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Generation and characterization of nanobodies against rhGH expressed as *sf*GFP fusion protein

Abdul Qader Abbady [a,*], Rasha Al-Shemali [b], Jana Mir Assaad [a], Hossam Murad [b]

[a] Division of Microbiology and Immunology, Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria
[b] Division of Human Genetics, Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria

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ABSTRACT

Growth hormone (GH) deficiencies are diagnosed in most children with short stature and treated with a long course of administrating expensive and daily doses of recombinant human GH (rhGH or Somatropin®). This work describes for the first time the production of several GH specific nanobodies with great potential in the field of GH production and detection. Nanobodies are the smallest intact antigen binders derived from heavy chain-only antibodies (HCAbs) of camelids. They are very stable, highly soluble and are produced as recombinant proteins in *Escherichia coli* at an affordable cost for various biotechnological applications.

To increase its solubility and immunogenicity, GH was produced as fusion with superfolder green fluorescent protein (*sf*GFP) and was used in this form to successfully immunize an adult camel. The active involvement of HCAbs in the specific camel immune response encouraged the preparation of large nanobody "immune" library. Phage display biopanning of this library against GH resulted in the isolation of five interesting and different nanobodies, referred to as NbGH01, 02, 03, 04 and 06. All nanobodies were able to recognize GH in its fusion and free formats and the detection sensitivity ranged from 0.5 to 10 ng/ml in sandwich ELISA. Pure rhGH was successfully purified by affinity chromatography, using immobilized NbGH06, from the cleavage reaction of fusion proteins with the tobaccos etch virus (TEV) protease. These specific molecular binders, especially NbGH06, provide valuable tools for rhGH diagnostic as well as for production purposes.

1. Introduction

Human growth hormone (hGH) is a single-chain polypeptide hormone synthesized mainly by the acidophilic somatotrophs of the anterior pituitary gland (Baumann, 2009). Somatotropin is the common name of the GH produced naturally in animals, whereas the term Somatropin (or rhGH) refers to the recombinant form of hGH (De Palo et al., 2006). Medical hGH is administered to patients whose pituitary glands generate insufficient quantities for normal growth and development (Vance and Mauras, 1999). Beside its licenced medical application, rhGH is widely abused by many athletes for its anabolic and lipolitic properties. Therefore, it is on the list of substances issued by World Anti-Doping Agency (WADA) as banned for competitive sports. However until recently, a standard test was lacking to detect rhGH abuse (McHugh et al., 2005) despite many assays for the measurement of GH levels in the blood of young patients (Popii and Baumann, 2004) or abusing athletes (He and Wu, 2009) were suggested. For *in vitro* bioassays, GH-measurement depends on its proliferative effect on cultured cell lines which display its specific receptor (Ishikawa et al., 2000, Maimaiti et al., 2012). Because of their simplicity and accessibility, immunoassays are still the methods of choice in the field of GH measuring in clinical laboratories (Bidlingmaier and Freda, 2010). For years, immunoassays depend on specific anti-GH binding molecules, like conventional antibodies (Thomas et al., 2012), recombinant antibody fragments (Bird et al., 1988) or even DNA aptamers (Bruno et al., 2011).

Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; GHBP, growth hormone binding protein; HCAb, heavy-chain antibody; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; NBT, nitro blue tetrazolium chloride; NHS, N-hydroxysuccinimide; Ni-NTA, nickel-charged nitrilotriacetic acid; PBS, phosphate buffered saline; scFv, single chain antibody variable fragment; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine; TEV, tobacco etch virus; WADA, World Anti-Doping Agency.

* Corresponding author. Fax: +963 11 6112289.
E-mail address: ascientific@aec.org.sy (A.Q. Abbady).

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Before rhGH became available, the hGH for therapeutic use was obtained from pituitary glands of cadavers and this unsafe practice led to some patients developing Creutzfeldt–Jacob disease (Abrams et al., 2011). These potential risks facilitated the introduction of recombinant DNA technology for safe and abundant production of rhGH in various heterologous systems, like *Escherichia coli* (Patra et al., 2000), yeast (Hahnm and Chung, 2001), insect cells (Lan et al., 2010) and plants (Shigemitsu et al., 2012). Most studies on recombinant protein production have described the use of N- or C- terminal 6×His tag for subsequent steps of purification and detection. Ligands that bind 6×His protein tags are commercially available in pre-immobilized forms ready to use for affinity chromatography purification (Lewis, 1984). However, rhGH and just like most other medical recombinant proteins needs to be tag-free before administration in humans, thus further steps for tag removal need to be accomplished. The presence of an extra tag in the protein native structure could affect its biological activity, or even worse, could stimulate the immune system, reducing the recombinant protein half-life in the blood circulation. An alternative solution for producing untagged rhGH implies the presence of more specialized ligands, such as specific antibodies that can be immobilized using one of the several commercially available activated affinity supports.

Beside their role as trapping molecules in affinity chromatography, conventional poly- and monoclonal antibodies are still indispensable reagents in basic research and diagnostics. However, both of them have some shortcomings including the low specificity of the polyclonal antibodies and the elevated costs and long time necessary for producing monoclonal antibodies. Furthermore, their large size and complexity is detrimental for some diagnostic and therapeutic applications. Because specific antibodies are expensive to produce or obtain commercially, they are seldom used for large scale purification of their antigens by affinity chromatography approach, even if their use is confined almost entirely to very small-scales. All these reasons urged the development of strategies aimed to produce alternative binders (Gebauer and Skerra, 2009), like recombinant antibodies of smaller dimensions that could be easily selected, produced, and manipulated using standard molecular biology techniques.

Advances in the field of recombinant antibody technology provide an alternative means to produce antibodies. One of the most successful recombinant antibodies is the single chain Fv antibody (scFv). It has several advantages over conventional antibody, including the low cost production by fermentation in *E. coli* (Ahmad et al., 2012). More importantly, its single chain makes it easy to fuse with other proteins or toxins, resulting in the formation of antibody molecules with two or more desired functions (Liu et al., 2010). Camelids produce antibodies called heavy chain antibodies (HCAbs) which are naturally devoid of light chains and are fully capable of binding antigen (De Genst et al., 2006). The recombinant variable domain of HCAb, referred to as nanobody®, is a monomeric structure that possesses a high physicochemical stability and solubility, with high production level obtained in *E. coli* or yeast (Dumoulin et al., 2002). Thus, nanobodies offer several advantages over intact antibodies for biotechnological or research purposes and medical applications (De Genst et al., 2006, Deckers et al., 2009, Muyldermans et al., 2009, Saerens et al., 2008a,b), and they might be an efficient alternative to scFv (Van Bockstaele et al., 2009, Wesolowski et al., 2009). Nanobodies were successfully generated against numerous antigens including various molecules or venoms and even intact pathogens (Abderrazek et al., 2009, Conrath et al., 2001, Deckers et al., 2009, El Khattabi et al., 2006, Harmsen et al., 2009, Lam et al., 2009, Stijlemans et al., 2004) as well as purified recombinant proteins (Bakhtiari et al., 2009, Conrath et al., 2001, Roovers et al., 2011). Furthermore, nanobodies represent a novel class of affinity ligands with potential applications in various fields, such as diagnostics, therapeutics, proteomics, etc. (Saerens et al., 2008a,b), and they have been used in immunoaffinity purification as capturing reagents and in biosensor technology (Bird et al., 1988, Ikeda et al., 2000). Because of the robust nature of nanobodies, they are expected to be tolerant to the covalent immobilization on purification column and the persistent steps of regeneration and elution.

E. coli expression system is an affordable method for rhGH production in large yields since no post translational modifications are reported in its protein structure. However, one major obstacle encountering the production process is related to the low solubility of rhGH, resulting in its accumulation in the inclusion bodies. In this structure rhGH is misfolded, and inactive, thus difficult and costly procedures are needed to refold the protein in order to recover its activity. The green fluorescent protein (GFP) from *Aequorea* jellyfish has become a common fusion tag because of its interesting spectral and structural features (Waldo, 2003; Waldo et al., 1999). Recently, Waldo and coworkers reported the engineering of superfolder GFP (*sf*GFP) that showed improved folding kinetics and increased solubility (Andrews et al., 2007; Fisher and DeLisa, 2008; Pedelacq et al., 2006, Wu et al., 2009). An enhanced solubility was observed for many proteins when fused to *sf*GFP, proving the usefulness of this tag as a mean to improve protein expression, detection and purification (Cabantous et al., 2005; Cabantous and Waldo, 2006). Before the final step of affinity purification, the removal of the N-terminal fusion tag from the recombinant protein could be achieved by cleavage with site-specific proteases, such as the tobacco etch virus (TEV) protease (Miladi et al., 2011).

Surprisingly, production of specific anti-GH genetically engineered antibodies, scFv or nanobody, has never been previously reported. Because of that and considering all nanobody advantages, the purpose of this study was to produce and characterize anti-rhGH nanobodies, which could be applied in the future in the field of GH production and detection. In this study, we report the production of rhGH as fusion with *sf*GFP to enhance its solubility and immunogenicity, and this fusion form of rhGH was successfully used to immunize an Arabian camel in order to establish new nanobody-phage display library. This "immune" library was sufficiently large and competent to retrieve five different anti-rhGH specific nanobodies. GH specific nanobodies have particular importance since there is a definite need for better diagnostic and cheaper tools for developing GH productive assays.

2. Materials and methods

2.1. Antigens and antibodies

For ELISA and immuno-blotting, detection of GFP and GH was performed using specific polyclonal antibody (homemade rabbit anti-GFP) and monoclonal antibody (mouse anti-GH; Santa cruz), respectively. Detection of antigen-bound nanobodies was performed with either rabbit anti-His antibody (Bethyl Laboratories Inc.) for ELISA or with rabbit anti-nanobody (homemade) for immuno-blotting. Detection of antigen-bound IgGs was performed with rabbit anti-camel antibody (Bethyl Laboratories Inc.). Subsequent detection of rabbit or mouse antisera was done using anti-rabbit or anti-mouse conjugated to horseradish peroxidase (HRP) for ELISA or to alkaline phosphatase (AP) for immuno-blotting (Bethyl Laboratories Inc.). Detection of antigen-bound phage particles presenting nanobodies was directly performed using mouse anti-M13-HRP conjugate (GE Healthcare). For nanobody preparation, pMES4 phagemid and *E. coli* strains (TG1 and WK6) were kindly provided by Prof. S. Muyldermans (VIB/VUB, Brussels, Belgium).