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Characterization of the glycosylphosphatidylinositol-anchor signal sequence of human Cryptic with a hydrophilic extension

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ABSTRACT

Epidermal Growth Factor-Cripto-1/FRL-1/Cryptic (EGF-CFC) proteins, including human Cripto-1 (hCFC2/hCR-1) and human Cryptic (hCFC1), are membrane-associated Nodal co-receptors, which have critical roles in vertebrate development. Most of the EGF-CFC proteins have been experimentally proven or predicted to be glycosylphosphatidylinositol (GPI)-anchored proteins. However, unlike other EGF-CFC proteins, hCFC1 does not exhibit a typical GPI-signal sequence, containing a 32-amino acid hydrophilic extension in its COOH-terminal end. Here we experimentally demonstrate that the COOH-terminal sequence of hCFC1 functions as a GPI-anchoring signal. Moreover, addition of a hydrophilic epitope tag of 55-amino acids (V5-His) after the GPI signal of hCR-1 interfered with generation of a GPI-anchored form of hCR-1. In contrast, addition of the same epitope tag to the end of GPI signal of hCFC1 did not affect the GPI-attachment of hCFC1. The COOH-terminal signal of hCFC1 could produce two different forms of the protein; a GPI-anchored form and an unprocessed form which was more prone to be secreted into the conditioned medium. The hydrophilic extension of hCFC1 negatively regulates the activity of hCFC1 as a Nodal co-receptor. These results demonstrate the presence of endogenous GPI-signal sequence with a hydrophilic extension, which can generate both GPI-anchored and soluble forms of the protein.

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1. Introduction

Epidermal Growth Factor-Cripto-1/FRL-1/Cryptic (EGF-CFC) family of proteins performs an essential role in vertebrate development and in tumor progression [1,2]. Two functional members of the EGF-CFC family proteins have been identified in human; Cripto-1 (hCFC2/hCR-1) and Cryptic (hCFC1). Knockout studies of mouse orthologous genes (mCr-1 and mcfc1) suggested that these two genes are essential during embryonic development [3–5]. Cr-1-null mice are embryonic lethal at day E8.5-10.5 mainly due to the failure to form appropriate germ layers [3]. On the other hand, mcfc1-null mice are not embryonic lethal but die within the first 2 weeks because of severe left-right laterality defects and cardiac malformations [4,5]. Both CR-1 and CFC1 can function as obligatory co-receptors for the TGFβ family ligand Nodal to facilitate their binding to the Activin type I receptors (ALK) 4/7 and Activin type II receptor [6]. Binding of Nodal to its receptors induces Smad2/3 phosphorylation which can heterodimerize with Smad4 and regulate gene transcription in the presence of the co-transcriptional cofactor Fast-1 (FoxH1) [6].

CR-1 and CFC1 proteins contain NH_2-terminal signal sequences, modified EGF-like domains that bind to Nodal, cysteine-rich CFC domains that bind to ALK4/7 and COOH-terminal hydrophobic domains [1,2]. The COOH-terminal hydrophobic domain of human and mouse CR-1/Cr-1 has been described as a glycosylphosphatidylinositol (GPI)-attachment signal [7–9] and the COOH-terminal hydrophobic domain of hCFC1 has been also shown to serve as a membrane-anchoring signal [10], even though the GPI-attachment of human CFC1 has not been experimentally demonstrated. The membrane-anchor domain of CFC1 is considered to be important since a frameshift mutation at the beginning of the membrane-associating domain in human *CFC-1* is related to human left-right laterality defects [10].

Biosynthesis of GPI-anchored proteins requires an NH_2-terminal leader sequence and a COOH-terminal GPI signal sequence which is recognized by the GPI transamidase complex and then cleaved during the addition of the GPI moiety [11–13]. The COOH-terminal signal sequences for GPI-attachment are usually 17 to 31 amino acids in length and, in most cases, consist of four regions; 1) an unstructured linker region, 2) a region of small amino acid residues including the ω-site for propeptide cleavage and GPI-attachment, 3) a hydrophilic spacer region,

Abbreviations: ALK, activin-like kinase; CR-1, Cripto-1; CFC1, Cryptic; EGF-CFC, Epidermal Growth Factor-Cripto-1/FRL-1/Cryptic; EV, empty vector; GPI, glycosylphosphatidylinositol

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and 4) a hydrophobic tail [11,14,15]. These sequences are recognized by the GPI-transamidase complex after translation of the protein core with subsequent addition of GPI and signal peptide cleavage which occurs in the endoplasmic reticulum (ER) [13,16]. Although previous studies have suggested that artificially generated, internally positioned GPI-signals can be substrates for the GPI-transamidase [17,18], all known endogenous GPI-anchored proteins contain hydrophilic extensions of no more than a few amino acids [14,17,19,20]. In this context, hCR-1 exhibits a typical sequence of GPI-signal in its COOH-terminus. In contrast, the COOH-terminal sequence of hCFC1 is not a typical GPI-signal sequence since the COOH-terminal sequence of hCFC1 contains an additional 32 amino acids hydrophilic extension (Fig. 1A).

We have previously shown that the GPI-anchor of hCR-1 is necessary for optimal biological activity of hCR-1 as a co-receptor to mediate Nodal signaling [9]. Thus, we hypothesized that hCFC1 protein might also be GPI-anchored even though hCFC1 exhibits an atypical GPI signal sequence. Here we experimentally demonstrate that both hCR-1 and hCFC1 proteins are GPI-anchored proteins and define the different properties of these two GPI-signals. These results describe the presence of an endogenous non-canonical GPI-signal sequence with a hydrophilic extension.

2. Materials and methods

2.1. Cell culture

HEK293T (293T) cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C, in 5% CO_2.

2.2. Expression vectors and transfection

The cDNA encoding the open reading frame of hCR-1 was previously described [8]. The cDNA encoding the open reading frame of hCFC1 was cloned from NTERA2/D1 embryonal carcinoma cells and validated by direct sequencing. The obtained sequence of hCFC1 completely matched the reported sequence (Accession number; AF312769). All hCR-1- or hCFC1-related constructs except for the FLAG-tagged constructs were cloned into the pEF6/V5-His TOPO TA expression vector (Invitrogen, Carlsbad, CA). A chimeric construct of hCR-1 with the COOH-terminal domain of hCFC1 (CR-CFC) was generated by PCR-based method using primers; hCR-1 start-F, ACCATGGACTGCAGGAAGATG; CR-CFC chimera-F,

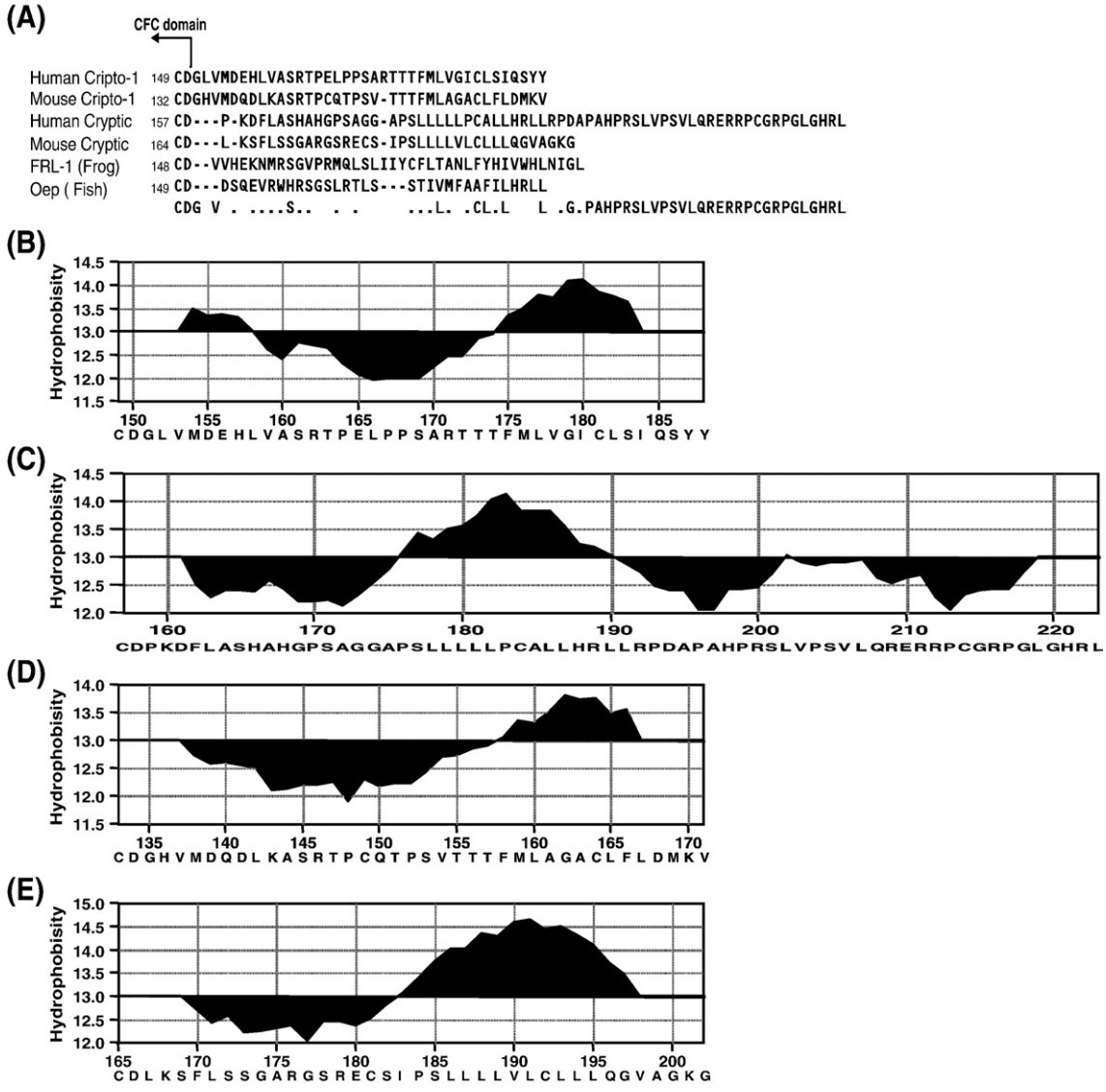

Fig. 1. Sequence analysis of COOH-terminal domains of EGF-CFC proteins. (A) Sequences of GPI signal of EGF-CFC proteins. COOH-terminal sequences of indicated gene products are aligned using MacVector software. Conserved amino acids are shown at the bottom. (B–E) Hydropathy plot of the human CR-1 (B), human CFC1 (C), mouse Cr-1 (D), and mouse cfc1 (E). Hydrophobic scores were determined using the Manavalan algorithm with a window size of 11.