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Identification of ligand-selective peptidic ActRIIB-antagonists using phage display technology

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ABSTRACT

ActRIIB (activin receptor type-2B) is an activin receptor subtype constitutively expressed in the whole body, playing a role in cellular proliferation, differentiation, and metabolism. For its various physiological activities, ActRIIB interacts with activin and multiple other ligands including myostatin (MSTN), growth differentiation factor 11 (GDF11), and bone morphogenetic protein 9 (BMP9). Notably, the protein-protein interaction (PPI) between ActRIIB and MSTN negatively controls muscular development. Therefore, this PPI has been targeted for effective treatment of muscle degenerative diseases such as muscular dystrophy and sarcopenia. Here, we report the identification of ligand-selective peptidic ActRIIB-antagonists by phage display technology. Our peptides bound to the extracellular domain of ActRIIB, inhibited PPIs between ActRIIB expressed on the cell surface and its ligands, and subsequently suppressed activation of Smad that serves as the downstream signal of the ActRIIB pathway. Interestingly, these peptidic antagonists displayed different ligand selectivities; the AR2mini peptide inhibited multiple ligands (activin A, MSTN, GDF11, and BMP9), AR9 inhibited MSTN and GDF11, while AR8 selectively inhibited MSTN. This is the first report of artificial peptidic ActRIIB-antagonists possessing ligand-selectivity.

1. Introduction

ActRIIB (activin receptor type-2B) is a receptor serine/threonine kinase that interacts with multiple ligands of the TGFβ superfamily, such as activin, MSTN (myostatin), GDF11 (growth differentiation factor 11), and BMP9 (bone morphogenetic protein 9) [1,2]. Upon interaction, ligand/ActRIIB complex recruits activin receptor type-1 (ALK) forming a ligand/ActRIIB/ALK (2:2:2) hexamer complex to activate intracellular Smad signaling (Smad is the downstream signal of the ActRIIB pathway) [3]. Though ActRIIB is constitutively expressed in the whole body, its various physiological functions are tissue-selective, depending on the distribution and timing of ligand/ALK expression. Among ActRIIB ligands, MSTN plays a dominant role in both developing and adult human skeletal muscle [4]. MSTN-knockout mice exhibit skeletal muscle hypertrophy, indicating that MSTN acts as a negative regulator of muscle development. Thus, inhibitors of the ActRIIB-MSTN protein-protein interaction (PPI) provide a novel strategy to treat muscle degenerative disorders like muscular dystrophy and sarcopenia.

Some approaches have been explored to reduce MSTN function by either blocking the interaction with its receptor or through MSTN natural inhibitor overexpression [5]. Among them, monoclonal antibodies neutralizing MSTN have been well studied (e.g., MYO-029, PF-06252616, LY-2495655, and REGN-1033) [6]. The injection of a soluble form of ActRIIB (ACE-536) has been reported to improve the amyotrophic phenotype in animal models [7]. In addition to blockers that mask MSTN, the ActRIIB antagonist antibody BYM-338 has been studied [8]. However, treatment with antibodies block multiple ligands due to the high molecular weight of antibodies, leading to undesirable side effects. For example, Suragani et al. reported that injection of GDF11 to mice causes anemia, suggesting that inhibition of the PPI between ActRIIB and GDF11 would have the side effect of increasing the population of red blood cells to abnormal levels [9]. Since, as described above, ActRIIB interacts with multiple ligands and exerts various physiological functions, ligand-selective antagonists would be advantageous.

Recently, peptides have attracted much attention as an alternative

Abbreviation: ActRIIB, activin receptor type-2B; MSTN, myostatin; GDF11, growth differentiation factor 11; BMP9, bone morphogenetic protein 9; PPI, protein-protein interaction; ALK, activin receptor type-1B

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to antibodies for PPI modulation because they exhibit high affinity and selectivity for target proteins compared to antibodies, in spite of their approximately 100 times smaller molecular weight [10]. Phage display is a powerful technology for the generation of PPI-modulating peptides [11–13], as it allows the construction of large and diverse peptide libraries (containing > 10 billion distinct peptides), and the identification of target-binding peptide sequences through an affinity selection technique called biopanning.

To generate peptidic ActRIIB antagonists, we panned random peptide libraries displayed on T7 phage against the extracellular domain of ActRIIB, which was fused to human IgG-Fc (ActRIIB-Fc). We successfully identified ActRIIB-Fc binding peptides that we classified into three types. Importantly, these peptides possess the desirable ligand-selective antagonist activity.

2. Materials and methods

2.1. Preparation of recombinant ActRIIB-Fc and MSTN

The mammalian pcDNA3.4 vector was used to create the construct pcDNA3.4/hACVR2B(1-135)-Fc, expressing a fusion protein comprising human ACVR2B-ECD (residues Met1–Leu135, NCBI Reference Sequence: NNM_001106) and the Fc region of human immunoglobulin G at the C-terminus. The construct was transfected into Expi293F cells (at a final density of 2.5×10^6 cells/mL) using the ExpiFectamine 293 Transfection Kit and Opti-MEM Reduced Serum Medium (Life Technologies, Carlsbad, CA, USA) in Expi293 Expression Medium. After 18 h, Enhancer 1 and Enhancer 2 were added and the cultures were expanded for 5 days. After incubation, the supernatant containing recombinant protein was separated from cells by centrifugation. To purify recombinant proteins, the supernatant was loaded onto an SP-Sepharose Fast-Flow column (GE Healthcare, Piscataway, NJ, USA) equilibrated in buffer A (50 mM Sodium Acetate, pH 4.0). The column was washed with buffer A and the protein was eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. The eluate was loaded onto a HiLoad 26/60 Superdex 200 pg column (GE Healthcare) using PBS (phosphate-buffered saline: 10 mM phosphate, 2.7 mM KCl, and 137 mM NaCl). Purified proteins were concentrated with AmiconUltra15 centrifugal filter units (MWCO = 30 kDa; Merck-Millipore, Billerica, MA, USA). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) using BSA (bovine serum albumin) as a standard.

The mammalian pcDNA3.4 vector was also used to create the pcDNA3.3/hMSTN-His construct that expressed human MSTN (NCBI Reference Sequence: NM_005259) as a fusion protein with a His-tag at its C-terminus. To generate an FS293F stable cell line secreting MSTN pro-peptide, FS293F cells were transfected with the construct using Neofection (ASTEC, Shime, Japan) according to the manufacturer's instructions. Transfected cells were selected by G418 treatment (500 μg/mL) for 14 days. The expression of recombinant MSTN pro-peptide in the stable cells was detected using western blotting analysis with anti-His6-peroxidase. The selected cell clones were cultured in FreeStyle expression medium (Life Technologies) containing 250 μg/mL G418 for 3 days. The supernatant containing recombinant protein was separated from the cultured cells by centrifugation. For protein purification, the supernatant was loaded onto a 5 mL Ni-NTA Superflow Cartridge (QIAGEN, Hilden, Germany). The Ni-NTA column was washed with 20 mM imidazole and then the protein was eluted with 250 mM imidazole in affinity buffer [50 mM Tris (pH 8.0) and 300 mM NaCl]. The eluate was loaded onto a HiLoad 26/60 Superdex 200 pg using SDX buffer [20 mM Tris (pH 7.5) and 150 mM NaCl]. To generate mature MSTN, the eluate was treated with the protease furin (produced internally) and the endoproteinase AspN at 37 °C for 16 h. The treated protein was bound to a reverse-phase PLRP-S column (Agilent Technologies, Santa Clara, CA, USA) and then eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). The eluted mature MSTN was freeze-dried and then dissolved in 10 mM HCl. Protein concentration was determined using the BCA Protein Assay Kit with BSA as a standard.

2.2. Chemical synthesis of peptides

AR2mini, AR2mut, AR8, and AR9 were synthesized internally by standard 9-fluorenylmethyloxycarbonyl (F-moc)-based solid-phase peptide synthesis (SPPS) using the Syro Wave microwave-assisted peptide synthesizer (Biotage, Uppsala, Sweden) with disulfide bond formation after cleavage from the solid support, followed by purification by reversed-phase high-performance liquid chromatography (RP-HPLC). Peptide purity was ascertained by analytical RP-HPLC, and the identity of the peptides was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

For disulfide bond formation in AR2mini, AR2mut, and AR8, a linear crude peptide containing two acetamidomethyl-Cys residues (AR2mini, AR2mut: 0.03 mmol; AR8: 0.02 mmol) was dissolved in 90% acetic acid (0.2–0.3 mM peptide). Iodine was added to the solution (10–13.3 eq.) and the mixture was stirred overnight at room temperature. After removal of excess iodine by activated charcoal, the solution was filtered. For disulfide bond formation in AR9, a linear crude peptide with two Cys residues (SH-free form, 0.02 mmol) was dissolved in 50% acetonitrile, then CLEAR-OXTM resin (1.5 eq.) was added to the solution. After stirring for 7 h at room temperature, the resulting solution was filtered. The obtained peptide solutions were applied to preparative RP-HPLC using appropriate columns [(AR2mini, AR8: Daisopak SP-100-5-ODS-P column, 20 × 250 mm; DaiSO, Osaka, Japan)(AR2mut and AR9: Kinetex 5 μm XB-C18 100 A AX column, 21.1 × 250 mm; Phenomenex. Torrance, CA, USA)]. Linear density gradient elution (60 min) was performed with 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B) at a flow rate of 8.0 mL/min, at the following ratios (A/B): AR2mini, 71/29–61/39; AR2mut: 79/21–69/31; AR8: 79/21–69/31; AR9: 76/24–66/34. The fractions containing the product peptides were collected and lyophilized. The obtained peptide amounts were 5.9 mg AR2mini, 5.8 mg AR2mut, 6.7 mg AR8, and 2.5 mg AR9.

2.3. Peptide screening by phage display

The T7 phage-displayed random peptide libraries; X_{12}, X_{16}, X_{20}, $CX_{7-10}C$, $X_3CX_{7-10}CX_3$ (X is a mixture of twenty natural amino acids), were internally constructed using mixed oligonucleotides as the DNA template and the T7Select 10-3 vector (Merck Millipore). Insert DNA were purified by QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) and ligated into the T7Select 10-3 vector, according to the manufacture's T7Select System Manual. The total library diversity was estimated to be 1.4×10^{10} by plaque forming unit (pfu). For screening of T7 phage libraries, 20 μg of ActRIIB-Fc was immobilized on 200 μL of Protein A Dynabeads (Invitrogen, Carlsbad, CA, USA) in PBS (Cat. No. 045-29795; Wako, Osaka, Japan) containing 0.5% BSA. After washing beads with PBS containing 0.1% Tween20 (PBST), they were incubated with the mixture of phage libraries for 1 h at room temperature and washed with PBST (3 times for the first round, 5 times for the second round, and 10 times for third–fifth rounds). Bound phages were eluted with 200 μL of 0.5% SDS and used to infect 80 mL of *E. coli* BLT5615