quantitative RT-PCR were performed as described previously (Kiyota *et al.*, 2001). The *XESR-1* primers have been described previously (Wittenberger *et al.*, 1999). Annealing temperatures of PCR conditions for *XESR-1* and *Histone H4* were 58°C and 55°C, respectively. Amplification cycles of PCR products for *XESR-1* and *Histone H4* were 28 and 24, respectively. For the quantitative analysis of RT-PCR products, electrophoresed gel images were measured by FluorImager SI (Amersham Biosciences), and the average density level was calculated from triplicate samples.

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