Cerberus-like Is a Secreted BMP and Nodal Antagonist Not Essential for Mouse Development

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Summary: Mouse cerberus-like (cer-l) is a member of the Cerberus/Dan family of secreted factors. As members of this family of proteins, Cer-l functions in the extracellular space, inhibiting signaling molecules. Here we show that the neural-inducing and mesoderm-inhibiting activities of Cer-l result from specific binding to BMP and Nodal molecules, respectively. These properties resemble the ones from the related factor Xenopus Cerberus. However, Xenopus Cerberus in addition to BMP4 and Nodal also binds to and inhibits Wnt proteins. We show that Cer-l does not directly inhibit Wnt signals. A null allele of the mouse Cer-l gene was generated by targeted inactivation in ES cells. Homozygous embryos show no anterior patterning defects, are born alive, and are fertile. Since mouse Cer-l and Xenopus Cerberus differ in biochemical activities, we propose the existence of additional members of this family of inhibitors, which may compensate for the loss of cer-l.

Key words: cerberus-like; Cerberus; nodal; AVE; head induction

Mouse cerberus-like mRNA has been shown to be a potent neural inducer in Xenopus assays (Belo et al., 1997; Biben et al., 1998), inducing the same set of anterior neural markers as the related head-inducing molecule Xenopus cerberus (Bouwmeester et al., 1996; Piccolo et al., 1999). It has been suggested that this property could result from the inhibition of BMP signaling (Biben et al., 1998; Hsu et al., 1998; Pearse et al., 1999), as it is the case for the Xenopus neural-inducing molecules Chordin and Noggin (Piccolo et al., 1996; Zimmerman et al., 1996).

To determine the molecular mechanism of the cer-l neural-inducing activity, we prepared soluble Cer-l protein by transfection of human 293T cells. The protein was secreted into the culture medium as a product of 40–45 kDa that forms dimers under nonreducing conditions (data not shown) and was able to block BMP4 protein signaling in a dissociated animal cap assay (Wilson and Hemmati-Brivanlou, 1995; Piccolo et al., 1996).

In this assay, the neutralization caused by the dispersal of the inner layer of the animal cap is prevented by the addition of BMP4, which acts as an epidermal (keratin) inducer. Animal caps were cut at stage 9, the outer layer manually removed, and the inner cells were dissociated in a calcium magnesium-free medium for 4 h in presence or absence of protein (Fig. 1A). After 4 h, the inner cells were reaggregated by addition of calcium and magnesium to the medium and cultured until stage 20 to be processed for RT-PCR (Fig. 1B). Dissociated cells express NCAM but not cytokeratin (Fig. 1B, lane 2). In the presence of 2 nM of BMP4 protein, the dissociated cells retain their epidermal state, expressing cytokeratin but not NCAM (Fig. 1B, lane 3). A mixture of Mcer-l and BMP4 proteins (10 nM and 2 nM, respectively) prevents BMP4 to act as an anti-keratin reagent and restores the NCAM expression in the animal cap cells (Fig. 1B, lane 4).

To test the level at which this inhibition takes place, we performed an epistatic experiment in which cer-l mRNA was co-injected into Xenopus embryos with either BMP4 or a constitutively active form of BMP4 receptor (CABMPR) mRNA. Injections into all animal blastomeres were done at 8-cell stage, animal caps were cut at blastula, harvested at stage 27, and analyzed by RT-PCR (Fig. 1C). Microinjection of cer-l mRNA (800 pg) induced the expression of NCAM in animal caps (Fig. 1C, lane 3). Co-injection with BMP4 mRNA did not prevent NCAM expression, whereas co-injection with CA-BMPR mRNA abolished NCAM expression (Fig. 1C, lanes 4, 5).

We conclude from these results that Cer-l acts extracel-
and upstream of the BMP4 receptor, probably by binding to BMP4. To test this directly, we constructed an expression vector in which Cer-l was tagged at the carboxy-terminus with the hemagglutinin (HA) epitope. Soluble Cer-l-HA protein was obtained by transfecting human 293T cells and used in co-immunoprecipitation experiments. A mixture of Cer-l-HA and BMP4 proteins (20 nM and 5 nM, respectively) was incubated at room temperature and immunoprecipitated with anti-HA antibody. BMP4 was indeed bound by Cer-l, as detected on a Western blot by a monoclonal anti-BMP4 antibody (Fig. 2A). To prove that the binding is direct, dithiobis (succinimidyl) propionate (DSP) was used to chemically cross-link Cer-l-HA/BMP-4 complexes. After separation in nonreducing SDS polyacrylamide gels, the products of the reaction were visualized on Western blots with anti-BMP4 monoclonal antibody. As shown in Figure 2B, Cer-l bound to BMP4 protein both as a monomer and as a dimer. This strongly suggests that the neutralizing activity of Cer-l results from direct binding to BMP4, thus preventing activation of the BMP4 receptor.

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Injection of Mcer-l mRNA in Xenopus embryos also results in enlarged dorso-anterior structures and shortened body axes (Belo et al., 1997), indicating some degree of mesoderm inhibition. The related cerberus (Xcer) protein has been shown to be a potent mesoderm inhibitor by blocking the Nodal and Wnt pathways (Piccolo et al., 1999). To determine whether Cer-l blocks the Nodal pathway, we assayed the effects of Xnr-1 protein (Xenopus Nodal related-1) in animal cap cells. When these cells were incubated in the presence of 2 nM Xnr-1, the mesodermal markers Xbra and Xwnt-8 were induced (Fig. 3A, lane 3). However, in cells incubated with 2 nM Xnr-1 and 5 nM mouse Cer-l or Xenopus Cerberus proteins, the induction of these mesodermal markers was abolished (Fig. 3A, lanes 4, 5).

To test whether the inhibition of Xnr-1 took place upstream or downstream of the nodal receptor, we performed epistatic experiments with a constitutively active form of the activin receptor (CA-ALK4; Chang et al., 1997). Microinjection of animal caps with mouse Nodal or Xnr-1 mRNA (Fig. 3B, lanes 4, 6, respectively) induced the expression of Xbra and Sox17β, a pan-endodermal marker. Co-injection of mouse cer-l mRNA completely blocked mouse Nodal activity and inhibited Xnr-1 as well (Fig. 3B, lanes 5, 7). Injection of CA-ALK4 also leads to the induction of Xbra and Sox17β. This activity could not be prevented by co-injection with mouse cer-l mRNA (Fig. 3B, lanes 8, 9). This experiment suggests that cer-l inhibits nodal signaling extracellularly, upstream of the receptor.

To investigate whether cer-l is also able to block the Wnt pathway, we first performed microinjections into Xenopus embryos. Although we observed that mouse cer-l mRNA antagonized secondary axis formation by Xwnt-8 (data not shown), we reasoned that this could be due to the mesoderm-inhibiting (anti-Nodal) activity of Cer-l, rather than to a direct effect on the Wnt pathway itself. To test this, we performed a set of epistatic experiments in animal caps using the direct downstream target of induction of the mesodermal marker Xbra. EF1α was used as a loading control. (b) cer-l mRNA is an inhibitor of mouse Nodal signaling in the Xenopus animal cap assay. Lane 1, control embryo. Lane 2, control animal caps. Lane 3, animal caps incubated with 2 nM Xnr-1. Lane 4, animal caps incubated with 2 nM Xnr-1 and 5 nM Xenopus Cerberus protein. Lane 5, animal caps incubated with Xnr-1 and 5 nM mouse Cer-l protein. Note the lack of induction of the mesodermal marker Xbra. EF1α was used as a loading control.

To determine the requirement of cer-l during mouse development, we generated a null allele of the gene by deleting most of the first and the entire second exon (Fig. 5A). Cell lines that had undergone homologous recombination were identified by Southern blot and one of them injected into C57BL/6 blastocysts. The chimeric males obtained were subsequently mated with wild-type 129/Sv or C57BL/6 females.

**cer-l**/− animals in the 129/Sv pure inbred background were intercrossed and the offspring genotyped by Southern and PCR analysis (Fig. 5B, C). Homozygous mutant pups were recovered at a mendelian ratio (28 of
METHODS

DNA Expression Constructs

An HA-tagged version of the full-length cer-l cDNA (pCS2-Mcrl-HA) was constructed by PCR. The sequence YPYDVPDYA-Stop was added after the last amino acid of cer-l and the entire sequence inserted into pCS2. To generate an active form of mouse Nodal, we used pCS2-proAct, an expression vector containing the pre-pro region and proteolytic cleavage site of activin-βB (Kessler and Melton, 1995; Piccolo et al., 1999). A PCR fragment encoding the mature region of mouse Nodal (starting at residue H242) was generated by PCR amplification using primers flanked by Xbol and Xbal sites and subcloned into pCS2-proAct, resulting in the pCS2-ProActMnodal plasmid. cer-l-Act5B-Mnodal sense mRNA were synthesized using SP6 polymerase after linearization with NotI (Bouwmeester et al., 1996). All other mRNAs used in microinjection experiments were generated and microinjected as described by Piccolo et al. (1999).

Cell Culture and Protein Binding

Soluble Cer-l and Cer-HA proteins were obtained by transfection of embryonic human kidney cells (293T) as described (Belo et al., 1997). Production of soluble Cer and Xnr-l proteins by secretion of manually defolliculated Xenopus oocytes, protein incubation, and binding experiments were performed as described (Piccolo et al., 1999).

Generation of Chimeric Mice

cer-l cDNA was used to screen a 129/Sv mouse genomic DNA λ phage library (Stratagene) and resulted in the isolation of two 6.0 kb EcoRI fragments. The targeting construct was generated in the pPNT plasmid (Tybulewicz et al., 1991) by replacing a 3.5-kb BglII/KpnI fragment containing most of the exon I and the entire exon II by an IRES-lacZ-Neo cassette. This was flanked by a homologous 2.5-kb 5’ region and a homologous 3.0-kb 3’ region. An HSV-TK cassette was inserted at the end of the 3’ arm for negative selection, generating the targeting vector pPNT-McrlKO. The IRES-lacZ-Neo cassette introduced novel endonuclease restriction sites. Regrettably, the IRES sequence used in this construction had a deletion of several nucleotides, and the targeted strain did not express lacZ.

pPNT-McrlKO was linearized at a unique NotI site and electroporated into RW-4 ES cells (Genome Systems). G418-resistant colonies were isolated and analyzed for correct homologous recombination. ES cell manipulation, recombination, and generation of chimeric mice were done as described (Robertson et al., 1987). Alcian blue-alizarin red skeletal staining was performed as previously described (Belo et al., 1998) and images were acquired using a Leica DC 200 digital camera.
FIG. 5. A targeted null mutation of the mouse cerberus-like gene. (a) Schematic representation of the targeted deletion of the cer-I gene. Restriction map of the 129/Sv murine cer-I genomic clone, of the targeting construct, and of the recombinant allele. The two targeted exons are depicted here as filled boxes (I and II). The targeting construct contained an IRES-LacZ-Neo cassette and was flanked by 5' and 3' homology regions. In addition, an HSV-TK cassette was inserted at the end of the 3' arm to allow for negative selection. The 5' and 3' external probes used in Southern blot analysis, after Pst I digest, are shown as white rectangles; the Southern blots confirmed that homologous recombination had taken place. Pairs of PCR primers used to genotype ES cells clones are indicated by arrows. White arrowheads represent PCR primers used for the genotyping of newborns. Bg, Bgl II; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I. (b) Southern blot analysis of DNA obtained from cer-I heterozygous intercrosses. DNA was digested with Pst I and hybridized with the 3' external probe. (c) PCR analyses of intercrosses. (d, e) Dorsal views of the base of the cranium of neonate mouse skeletons stained with Alcian blue and alizarin red: D, low-power view of wild-type neonate; E, same view of a cer-I−/− littermate. BO, basioccipital; BS, basisphenoid; PS, presphenoid.
DNA Analysis

DNA was extracted from ES cell clones, mouse newborns or adult tails using standard procedures. Clones presenting proper homologous recombination at the cerl locus were identified by PCR (see Fig. 5) using the following primers: P1-cer-l-F (5'-GCT TGG GCT TGT TTC CTT GTG ATG ACT GGT-3') and P3 IRES-R (5'-TGC TTC CTT CAC GAC ATT CAA CAG ACC TT-3') for 5' recombination; P6-Neo-F (5'-GTT CCA CAT ACA CTT CAT TCT CAG-3') and P4-cer-l-R (5'-TAA GGC TTC CAA ACA CAT AGT AAA GTC TCA-3') for 3' recombination. Correct targeting was confirmed by Southern blot analysis after PstI digestion using the two external probes indicated in Figure 5.

Genotyping of mouse offspring was routinely determined by PCR using the three oligonucleotides: Mcerl-Forward (5'-ACC CAC CTG CTG ACC ACC TGC TTC), Mcerl-L4-Reverse (5'-CTC TTT CTA TTT TGC CGT), and P3-IRES-R (indicated as white triangles in Fig. 5), which resulted in bands of 700 and 500 bp for the wild-type and mutant alleles, respectively.

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LITERATURE CITED


