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Single-domain antibodies that compete with the natural ligand fibroblast growth factor block the internalization of the fibroblast growth factor receptor 1

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ABSTRACT

Single-domain antibodies in VHH format specific for fibroblast growth factor receptor 1 (FGFR1) were isolated from a phage-display llama naïve library. In particular, phage elution in the presence of the natural receptor ligand fibroblast growth factor (FGF) allowed for the identification of recombinant antibodies that compete with FGF for the same region on the receptor surface. These antibodies posses a relatively low affinity for FGFR1 and were never identified when unspecific elution conditions favoring highly affine binders were applied to panning procedures. Two populations of competitive antibodies were identified that labeled specifically the receptor-expressing cells in immunofluorescence and recognize distinct epitopes. Antibodies from both populations effectively prevented FGF-dependent internalization and nuclear accumulation of the receptor in cultured cells. This achievement indicates that these antibodies have a capacity to modulate the receptor physiology and, therefore, constitute powerful reagents for basic research and a potential lead for therapeutic applications.

1. Introduction

Fibroblast growth factor receptor 1 (FGFR1) participates in the regulation of a broad range of molecular mechanisms involved in metabolism homeostasis [1] and its uncontrolled expression and activity leads to the alteration of signaling pathways [2], resulting in different pathological conditions among which angiogenesis and tumorigenesis [3–5]. Canonical FGFR1 activation occurs upon binding of the cognate ligand named fibroblast growth factor (FGF), but neural cell adhesion molecule (NCAM) also acts as an unconventional FGFR1 ligand [6–8]. In combination with heparin, FGF can induce the translocation of the receptor into the nucleus where it acts as a transcriptional modulator [7–11]. Therefore, molecules able to interfere with the binding of FGF can be exploited for modulating the signaling mechanisms triggered by FGFR1 and, therefore, may have important therapeutic implications.

Given their elevated specificity, antibodies are preferred candidates for recognizing specific epitopes. Non-immune libraries are optimal tools for recovering monoclonal antibodies for suitable sequences such as the binding regions recognized by ligands. This is feasible since the sub-population of recombinant antibodies that binds to a given epitope can be specifically eluted by adding an excess of the natural binder for the same site.

In this report, we describe the identification of single-domain antibodies (VHH) selected from a phage displayed llama naïve library [12] that compete with FGF-2 for the binding to the FGFR1 ectodomain and demonstrate that such VHHs can inhibit the internalization of FGFR1 promoted by FGF-2 and heparin [13].

2. Materials and methods

2.1. Panning of the non-immune library

The pRK5tkNEO vector encoding the ectodomain of human FGFR1 fused to human Fc was kindly provided by A. Gurney (Genentech, San Francisco, CA). FGFR1-Fc was expressed in HEK 293 cells and the protein accumulated in the medium was purified by Protein A affinity chromatography [12]. The cDNA for FGF-2 (kindly provided by M. Presta and P. Dell'Era, Brescia, Italy) was expressed in bacteria and purified using first a HiPrep 16/10 Heparin column followed by a step of cation exchange chromatography. The VHH library and the panning protocol have been utilized as described previously [12], with the difference that, before the chemical elution in the presence of triethylamine, the phages bound to FGFR1 were incubated in the presence of FGF-2 at the concentration of 57 μg/mL. Given the high affinity of the FGF-2/FGFR1 interaction

Abbreviations: FGF, fibroblast growth factor; FGFR1, fibroblast growth factor receptor 1; VHH, heavy chain variable domain.

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[2], this step should remove by competition the binders that share with FGF-2 the same binding site on the FGFR1 ectodomain.

The VHHs recovered by both competitive and chemical elutions were tested by ELISA to confirm their specificity for FGFR1 and sequenced [12].

2.2. VHH subcloning, production, and activity tests

Unique VHH sequences were subcloned into the pFUSE-VHH-Fc mouse vector [14] and expressed in HEK 293 cells to obtain reconstituted IgG-like antibodies. After affinity purification on Protein A, the anti-FGFR1 antibodies were assessed in an immunofluorescence assay using HeLa cells and a FGFR1-GFP (see below) [13,14]. The antibody affinity for the antigen was determined by ELISA performed in the presence of decreasing antibody concentrations.

2.3. ELISA competition assays

The Fc-constructs corresponding to the VHHs 2B10, 2F5, G12, 2G6, 2E2 were assessed by competitive ELISA [15]. Immunoplates (Nunc A/S) were coated overnight at 4 °C with 100 μL/well of 0.5 μg/mL (corresponding to $7.32e^{-13}$ mol) of human Fc-FGFR1 in Na_2CO_3 0.1 M, pH 9.6. Unspecific binding was blocked incubating the plates for 2 h at room temperature with 300 μL/well of PBS plus 3% BSA. Each individual VHH fused to mouse Fc was used at a concentration sufficient to saturate the immobilized FGFR1 (0.32–1.28 μg/mL) and mixed with any of the other VHHs tagged with rabbit Fc at molar ratios varying between 128 and 0.00312 or with FGF-2 at concentrations between 5.6 and 0.00268 mM. The total volume of each solution was 100 μL. Wells were washed three times with 350 μL of PBST and 100 μL of HRP-conjugated goat anti-mouse antibodies were added and incubated 1 h at room temperature. Following six washing cycles in PBST, the chromogenic reaction was induced by adding 100 μL/well of ABTS 0.2 mg/mL containing 18 mM hydrogen peroxide in citrate buffer (pH 4.0), and the reaction was allowed to proceed for 30 min in the dark at room temperature. The absorbance was read at 405 nm using an Infinite M200 plate reader (Tecan). All the experiments were performed in triplicate and repeated at least three times.

2.4. Immunofluorescence

HeLa cells were transfected with the plasmid encoding for the human FGFR1 ectodomain C-terminally fused to GFP (FGFR1-GFP), kindly provided by J. Stow (Brisbane, Australia). Briefly, coverslips were coated with 2 mL/well of PBS containing 0.1% gelatin and incubated in 6% CO_2 for 1 h at 37 °C before removing the gelatin solution and seeding HeLa cells at the density of 2×10^5 cells/coverslip. Cells were allowed to grow up to the 70–80% of confluence before transfection with FGFR1-GFP (2.5 μg/mL) and lipofectamine (5 μL/mL) (Invitrogen) resuspended in serum free MEM. Cells were cultured 5 h at 37 °C and 6% CO_2, the medium was replaced with fresh medium before overnight incubation in the same conditions. Finally, cells were fixed in 4% PAF, permeabilized in the presence of 0.5% Triton X-100, blocked with 3% BSA, and incubated for 1 h with 2.5 μg/mL of mouse Fc-tagged VHHs before the addition of Cy3-conjugated donkey anti-mouse secondary antibodies. After DAPI staining, the samples were analyzed using an Olympus AX70 Microscope (Olympus Corporation).

2.5. FGFR1 internalization

The VHH clones G12 and 2G6 were evaluated for their capacity to inhibit the FGF-2-mediated internalization of FGFR1 according to the protocol described in detail by Bryant et al. [13]. As a negative control, an unrelated VHH direct to the SNAP protein [14] was used.

Coverslips were coated with 0.1% gelatin/PBS and incubated in 6% CO_2 for 1 h at 37 °C before removing the gelatin solution and seeding MCF7 cells at the concentration of 250×10^3 cells/mL. Cells were allowed to grow up to the confluence of 70–80%. FGFR1 internalization was stimulated by adding 2 mL/well of freshly prepared serum free MEM medium containing 5 μg/mL heparin and 20 ng/mL human FGF-2. The VHH-Fc mouse constructs were added at the concentration of 5 μg/mL, cells were fixed at different times in the subsequent 24 h by 15-min incubation in a 4% PAF solution, VHHs were labeled with donkey anti-mouse Cy3-conjugated antibodies, and the samples were finally observed by fluorescence microscopy.

3. Results

The FGFR1 panning procedure followed by competitive elution of the antibodies with FGF-2 enabled us to isolate seven antibodies with unique sequence, whereas 11 unique binders were selected by triethylamine elution subsequent to the competitive elution step. Some of these VHHs are very similar, suggesting that they belong to a single germline (Supplementary Fig. 1). All together, VHHs corresponding to 12 diverse germlines were identified and one representative antibody for each of them was subcloned for large-scale expression. However, because of low production yields, only a subset of the initial antibody pool was used for further characterization. Interesting, one VHH (2F5) share the same sequence of C8, a binder selected by triethylamine elution in a previous panning procedure using the same library [12], demonstrating the reproducibility of the panning method.

The apparent affinity of the selected VHHs for the antigen FGFR1 was measured by ELISA recording the signal intensity at decreasing antibody concentrations and resulted extremely variable (Fig. 1). Specifically, the competitive elution allowed for the recovery of binders with low affinity in comparison to those selected by chemical denaturation. However, all the antibodies resulted effective and specific when used for detecting their antigen in immunofluorescence (Fig. 2 and Supplementary Fig. 2).

As expected, a competitive ELISA test confirmed that the VHHs eluted by FGF-2 competition (2E2, 2G6) share the same epitope on the FGFR1 ectodomain with FGF-2 (Fig. 3). The C8 and its homolog 2F5 behaved as previously reported [12], namely they were not displaced from FGFR1 by FGF-2 even at high concentration, indicating that their epitope is distinct form the region recognized by the natural ligand. In contrast, two of the triethylamine-eluted binders (2B10, G12) behaved as FGF-2 competitors.

The effectiveness of the competitive elution depends on the relative affinity and concentration of both the antibody and the ligand. The 2B10 and G12 VHHs have affinity significantly higher for FGFR1 than 2E2 and 2G6. Therefore, we assumed that the FGF-2 concentration used during the panning procedure was too low for displacing 2B10 and G12 and considered the hypothesis that the two groups of competitive VHHs bind different FGFR1 regions. Consequently, we looked for overlapping epitopes among all the binders performing another round of competitive ELISA experiments to identify the relative epitopes of all the VHHs. Each of them, engineered into an IgG-like structure with a mouse Fc [14], was challenged by any of the others, reconstituted into the same format, but with a rabbit Fc. The results clearly indicated that 2B10, G12, and 2E2 actually possess an overlapping epitope (Supplementary Fig. 3A) that is partially shared by FGF-2 and partially by C8/2F5. In contrast, 2G6 recognizes an independent epitope with respect to the other VHHs. The schematic distribution