 Requirement of Glycosylphosphatidylinositol Anchor of Cripto-1 for trans Activity as a Nodal Co-receptor*

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Cripto-1 (CR-1) has an indispensable role as a Nodal co-receptor for patterning of body axis in embryonic development. CR-1 is reported to have a paracrine activity as a Nodal co-receptor, although CR-1 is primarily produced as a glycosylphosphatidylinositol (GPI)-anchored membrane protein. Regulation of cis and trans function of CR-1 should be important to establish the precise body patterning. However, the mechanism by which GPI-anchored CR-1 can act in trans is not well known. Here we confirmed the paracrine activity of CR-1 by fluorescent cell-labeling and immunofluorescent staining. We generated COOH-terminal-truncated soluble forms of CR-1 based on the attachment site for the GPI moiety (ω-site), which we identified in the present study. GPI-anchored CR-1 has a significantly higher activity than COOH-terminal-truncated soluble forms to induce Nodal signal in trans as well as in cis. Moreover, transmembrane forms of CR-1 partially retained their ability to induce Nodal signaling only when type I receptor Activin-like kinase 4 was overexpressed. NTERA2/D1 cells, which express endogenous CR-1, lost the cell-surface expression of CR-1 after phosphatidylinositol-phospholipase C treatment and became refractory to stimulation of Nodal. These observations suggest that GPI attachment of CR-1 is required for the paracrine activity as a Nodal co-receptor.

Establishment of the basic body plan of vertebrates is strictly regulated by a number of morphogens and signaling molecules such as Wnts, Hedgehog, fibroblast growth factors, and members of transforming growth factor β family ligands (1–4). Among them, a transforming growth factor β family ligand, Nodal, is known to be indispensable for early embryonic development. Nodal acts as a morphogen to initiate gastrulation and to establish the anterior-posterior and the left-right body axis (5, 6). Nodal utilizes a shared signaling pathway with other transforming growth factor β family ligands such as Activin, which activates Smad2/3 through phosphorylation to interact with Smad4. This oligomeric complex then translocates into nucleus to regulate transcription of target genes through a tran-scriptional co-factor, FOXH-1 (Fast-1) (5, 7). Unlike other transforming growth factor β family ligands, Nodal requires epidermal growth factor-Cripto-1/FRL-1/Cryptic (EGF-CFC) family proteins to bind its type I receptor Activin-like kinase 4 (ALK4) (5, 8). To regulate precise formation of body axis, the spatiotemporal activity of these extracellular molecules should be strictly regulated. Expression and localization of Nodal itself are known to be regulated by several mechanisms (5). For example, Nodal gene expression is strictly regulated by node-specific enhancer and left-side-specific intronic enhancer (9, 10). In addition, laminar leftward flow, which is generated by constant movement of cilia of embryonic cells, contributes to the left-side localization of Nodal ligand (11, 12). Requirement for EGF-CFC proteins as co-receptors for Nodal suggests that the activity of Nodal signaling could also be regulated by the spatiotemporal localization and function of EGF-CFC proteins.

EGF-CFC proteins contain several domains that include an NH₂-terminal signal peptide, a variant EGF-like domain, a cysteine-rich CFC domain, and a hydrophobic membrane-associated domain at the COOH terminus. Most of EGF-CFC proteins including human/mouse Cripto-1 (CR-1/Cr-1) have been experimentally shown (13) or predicted (14) to possess a glycosylphosphatidylinositol (GPI) anchorage. Although EGF-CFC proteins are primarily synthesized as GPI-anchored membrane proteins, several studies have shown trans activity of EGF-CFC proteins in vitro and in vivo (15–19). A chimeric mouse model revealed that cells derived from Cr-1-null embryonic stem (ES) cells can contribute to the formation of mesendoderm in the presence of Cr-1 wild-type cells (15, 17). In addition, COOH-terminal-deleted forms of Cr-1 or recombinant Cr-1 protein could rescue the One eye pinhead (oep)-mutant phenotype in zebrafish (18) or could induce cardiomyocyte differentiation in Cr-1 null ES cells (19). However, the mechanism by which GPI-anchored forms of EGF-CFC proteins can act as soluble co-receptors for Nodal signaling is not known.

In the current study we generated several variants of human CR-1 including soluble and transmembrane forms based on the

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identified ω-site of human CR-1 for GPI attachment. A comparative analysis of these variants revealed that the GPI-signal sequence of CR-1 is necessary to induce optimum Nodal signaling in trans as well as in cis.

EXPERIMENTAL PROCEDURES

Cells—HEK293T (293T) cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. NTERA2/D1 cells (ATCC) were grown in Macoy’s 5A medium with 15% fetal bovine serum. 293T cells stably transfected with wild-type CR-1 were grown in Dulbecco’s modified Eagle’s medium containing 0.5 mg/ml G418 and 10% fetal bovine serum. NTERA2/D1 cells (20). All CR-1-related constructs were generated by PCR-based methods and cloned into pCI neo vector (Promega, Fitchburg, WI) or into pEF6/V5-His TOPO TA expression vector (Invitrogen). Experiments related to Fig. 8 were performed using pEF6/V5-His-based constructs, and all other experiments were performed with pCI neo-based constructs. A stop codon (TGA) was inserted just after Ser-161 or Ser-169 of the transmembrane portion of ErbB4 (amino acids 651–683) with a FLAG tag was inserted just after Ser-161. Point mutations were inserted using QuikChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) using primers shown in Table 1. The pEF6/V5-His-related constructs were generated by the TOPO TA cloning method. DNA sequences were validated by direct sequencing. DsRed-Monomer-Golgi expression vector was purchased from Clontech (Mountain View, CA). Other expression vectors were described previously (20). Transfections were performed using Lipofectamine 2000 (Invitrogen).

Immunofluorescence and Fluorescent Cell Labeling—Cells were seeded in 4-well-chambered coverglass (Nunc, Rochester, NY) that had been coated with poly-L-lysine (Sigma-Aldrich) to avoid detachment of cells. For cell-labeling and co-culture assay, 293T cells stably transfected with wild-type CR-1 or NTERA2/D1 cells that express endogenous CR-1 were labeled with CellTracker green 5-chloromethylfluorescein (Invitrogen) before co-culture. After co-culturing overnight in the regular growth medium, cells were serum-starved for 24 h, and a 200 or 50 ng/ml final concentrations of Nodal or Activin, respectively, were added without changing the medium and stimulated for 1 h. After washing with phosphate-buffered saline, cells were fixed in 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. After blocking with phosphate-buffered saline containing 10% bovine serum albumin and 10% normal goat serum, samples were incubated with 1:200 dilution of anti-total Smad2 antibody overnight at 4 °C. Primary antibody was then labeled with Alexa Fluor 596-conjugated secondary antibody (1:200, Invitrogen). For co-staining of CR-1 and Smad2, CR-1 staining was carried out for 1 h at room temperature with 5 µg/ml MAB2771 that had been labeled with Alexa Fluor 488 using Zenon tricolor mouse IgG1 labeling kit (Invitrogen). Counterstaining for nuclei was performed with DAPI. For quantification of nuclear Smad2-positive cells, images were taken with a fluorescent microscope equipped with a 40× objective lens, and only cells with clear nuclear staining of Smad2 were counted as nuclear Smad2-positive cells. Counting was performed in blinded manner. For each experiment total 177–249 cells/field were counted in two different fields, and three independent experiments were performed.

For studies on the intracellular localization of CR-1 mutants, 293T cells were transiently transfected with each CR-1 mutant vector and the DsRed-Golgi expression vector. After 24 h of transfection, fixation and permeabilization were performed as described above, and CR-1 proteins were stained with 5 µg/ml MAB2771 and Alexa Fluor 488-conjugated secondary antibody. Endoplasmic reticulum (ER) was visualized with anti-calnexin rabbit polyclonal antibody (Santa Cruz, Santa Cruz, CA) at 1:250 dilution and Alexa Fluor 596-conjugated secondary antibody (1:200, Invitrogen). Images were taken with a Zeiss LSM 510 NLO Meta confocal system (Carl Zeiss, Göttingen.

TABLE 1

| Mutant          | Mutagenesis primer sequence |
|-----------------|-----------------------------|
| S161L           | GATGGACACCTCTGGCTCCTAGACCTCAAGAATCCAACCCG |
| S161L/R162L     | GATGGACACCTCTGGCTCCTGTGACCTCAGAATCCAACCCG |
| S161L/R162L/T163L | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| S169L           | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| S169L/A170L     | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| S161N           | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| T88A            | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| F94A            | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |

Primer sequences used for site-directed mutagenesis

Mutated nucleotides are underlined.

| Mutant          | Mutagenesis primer sequence |
|-----------------|-----------------------------|
| S161L           | GATGGACACCTCTGGCTCCTAGACCTCAAGAATCCAACCCG |
| S161L/R162L     | GATGGACACCTCTGGCTCCTGTGACCTCAGAATCCAACCCG |
| S161L/R162L/T163L | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| S169L           | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| S169L/A170L     | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| S161N           | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| T88A            | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
| F94A            | ACTCCGAACACTACCAACCCGTTTGCACTTACTACCACTTTTTGTTAATACTA |
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Germany) with an Axiovert 200M inverted microscope equipped with a 63× NA 1.4 Plan–APOCHROMAT oil immersion objective lens. Images were collected with Zeiss AIM software using a multi-track configuration.

Western Blot Analysis—Western blot analysis was performed using 16% (for detection of CR-1 protein) or 10% (for detection of phospho- and total Smad2) SDS–PAGE gels (Invitrogen). Depending on the experiments, 30–50 μg of total cell lysates or 40 μl of conditioned medium were loaded. CR-1 protein was detected with B3F6 mAb at a 1:5000 dilution and anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:3000; Amersham Biosciences). All images of Western blot analysis in this work were visualized, processed, and quantified with an Image Analyzer equipped with LabWorks software (Ultra Violet and Laboratory Products, Upland, CA).

Phase Separation—Phase separation by Triton X-114 was performed as previously described (23). Cells were lysed in TX-114 solution (20 mM Tris, pH 8.0, 150 mM NaCl, 2% TX-114) for 1 h on ice. Phase separation was carried out by warming up to 37 °C and subsequent centrifugation at 3000 × g at 25 °C. The upper aqueous phase and the lower detergent phase were collected with micropipettes carefully. Before Western blotting, proteins were precipitated with chloroform-methanol (1:4) to remove the detergent.

Preparation of Conditioned Medium—293T transfectants were incubated with Opti-MEM serum-free medium (Invitrogen) for 24 h. Conditioned medium was collected and centrifuged at 3000 × g for 5 min to remove cellular debris. Recombinant Nodal protein was then added at a final concentration of 200 ng/ml and immediately used for stimulation of cells.

Dual Luciferase Assay—293T cells, which had been plated in 24-well culture plates, were transfected with an optimized amount of expression vectors ((n2)7-Luc, 50 ng/well; TK-renulla, 5 ng/well; mFast-1, 25 ng/well; mNodal-V5, 100 ng/well; ALK4-HA, 50 ng/well; each CR-1 mutant, 5–1000 ng/well). Depending on the experiments, recombinant Nodal or Activin B (R&D Systems) were used instead of mNodal-V5 expression vector. pCINeo or pEF6/V5-His empty vector was added to adjust for total amount of DNA. After 24 h of transfection or addition of ligands, dual luciferase assays were carried out using a kit provided by Promega according to the manufacturer’s instructions.

Co-immunoprecipitation Assay—Co-immunoprecipitation of CR-1 mutants and ALK4 or Nodal was performed as described previously (16, 21). 293T cells were transfected with CR-1-related expression vectors and ALK4-HA or Nodal-V5. Whole cell lysates were prepared 24 h after transfection using modified radioimmune precipitation assay buffer (50 mM NaF, 20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 5 mM sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 mM EDTA) with complete protease inhibitor mixture (Roche Applied Science) and subjected to immunoprecipitation using an anti-HA or anti-V5 antibody. For detection of the association with Nodal-V5 on the cell membrane, cross-linking with membrane-impermeable reversible cross-linker, 3,3′-dithiobis(sulfosuccinimidyl-propionate) (DTSSP; 0.5 mM, Calbiochem) was carried out 1 h before preparation of cell lysates.

Fluorescence-activated Cell Sorting (FACS) Analysis—293T transfectants or NTERA2/D1 cells were collected with enzyme-free cell dissociation buffer (phosphate-buffered saline containing 4 mM EDTA). After washing with ice-cold FACS buffer (phosphate-buffered saline with 0.1% bovine serum albumin), 1.0 × 105 cells were incubated for 20 min with anti-human CR-1 phycoerythrin (PE)-conjugated antibody (FAB2772P) at a dilution of 1:50. Cells were then pelleted, resuspended in 500 μl of ice-cold FACS buffer, and analyzed using a FACSScan instrument (BD Biosciences).

Statistical Analysis—Student’s t test was used to determine the statistical significance of the quantitative results. Results with a p value <0.05 were considered statistically significant.

RESULTS

Paracrine Activity of Wild-type CR-1—To confirm the paracrine function of human CR-1, we demonstrated the direct visualization of the trans activity of CR-1 in cell culture using immunofluorescence and fluorescent cell-labeling. Transient transfection of wild-type (WT) CR-1 into 293T cells that do not express CR-1 (16) achieved up to 50–60% transfection efficiency resulting in the mixed population of CR-1 positive (transfected) and negative (untransfected) cells. We assessed the effect of Nodal and Activin on translocation of the Nodal/Activin intracellular mediator, Smad2, into nuclei by immunofluorescent staining. The localization of Smad2 in 293T cells that had been transfected with empty vector (EV) was mainly cytosolic (data not shown), and these cells did not respond to Nodal stimulation (Fig. 1A). 293T cells transfected with WT CR-1 also showed cytosolic localization of Smad2 without stimulation (Fig. 1B). In contrast to EV-transfected cells, stimulation of WT CR-1-transfected cells with Nodal induced Smad2 nuclear translocation in ~70–80% of the total population (Fig. 1C). Smad2 nuclear localization was observed both in CR-1-staining-positive cells and in CR-1-staining-negative cells (Fig. 1C, arrowheads). Activin stimulated Smad2 nuclear translocation in almost 90–100% of the WT CR-1-transfected cells (Fig. 1D). This effect of Activin was also observed in EV-transfected or untransfected 293T cells (data not shown), demonstrating the CR-1-independent activity of Activin on Smad2 nuclear translocation.

These data strongly indicate the paracrine activity of CR-1 in Nodal signaling. However, it was possible that WT CR-1-transfected cells, which did not show positive staining for CR-1, might express an undetectable but functional level of CR-1 protein. To exclude this possibility, we performed co-culture experiments of CR-1-stably transfected 293T cells and wild-type 293T cells (Fig. 1E) using a cell-labeling technique to segregate the CR-1-positive and negative cells (Fig. 1F). Consistent with the observation in transiently transfected 293T cells (Fig. 1A), a mixture of untransfected 293T cells and labeled stable transfectants of EV did not respond to Nodal (Fig. 1G). In contrast, Nodal stimulation of the mixture of untransfected 293T cells and labeled stable transfectants of WT CR-1 significantly induced Smad2 nuclear translocation not only in CR-1-positive cells but also in ~16% of the CR-1-negative cells even though only 10% of the mixed population was the labeled, WT CR-1 stable transfectants (Fig. 1, H and J). Activin was more potent
FIGURE 1. Visualization of paracrine activity of CR-1. A–D, 293T cells that had been transiently transfected with EV or wild-type CR-1 expression vector were cultured in serum-free medium for 24 h and stimulated with Nodal or Activin for 1 h. Nodal or Activin addition was performed without changing the medium at final concentrations of 200 or 50 ng/ml, respectively. Cells were stained with anti-CR-1 antibody (A1–D1) and anti-Smad2 antibody (A2–D2) and analyzed by a fluorescent microscope. Arrowheads in C, nuclear Smad2-positive, CR-1-negative cells. Nuclear staining with DAPI is shown in 1–D3. Scale bar = 15 μm. E, validation of stable transfectants of EV or WT of CR-1 by FACS analysis. Cell surface expression of CR-1 in EV- or WT CR-1-stably transfected 293T cells was analyzed by live cell staining of the cells with PE-conjugated anti-CR-1 antibody followed by FACS analysis. Staining of WT CR-1-transfectants with PE-conjugated normal mouse IgG1 (IgG1-PE) is shown as a negative control. F, method for co-culture experiments. G–I, 293T cells that had been stably transfected with an EV or WT CR-1 expression vector were labeled with CellTracker green (G1–I1). After 24 h of co-culture, 293T cells were stimulated with Nodal or Activin as described for A–D and stained with anti-Smad2 antibody (G2–I2). Nuclear staining with DAPI is shown in G3–I3. Solid arrowheads, nuclear Smad2-positive, unlabeled cells. Open arrowheads, nuclear Smad2-positive, labeled cells. Scale bar = 20 μm. J, quantification of nuclear Smad2-positive cells in co-culture assay of 293T cells (Unlabeled) and CR-1-transfected 293T cells (Labeled). Control, no stimulation. Values represent the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.
than Nodal in inducing Smad2 nuclear translocation both in labeled CR-1-positive and in unlabeled CR-1-negative cells (Fig. 1, I and J). To ascertain if this observation could be reproduced in cells that express endogenous CR-1 protein, we performed co-culture experiments using CR-1-deficient 293T cells and human embryonal carcinoma NTERA2/D1 cells from which CR-1 was isolated and cloned and which express a relatively high level of endogenous CR-1 (14) (Fig. 2A). CellTracker-labeled NTERA2/D1 cells responded to Nodal almost equally to Activin (Fig. 2, B–D). A significant population of unlabeled 293T cells responded to Nodal stimulation especially in the cells that were located proximally to NTERA2/D1 cells (Fig. 2, C, arrowheads, and E). From these results, we concluded that ectopically expressed or endogenously expressed wild-type CR-1 can act as a Nodal co-receptor in trans.

Identification of a Functional \( \omega \)-Site of CR-1—We have recently demonstrated that the GPI moiety is attached to Ser-161 of CR-1 in the COOH-terminal region by mass spectrometric analysis of “shed” CR-1 in the conditioned medium (22). To confirm that Ser-161 is a functional \( \omega \)-site for GPI attachment, we performed a mutational analysis of the COOH-terminal sequence of CR-1. Because Ser-169 of human CR-1 had also previously been predicted as a possible \( \omega \)-site for GPI-anchorage (“big-II predictor”), we introduced point mutations in these two possible \( \omega \)-sites and also at the \( \omega_1 \) and \( \omega_2 \) sites. Because previous reports have described that the \( \omega \)-site should be a small hydrophilic amino acid such as Ser, Asn, Ala, Asp, Gly, and Cys (23), possible \( \omega, \omega+1, \) or \( \omega+2 \) sites were substituted by a hydrophobic amino acid, Leu (S161L, S161L/R162L, S161L/R162L/T163L, S169L, S169L/A170L, S169L/A170L/R171L) or a small hydrophilic amino acid, Asn (S161N). Western blot analysis of cell lysates revealed that the WT CR-1 protein was found primarily as the 26- and 24-kDa forms and to a lesser extent as a 18-kDa form, which may be due to different glycosylated forms (Fig. 3A) (14, 24). The 26-kDa form of CR-1 was not present in S161L series (S161L, S161L/R162L, and S161L/R162L/T163L) mutants, whereas the S169L series (S169L, S169L/A170L, S169L/A170L/R171L) and S161N mutants contained the 26-kDa form and showed similar band

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**FIGURE 2. Paracrine activity of endogenous CR-1.** A, method for co-culture experiments. B–D, NTERA2/D1 cells were labeled with CellTracker green (B1–D1) and co-cultured with 293T cells for 24 h. Culture medium were then replaced with serum-free medium and incubated for 24 h. Co-cultured cells were stimulated with Nodal or Activin for 1 h. Nodal or Activin addition was performed without changing medium at final concentrations of 200 or 50 ng/ml, respectively. Cells were stained with anti-Smad2 antibody (B2–D2). Arrowheads in C, nuclear Smad2-positive, unlabeled cells. Nuclei were stained with DAPI (B3–D3). Scale bar = 20 \( \mu \)m. E, quantification of nuclear Smad2-positive cells in each treatment. Values represent mean ± S.D. of three independent experiments. *, \( p < 0.05; **, p < 0.01.\)
were collected 24 h after transfection and analyzed by Western blotting. S161L series mutants significantly lost the ability to induce Nodal signaling in these two assays, whereas S169L series and S161N mutants retained this ability (Fig. 4, H and I). These results suggest that Ser-161 is a functional ω-site for GPI-anchoring in CR-1 and that substitution of Ser-161 with a hydrophobic amino acid such as Leu, but not with a small hydrophilic amino acid such as Asn, affects the correct processing, localization, and function of CR-1 as a co-receptor.

Inability of Soluble Forms of CR-1 to Induce Nodal Signaling—

The paracrine function of CR-1 suggested that CR-1 can act as a soluble co-receptor of Nodal. Therefore, we designed variant forms of CR-1 in its COOH terminus based on the identified ω-site Ser-161 and compared the ability of these variant forms
to induce Nodal signaling (Fig. 5A). We generated a soluble CR-1 ΔC (Ser-161) that lacks the GPI signal as described above. We constructed a TM form of CR-1 in which the transmembrane domain of the EGF receptor-related receptor ErbB4 was attached just after Ser-161. We also prepared a ΔC (Ser-169) construct in which a stop codon was inserted after Ser-169. The
Nodal-dependent (n2)7-Luc reporter assay was performed using these CR-1 variants. WT and TM forms of CR-1 significantly induced the (n2)7-Luc activity, whereas both ∆C mutants of CR-1 did not activate Nodal-responsive reporter at a detectable level after co-transfection with mFast-1, ALK4-HA, and mNodal-V5 expression vectors (Fig. 5B). We also confirmed the dose-dependent effect of ligands using either recombinant Nodal or Activin instead of mNodal-V5 expression vector (Fig. 5C and D). Recombinant Nodal was only able to induce (n2)7-Luc activity dose-dependently in cells transfected with WT or TM CR-1 but not in ∆C (Ser-161)-transfected cells (Fig. 5C). In contrast, Activin was able to induce the (n2)7-Luc activity regardless of the type of CR-1 expression vectors (Fig. 5D), confirming the CR-1-independent activity of Activin. To address the interactions of the CR-1 variants with ALK4 or Nodal, co-immunoprecipitation experiments were performed using the CR-1 variants with epitope-tagged ALK4 and Nodal (Fig. 6). Although all three CR-1 variants could bind to ALK4-HA to a similar extent (Fig. 6A), the ability of ∆C (Ser-161) to form a complex with mNodal-V5 on the cell surface was markedly lower than that of WT or TM forms of CR-1 as assessed by co-immunoprecipitation after reversible cross-linking with the membrane-impermeable cross-linker DTSSP (Fig. 6B). A similar result was observed in the ∆C (Ser-169) form of CR-1 (data not shown).

We then directly compared the trans activity of GPI-anchored, soluble, and transmembrane forms of CR-1. Smad2 phosphorylation status after stimulation with recombinant Nodal was significantly enhanced in WT CR-1-transfected 293T cells as compared with EV-transfected cells (Fig. 7A). However, ∆C (Ser-161)-transfected cells did not show any increase of Smad2 phosphorylation over EV-transfected cells (Fig. 7A), which is consistent with the results of (n2)7-Luc assay (Fig. 5). Conditioned medium obtained from WT CR-1-transfected 293T cells significantly induced Smad2 phosphorylation in CR-1-deficient wild-type 293T cells in the presence of recombinant Nodal, but the conditioned medium from ∆C (Ser-161)-transfected cells did not show any difference compared with the conditioned medium from the EV-transfected cells even though...
the amount of immunoreactive ΔC (Ser-161) form of CR-1 protein in the conditioned medium was ~30-fold higher than WT CR-1 protein found in the conditioned medium (Fig. 7B). Conditioned medium of WT CR-1-transfected 293T cells could not induce Nodal signaling in the absence of Nodal (data not shown). Results that were similar to the ΔC (Ser-161) were also observed with the ΔC (Ser-169) form (data not shown). These results were also confirmed by the (n2)7-Luc reporter assay (Fig. 7C).

To compare differences in physiochemical properties between the released GPI-anchored form and COOH-terminal-deleted soluble form in the conditioned medium, Western blot analysis under reducing and non-reducing conditions was carried out (Fig. 7D). Because the amount of the GPI-anchored form released in the conditioned medium is markedly lower than that of the soluble form (Fig. 7B), concentrated medium was analyzed for the GPI-anchored form of CR-1. As shown in Fig. 7D, the released GPI-anchored proteins were detected as major two species of 30- and 28-kDa, which were also found in the PI-PLC-treated samples of the cell lysates. On the other hand, the ΔC (Ser-161) protein in the conditioned medium was found as a major band of 22–24 kDa. There is a clear difference in size and pattern between the released WT and ΔC (Ser-161) forms of CR-1 in the conditioned medium even though the released GPI-anchored form and ΔC (Ser-161) form should theoretically contain the identical amino acid sequence. Treatment with a reducing agent did not affect the SDS-PAGE mobility of each band in both WT and ΔC (Ser-161) of CR-1. However, the antigenicity of the released WT of CR-1 was completely lost under non-reducing conditions, whereas the ΔC (Ser-161) protein in the conditioned medium was still detected under the same conditions. This finding may suggest the presence of structural or conformational differences between the released GPI-anchored form and the soluble form of CR-1 in the conditioned medium.

These results are at variance with the previous reports that have demonstrated the activity of different COOH-terminal-truncated forms of mouse Cr-1 as summarized in Fig. 8A and Table 2. Therefore, we introduced similar COOH-terminal truncations into human CR-1 as had been introduced into mouse Cr-1 in the previous reports (18, 19, 26, 27) (Fig. 8A and Table 2). Western blot analysis of cell lysates revealed that ΔC (T174) and ΔC (T174)-V5His constructs generated some additional species with slower mobility in SDS-PAGE (Fig. 8B). These species were likely to be highly glycosylated forms in the COOH-terminal extension beyond the ω-site since there are several possible O-linked glycosylation sites including Ser-161 (the ω-site for GPI-anchored form), Ser-169, and Thr-172 to Thr-174. These highly glycosylated species of ΔC (T174) and ΔC (T174)-V5His mutants were also found in the conditioned medium (Fig. 8C). The GPI-V5His mutant in which V5-His$_5$ tag had been added at the end of COOH-terminal hydrophobic domain showed an expected SDS-PAGE mobility from its amino acid sequence (Fig. 8A) and was difficult to detect in the conditioned medium (Fig. 8B).

We then tested the activity of these COOH-terminal variant forms of CR-1 in Nodal signaling in transiently transfected 293T cells. None of the COOH-terminal-truncated variants of CR-1 such as ΔC (Ser-161), ΔC (Ser-161)-V5His, ΔC (T174), or ΔC (T174)-V5His were able to mediate significant Nodal signaling, as assessed by (n2)7-Luc assay (Fig. 8D) and by Smad2 phosphorylation (data not shown). On the other hand, GPI-V5His retained some reduced activity to induce Nodal signaling in transiently transfected 293T cells with co-transfection of mFast-1, mNodal-V5, ALK4-HA expression vectors (Fig. 8D). The GPI-V5His construct corresponds to the COOH-terminal FLAG-tagged construct of mouse Cr-1 which was used in a
previous study and was shown to interact with ALK4 and Nodal (26). GPI-V5His was also able to induce significant activities of the (n2)7-Luc reporter with exogenous recombinant Nodal protein (Fig. 8E).

Reduced Activity of Transmembrane Forms of CR-1 in Nodal Signaling—We then assessed the difference in the activity of the GPI-anchored and transmembrane forms of CR-1 to induce Nodal signaling. As shown in Fig. 5, the activity of the TM form was reduced up to ~30–40% compared with WT CR-1. We confirmed this reduction of activity by titration of the DNA amount used for transfection (Fig. 9A). The reduced activity of this artificial TM construct of CR-1 could be due to the distance and/or flexibility of linker region between the transmembrane domain and the EGF-CFC domain. Therefore, we introduced several different lengths of flexible poly-glycine-serine (poly-GS) spacer linkers ((GGGGS)ₙ) between the functional ω-site (Ser-161) and the ErbB4 transmembrane domain (Fig. 9B). These TM variants with different lengths of the poly-GS linker did not show any significant difference in their activity to induce Nodal signaling (Fig. 9C), suggesting that the length and flexibility of the linker region may not be critical for interaction with Nodal and ALK4. We then tested if the TM forms of CR-1 could induce Nodal signaling without ALK4 overexpression in transiently transfected 293T cells. (n2)7-Luc assays without overexpression of ALK4-HA revealed that transmembrane forms of CR-1 but not WT CR-1 require ALK4 overexpression to induce Nodal signaling (Fig. 9D).

Thr-72 of mouse Cr-1, which corresponds to Thr-88 in human CR-1 has been reported as a site of O-fucosylation and is necessary for Nodal signaling (24, 28). Phe-78 of mouse Cr-1, which corresponds to Phe-94 in human CR-1, has also been reported to be necessary to rescue the oep-mutant phenotype in zebrafish (18). We, therefore, substituted these two residues with Ala by point mutations in the GPI-anchored (WT) and TM forms of human CR-1 (T88A and F94A, respectively, Fig. 9, E–G). GPI-anchored, WT CR-1 protein was found primarily as 26- and 24-kDa forms (Figs. 3A and 9E). In the TM forms of CR-1, a similar pattern was observed with the 34- and 32-kDa forms (Fig. 9E). The F94A mutant forms showed an identical band pattern to the non-mutated forms of both the GPI-anchored and TM forms of CR-1. On the other hand, in T88A mutant forms the larger bands (26-kDa in GPI-anchored forms or 34-kDa in the TM forms) were weaker than the non-mutated forms (Fig. 9E). FACS analysis revealed that the F94A mutants exhibited comparable cell-surface expression of immunoreactive CR-1 as compared with the non-mutated forms of both WT and TM forms (Fig. 9, F and G). However, the cell-surface expression of the T88A mutants was decreased by ~50% (Fig. 9, F and G). We then assessed the activity of these mutants to induce Nodal signaling using (n2)7-Luc assay in 293T cells co-transfected with mFast-1, mNodal-V5, and ALK4-HA. In accordance with previous reports (24, 28), the T88A mutant of the GPI-anchored form completely lost the ability to induce Nodal signaling (Fig. 9H), whereas the F94A mutant of the GPI-
anchored form still retained some activity. Because the EGF-like domain of CR-1 is known to bind to Nodal (14), these results suggested that the T88A mutant was not able to bind to Nodal, and the F94A mutant still retained some ability to bind Nodal but possibly with weaker affinity. In contrast to the GPI-anchored form, the F94A mutant of the TM form completely lost the ability to induce Nodal signaling, suggesting that the TM form of CR-1 could induce Nodal signaling only when the Nodal binding capacity is totally intact. These results suggest that the TM form of CR-1 requires high ALK4 expression as well as an intact Nodal binding capacity to function.

To ascertain the importance of GPI attachment of endogenous CR-1, we examined the effect of PI-PLC treatment on Nodal signaling using NTERA2/D1 cells. FACS analysis revealed that PI-PLC treatment almost completely abolished the cell-surface expression of CR-1 in NTERA2/D1 cells (Fig. 8).
This result suggests that GPI attachment of endogenous CR-1 is
decreased the ability of Nodal to induce Smad2 phosphoryla-
tion but PI-PLC treatment did not affect the activity of Activin.

Discussion

In the present study we confirmed the paracrine activity of
GPI-anchored CR-1 in transfected 293T cells, which is consist-
ent with the previous report (16). We further demonstrated that cells which express endogenous CR-1 are also able to
induce Nodal signaling in CR-1-deficient cells by co-culture.
The fact that the inability of Cr-1-null mouse ES cells to form
mesoendoderm can be rescued in a chimeric mouse of Cr-1-
null ES cells and wild-type embryonic cells (15, 17) strongly
suggests that the trans activity of GPI-anchored CR-1 is not
negligible. However, previous reports are controversial as to the
activity of different artificially generated soluble forms of CR-1
(18, 19, 27) (Table 2). To design a suitable soluble human CR-1
construct, we identified the functional ω-site Ser-161 by struc-
tural and mutational analysis (22). Therefore, the ΔC (Ser-161)
protein should have an identical amino acid sequence with GPI-
anchored CR-1 after cleavage of the GPI signal. However, the
ΔC (Ser-161) protein almost completely lost the ability to
induce Nodal signaling. Our results are consistent with a previ-
ous report which demonstrated the inability of a COOH-termin-
ally truncated form of mouse Cr-1 using a similar (n2)7-Luc
reporter assay in Xenopus embryos (27), although their conclu-
sion that Cr-1 functions solely as a “cell-autonomous” factor in
Nodal signaling may not be correct. There are still some dis-
crepancies between the present study and some of the previous
reports which have demonstrated that COOH-terminal-de-
leted forms of mouse Cr-1 or zebrafish oep still retained biolog-
ical activities (18, 19, 29, 30). In these reports the activity of
truncated forms of Cr-1 (or oep) were mainly measured by res-
cue experiments of the oep-mutant phenotype in zebrafish (18,
29, 30) or of the lack of the ability of mouse Cr-1-null ES cells
to differentiate into cardiomyocytes (19). It might be possible that
soluble forms of CR-1 could have an additional or different
function(s) other than as a Nodal co-receptor. Additionally, this
discrepancy might be explained by the difference in the sensi-
tivity of the various assays used for the functional analysis
(Table 2). For example, the presence of extracellular matrix
proteins that contain heparin sulfate-containing proteoglycans
and that may sequester or locally concentrate a soluble form of
CR-1 and/or expression of endogenous inhibitors of CR-1
such as lefty proteins (21) might account for these differences.
However, the present findings at least demonstrate that the
deletion of GPI-signal sequence can reduce the activity of CR-1
as a Nodal co-receptor. Our conclusion is also supported by the
clinical observation of human Cryptic (hCFC1), a homologue of
human CR-1. A frameshift mutation at the beginning of the
hydrophobic COOH-terminal domain in CFC-1 is related to
human left-right laterality defects (31). In fact, this hCFC1
mutant was absent from the cell surface and failed to rescue a
zebrafish oep-mutant phenotype (31) or to mediate Nodal sig-
aling in vitro (16).

A previous study successfully demonstrated the interaction of
Cr-1 with Nodal and its receptors using a construct of mouse
Cr-1 that contained an epitope tag at the end of the GPI signal
(26). A similar construct of human CR-1 that we generated in
the present study (GPI-V5-His) retained some activity to
induce Nodal signaling. However, the reduced activity of this
COOH-terminal-tagged construct may not reflect the full
physiological activity of wild-type CR-1 in mediating Nodal sig-
aling. This is especially critical for immunoprecipitation
assays that were performed using an epitope tag introduced
after the GPI-signal sequence (26), since the hydrophobic
COOH-terminal signal of wild-type CR-1 should be cleaved off
during processing to the mature form of GPI-anchored CR-1.

The activity of the GPI-anchored form of CR-1 to induce
Nodal signaling in trans was significantly higher than that of
soluble forms of CR-1, since a nearly 30-fold larger amount of a
soluble form of CR-1 in the conditioned medium failed to
induce Nodal signaling. There are several possibilities that
could explain the differences in biological activity to function as
a Nodal co-receptor between the secreted GPI-anchored and
soluble forms of CR-1. First, post-translational modification
such as glycosylation of a soluble CR-1 protein might be differ-
ent from that of the GPI-anchored form. The difference in the
SDS-PAGE mobility as well as the loss of immunological detec-
tion of the secreted GPI-anchored form of CR-1 and the retention
of immunoreactivity of the soluble form under non-reducing
conditions may suggest that structural differences exist
between these two forms. Indeed, differences in the glycosyla-
tion status and biological activity between GPI-anchored and
soluble forms have been described for GPI-anchored proteins.

### Table 2
Summary of COOH-terminally truncated forms of CR-1 in the literature

| Constructs | References | Species | Activity | Assays | Cells/animals |
|------------|------------|---------|----------|--------|--------------|
| Ref. 1 (Sakuma et al.) | 27 | Mouse | No | (n2)7-Luc; Rescue of oep-mutant phenotype | Xenopus embryo |
| Ref. 2 (Minchiotti et al.) | 18, 19 | Mouse | Yes | Cardiomyocyte differentiation; Smad2 phosphorylation* | Zebrafish embryo; Mouse ES cell |
| Ref. 3 (Yeo et al.) | 26 | Human | Yes | Smad2 phosphorylation | Xenopus embryo |
| ΔC(S161) | | | No | | |
| ΔC(T174) | | | No | | |
| ΔC(T174)-V5His | | | No | | |
| GPI-V5His | | | Yes (reduced) | | |

* Using a recombinant protein at the concentration of 10 μg/ml.
such as CD59 and prion protein (32, 33). Second, GPI-anchor- ing of CR-1 might be necessary for the trans activity of CR-1. For example, potential CR-1-containing microvesicles released from the cell membrane might have a functional activity. Several reports have described that intact GPI-anchored proteins can be released in the form of membrane vesicles (34) and can

![Figure 9](https://example.com/figure9.png)

**FIGURE 9.** Partially retained activity of transmembrane forms of CR-1. A, (n2)7-Luc reporter assay was performed under the same condition as in Fig. 58. Titration of WT and TM are shown. B, Western blot analysis of variant TM forms of CR-1 with or without poly-GS linker (pGS5–15) expressed in 293T cells. C, (n2)7-Luc reporter assay was performed for the variant TM forms of CR-1 under the same conditions as in A. 200 ng DNA/well of EV or indicated CR-1 expression vectors were used. D, (n2)7-Luc reporter assay was performed without overexpression of ALK4-HA. Other conditions were identical with the condition described in C. E–G, Western blot analysis (E) and FACS analysis (F and G) of point mutants in EGF-like domain of WT or TM forms of CR-1 expressed in 293T cells. H, (n2)7-Luc reporter assay was performed for indicated CR-1 mutants in the same conditions with C.
activity of the TM CR-1 constructs in Nodal signaling was reduced in comparison to the GPI-anchored form of CR-1. The different properties of these two membrane anchors may account for the different activities of these two forms. For example, GPI-anchored proteins predominantly localize in lipid raft microdomains on the plasma membrane, and the GPI-anchored form of CR-1 is cleavable by the activity of mammalian phospholipases including GPI-phospholipase D, whereas transmembrane forms are not (22). Recent reports have shown that GPI-signal sequences and glycosylation can play a critical role as determinants of biological activity (38, 39), suggesting that the structure of the GPI membrane anchor in conjunction with glycosylation could affect the biological function of membrane proteins. For example, N-glycosylation of mouse Nodal increases stability of the mature secreted form and thereby enhances the biological activity in vitro (40).

We have recently found that the same COOH-terminal-deleted constructs ΔC (Ser-161) and ΔC (Ser-169) can induce Nodal-independent, c-Src/MAPK/PI3k-Akt-dependent signaling and can promote endothelial cell migration (22). This suggests that the mechanism by which CR-1 acts in Nodal-dependent and Nodal-independent signaling pathways may be different. CR-1 is known as a multifunctional molecule and is important both in embryonic development and in tumor progression (14). Recent studies have also shown that CR-1 is a potential candidate as a tumor marker and as a target for tumor immunotherapy (8, 41, 42). This study provides additional information for understanding the molecular mechanisms of the action of CR-1.

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