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ORIGINAL PAPER

Immunodetection of the recombinant GroEL by the Nanobody NbBruc02

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Abstract *Brucella* has a great impact on health and economy in Syria, thus much effort is being placed on the development of diagnostics and vaccines. In this context, a wide Nanobody "immune" library was previously established, from which several *Brucella*-specific binders were isolated. One of these camel genetically engineered heavy-chain antibody fragments was referred to as NbBruc02. The precise antigen of NbBruc02 was presumed to be, according to proteomic approaches, the *Brucella* heat shock protein of 60 kDa (HSP-60). HSP-60, or alternatively named GroEL, is an interesting *Brucella* immunodominant antigen with important roles in the parasite life cycle, mainly adhesion and penetration during the infection of macrophages. In the present work, the capacity of NbBruc02 to filtrate the native GroEL from *Brucella* total extract was tested by immunochromatography approach. The interaction between NbBruc02 and its antigen was further confirmed using recombinant GroEL from *Brucella*. Interestingly, NbBruc02 was able to immunodetect the native as well as the denatured forms of the rGroEL in ELISA and immunoblotting, respectively. In agreement with previously reported data, NbBruc02 was able only to detect the denatured *Yersinia* rGroEL. Using surface plasmon resonance (SPR) biosensor, NbBruc02 showed a strong interaction, with nanomolar affinity ($K_D = \sim 10^{-8}$ M), with the native rGroEL of *Brucella* and not of *Yersinia*. Because the casual conformational changes in the GroEL 3D structure make the base of its function, NbBruc02 by its ability to recognize a "conformational epitope," could open wide perspectives to study the role of GroEL in *Brucella* physiology.

Keywords *Brucella* · *Yersinia* · GroEL · Nanobody

Abbreviations

AEC	3-amino-9-ethylcarbazole
FPLC	Fast protein liquid chromatography
HCAb	Heavy-chain antibody
HSP-60	Heat shock protein of 60 kDa
IPTG	Isopropyl β-D-thiogalactoside
LPS	Lipopolysaccharide
Ni-NTA	Nickel-charged nitrilotriacetic acid
SPR	Surface plasmon resonance
TMB	3,3′,5,5′-tetramethylbenzidine

Introduction

Brucellosis is a zoonotic disease caused by Gram-negative bacteria belonging to the genus *Brucella,* which are facultative intracellular pathogens. The disease is characterized by abortion and reduced fertility in cattle, and by chronic infections with symptoms such as undulant fever, arthritis and osteomyelitis in humans (Pappas et al. 2005). Thus, the development of a rapid and accurate diagnostic test for brucellosis in humans and animals remains an elusive target.

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Currently, there are many serological tests relying on the use of lipopolysaccharide (LPS) or whole-cell bacterial extracts as antigens for ELISA or agglutination tests using colorimetry (Pappas et al. 2005). Beside their false positives due to serological cross-reactivity (Alonso-Urmeneta et al. 1998), the production of these antigens has many other problems including the difficulties associated with the biohazard risk since *Brucella* species are class III pathogens (Pappas et al. 2006). Moreover, serum cross-reactivities are regularly observed between the LPS of some other bacteria (i.e. *Yersinia enterocolitica O:9*, *Vibrio cholerae*, *Salmonella typhimurium and Escherichia coli O157*). A possible strategy for circumventing the cross-reaction caused by anti-LPS antibodies is to base the diagnosis on selected *Brucella* immunodominant proteins. Such antigens were identified and characterized by proteomic methods from various *Brucella* preparations (Connolly et al. 2006; Teixeira-Gomes et al. 1997a, b; Lin and Ficht 1995). One of these methods was recently developed using recombinant camel heavy-chain antibody (HCAb) fragment, or Nanobody. This genetically tailored variable single domain is able to bind firmly and specifically the antigen. As an initiative act against *Brucella*, a highly diverse Nanobody library was recently created from a camel immunized with a heat-killed *B. melitensis* Rev.1. strain (Abbady et al. 2011). Phage display panning of this library using *Brucella* total lysate resulted in the identification of several Nanobodies for unspecified antigens within this lysate. One of these Nanobodies, named NbBruc02 (GenBank accession no. JF313902), was highly reactive even at very low concentrations and able to distinguish *Brucella* lysate from other bacteria including *Yersinia* in ELISA testes. By advanced proteomic techniques, the specific antigen of NbBruc02 was isolated and identified as the chaperonin GroEL (Abbady et al. 2012). This protein, which is also known as the heat shock protein of 60 kDa (HSP-60), represents one of the most immunogenic proteins of *Brucella* with a great potential in the serological assays of the disease (Al Dahouk et al. 2006).

In fact, *Brucella* are facultative intracellular pathogens that infect phagocytic cells, such as macrophages, and multiplies within (Pappas et al. 2005). *Brucella* synthesis GroEL protein in response to macrophage phagocytosis that helps denaturated proteins under acid conditions to refold, therefore enhances the ability of *Brucella* to survive (Basharov 2003; Lin and Ficht 1995). GroEL proteins are the major antigens involved in T-lymphocyte activation and induction of protective immunity against various intracellular pathogens (Kaufmann et al. 1991; Retzlaff et al. 1994). Furthermore, GroEL was found as an immunodominant antigen among many other proteins from the outer membrane of *B. abortus* and may play a critical role in the virulence to both human and bovines (Connolly et al. 2006; Al Dahouk et al. 2006). GroEL reacts with serum from humans, cattle and sheep with naturally acquired brucellosis (Connolly et al. 2006; Amirmozafari et al. 2008).

Under appropriate and native (undenatured) conditions, NbBruc02 showed a clear ability to distinguish several *Brucella* sps. from many other *Bacteria* in ELISA test using total protein lysates. However, it is not yet clear why NbBruc02 lost this ability and exhibited a confusing behavior toward its antigens, from *Brucella* and *Yersinia*, under denatured conditions (Abbady et al. 2012). Since GroEL is a highly conserved protein, it was concluded that NbBruc02 could sense a conserved epitope that can adapt easily conformational changes between native and denatured conditions. In this context, this work aimed at characterizing the interaction between NbBruc02 and its pure recombinant antigen from two different sources, *Brucella* and *Yersinia*. The results of this work could be of a great importance for future studies about the role of GroEL in *Brucella* life cycle, mainly the adhesion and invasion, and the possibility to interfere in these phenomena using NbBruc02.

Materials and methods

Bacterial strains, growth conditions and plasmid

Brucella and *Yersinia* strains were provided by (Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium). Prior to inactivation, *Brucella* strains were grown in *Brucella* broth medium (HIMEDIA Laboratories) supplemented with 5 % heat-inactivated horse serum (PAN Biotech GmbH) for at least 48 h in a 37 °C incubator. The inactivation step was performed by washing bacteria twice with cold PBS, followed by heat incubation at 60 °C for 30 min. Inactivation was confirmed by inoculating 1 ml of bacterial suspension on *Brucella* agar solid media. To prepare *Brucella* soluble lysate, 1 g of wet weight bacteria was resuspended in 5 ml ice-cold PBS and protease inhibitor solution (1 mM PMSF, 1 × complete-protease inhibitor from Roche supplemented with 1 mg/ml of lysozyme) and incubated for 30 min on ice followed by 5 times sonication (15 s pulses) on ice. Bacterial debris was eliminated by centrifugation (15,000 *g*, 15 min, 4 °C), and the supernatant was saved.

Escherichia coli WK6 strain, which was used in cloning and protein expression, was transformed by electroporation with the recombinant plasmid pHEN6 (kindly provided by Prof. Serge Muyldermans, Department of Cellular and Molecular Interactions, VIB, Brussel, Belgium). Generally, *E. coli* were grown in Luria Broth (LB) (Bio Basic INC) with the required antibiotic at 100 mg/ml (streptomycin or

ampicillin; Applichem) at 37 °C. Protein expression of positive clones was performed in Terrific Broth (TB) containing 100 mg/ml ampicillin, 2 mM $MgCl_2$ and 0.1 % glucose according to (Arbabi Ghahroudi et al. 1997). PCRs were achieved directly on 100 ng of genomic DNA, which was extracted from heat-killed bacteria according to standard CTAB/NaCl method (Sambrook and Russel 2001).

Immunochromatography assay

The ability of NbBruc02 to capture the antigen from *Brucella* total lysate was tested by immunoaffinity chromatography assay. In this respect, nickel-charged nitrilotriacetic acid (NTA) superflow Sepharose 1 ml column (Qiagen) was used to trap NbBruc02 by means of its C-terminal 6 × His tag. AKTAprime (GE lifescience) fast protein liquid chromatography system (FPLC) was used to monitor the purification procedure. Prior to purification, NTA column was equilibrated with a flow of elution (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) and wash (same as elution but with 20 mM imidazole) buffers. 1 ml of *Brucella* protein lysate (1 µg/µl) was incubated for 1 h at 4 °C under slow rotation, in the absence or presence of NbBruc02 (50 µg). Samples were injected at a flow rate of 1 ml/min in the column. The flow-through was collected, and the column was washed. Finally, trapped protein complexes (NbBruc02/antigen) were eluted with the elution buffer. Monitoring the UV absorption at 280 nm facilitated the collection of samples from the different steps of purification. Samples were separated on SDS-PAGE and revealed by blue gel staining and immunoblotting with NbBruc02 (1:500) followed with mouse anti-6 × His tag-HRP (1:1,000) conjugated antibody (Roche).

Cloning, expression and purification of rGroEL

Genomic DNA, extracted from *B. abortus* strain S19 and *Y. enterocolitica* strain $O9^-$, was used as a PCR template to amplify GroEL genes using specific primers; for *Brucella* (accession: NC_006933.1): BF-*Xba*I (5′-TCCGCA**TCTAGA**GCTGCAAAAGACGTAAAATT-3′) and BR-*Bst*EII (5′-TATATA**GGTCACC**CCGAAGTCCATGCCGCCCATGC-3′), and *Yersinia* (accession: NC_008800.1): YF-*Xba*I (5′-TCCGCA**TCTAGA**GCAGCTAAAGACGTAAAATT-3′) and YR-*Bst*EII (5′-TTATTA**GGTCACC**CCCATCATGCCGCCCATACCGC-3′). Primers were designed to amplify the full-length gene, but without the start and stop codons, and to add *Xba*I and *Bst*EII (Fermentas) restriction sites at the 3′ and 5′ ends, respectively. For cloning purposes, AccuPrim™Taq Polymerase High fidelity Kit (Invitrogen) was used in the PCR. PCR-amplified GroEL genes and pHEN6 plasmid were digested by restriction enzymes and then ligated using Ready-To-Go™ T4 DNA ligase Kit (GE life science). Freshly prepared electrocompetent *E. coli* strain WK6 cells were transformed with the ligated products through electroporation. Successful cloning of these plasmids was confirmed by sequencing the full-length of the cloned GroEL genes. Expression of rGroEL from confirmed plasmid constructs was performed in 250-ml shake flasks by growing the bacteria in a complete TB medium till an optical density of 0.6–0.9 was reached, and then expression was induced with 1 mM IPTG (Isopropyl β-D-thiogalactoside; Promega) for 16 h at 37 °C. After pelleting the cells, periplasmic proteins were extracted by osmotic shock according to Skerra et al. (Skerra and Pluckthun 1988). Using AKTAprime system, periplasmic extracts were loaded on a 1 ml NTA column. After washing, the bound proteins were eluted with 250 mM imidazole buffer. The eluted fraction was concentrated on Vivaspin concentrators with a molecular mass cutoff of 30 kDa (Vivascience) and loaded on a Superdex-75 10/30 gel filtration column (GE life science). Protein concentration of the purified proteins was measured by spectrophotometer at 280 nm.

Immunoblotting and coomassie blue staining of SDS-PAGE

SDS-PAGE was carried out using Bio-Rad mini-Protean II system following the manufacturer's instructions. Gels were prepared using 4 % stacking gel and 10 % running gel. After electrophoresis, gels were stained by coomassie blue for 2 h followed by distaining. For blotting, proteins in acrylamide gel were electrically transferred onto 0.45-µm nitrocellulose membranes (GE life science) using blotting buffer (25 mM Tris–HCl, 200 mM glycine, 0.1 % SDS and 20 % methanol, pH 8.3). After incubation in the blocking buffer (3 % skimmed milk, 1 % BSA in 1 × PBS), blots were incubated with Nanobodies (NbBruc02 or control Nanobody), rabbit anti-SP41 (1:1,000, home made) or with mouse anti-6 × His tag-HRP antibody (1:1,000). Detection of bound Nanobodies was performed either with the monoclonal anti-6 × His antibody or with a rabbit anti-camel antibody (1:4,000, Bethyl laboratories). Final detection of the rabbit antibodies was done by goat anti-rabbit-HRP conjugate (1:4,000, Bethyl laboratories), and subsequent revelation was obtained using AEC (3-amino-9-ethylcarbazole) chromagen substrate in phosphate citrate buffer in the presence of hydrogen peroxide.

ELISA assay

Maxisorb 96-well plates (Nunc) were coated overnight at 4 °C with pure rGroEL, at 0.3 µg/well in PBS. Residual

protein binding sites in the wells were blocked for 2 h at 37 °C with 5 % skimmed milk powder in 1 × PBS. Nanobodies (NbBruc02 or control Nanobody) were added to the wells at the indicated concentrations. Detection of bound Nanobodies was performed either with the monoclonal anti-6 × His-HRP antibody or with a rabbit anti-camel antibody (1:4,000) followed by anti-rabbit-HRP conjugate (1:4,000). The absorption at 450 nm was measured after adding the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB ready-to-use from Sigma) followed by the stopping buffer (1 M H_2SO_4).

Biosensor analysis

The capacity of NbBruc02 to bind specifically its antigen was evaluated by Surface Plasmon Resonance (SPR) using a SR7000DC instrument (Xantec). The assay was implemented by immobilizing 200 µl of NbBruc02 (0.1 µg/µl), dissolved in 10 mM acetate buffer pH 5, onto a research grade CMD200 M sensor chip according to the manufacturer's instructions. All binding experiments were performed at 25 °C in HBS buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 3.5 mM EDTA and 0.005 % Tween-20) at a flow rate of 50 µl/min. A blank uncoated channel was used as an online reference during all injections. The high capacity of binding of NbBruc02 was demonstrated by consecutive injection of five aliquots (100 µl) of *Brucella* rGroEL (1,600 nM) over the two flow cells and followed by a dissociation step of 200 s. For specificity determination, 100 µl injections of rGroEL (of *Brucella* or *Yersinia*) (1,600 nM) or *Brucella* protein lysate (1 mg/ml) were run over immobilized NbBruc02 and control channels. Association kinetics measurement of NbBruc02/GroEL binding was taken using injections (100 µl) of various concentrations (0, 80, 160, 400, 800 and 1,600 nM) of *Brucella* rGroEL. The sensorgrams were fitted by subtracting the signal from the reference flowcell and were globally treated using Scrubber 2 software (www.cores.utah.edu). Short pulses (15 µl) of regeneration buffers (e.g. 6 M guanidine HCl, 0.1 M HCL or 10 mM glycine–HCL pH 2.0) were used to regenerate the Nanobody-immobilized chip between the injections without any detectable effect on its binding capacity (data not shown).

Results

NbBruc02 interacts and binds specifically to GroEL in *Brucella* lysate

In previous study, the antigen of NbBruc02 was isolated from *Brucella* extract and identified as the GroEL protein (Abbady et al. 2012). An immunoaffinity chromatographic approach using NbBruc02 was exploited in order to confirm this interaction. NbBruc02 was incubated with the soluble *Brucella* lysate, and Nanobody/antigen complexes were then captured from the mixture by filtration through nickel-NTA column installed on an AKTAprime system. This column can trap the recombinant Nanobody by means of its 6 × His tail. After several washes, the protein complexes were eluted from the column, separated into SDS-PAGE and analyzed by staining and immunoblotting with NbBruc02. The UV detector, supplemented with this system, enabled real-time monitoring of the different steps of purification, from which several protein samples were analyzed by SDS-PAGE (Fig. 1). The purification chart (Fig. 1a) demonstrated the capacity of NbBruc02 to bind and to purify its ∼ 60 kDa antigen (sample 5), which was observed in the stained gel (Fig. 1b, left) and redetected by NbBruc02 in the blotted membrane (Fig. 1b, right). Purification was accompanied with a decrease in GroEL amount in the flow-through (sample 4). This was only observed when *Brucella* lysate was incubated with NbBruc02 (samples 3, 4 and 5) and not without it (samples 1 and 2). Two unspecific bands were co-purified (sample 5) during immunochromatography as shown in the stained gel. However, in contrast to GroEL, they could not be detected by NbBruc02 in immune blotting. These results demonstrated the capacity of NbBruc02 to bind and purify the native form of *Brucella* GroEL and to detect the denatured form of this antigen in immunoblotting.

Cloning and expression of the GroEL in pHEN6

In previous study, NbBruc02 was able in ELISA assay to distinguish protein extracts of *Brucella* from those of *Yersinia* under native conditions. In contrary, under denatured conditions, as in the immunoblotting after SDS-PAGE, NbBruc02 recognized the same antigen in both extracts (Abbady et al. 2012). To study NbBruc02 behavior toward its antigen, the recombinant GroELs (rGroELs) from *Brucella* and *Yersinia* were produced using pHEN6 plasmid that enables the expression of soluble C-terminal-6 × His-tagged proteins in the periplasm of the transformed *E. coli* due to the pelB leader signal peptide added to their N-terminal. Pure rGroEL, from *Brucella* or *Yersinia*, when immunoblotted with anti-6 × His-HRP-conjugated antibody, resulted in a relatively big protein consisting of 587 amino acid residues with apparent molecular weight of 60 kDa (Fig. 2a, a-6 × His).

Recognition of the rGroEL by NbBruc02

The interaction of NbBruc02 with its specific antigen, the rGroEL, was further confirmed by immunoblotting and ELISA assays using approximate amounts of the purified

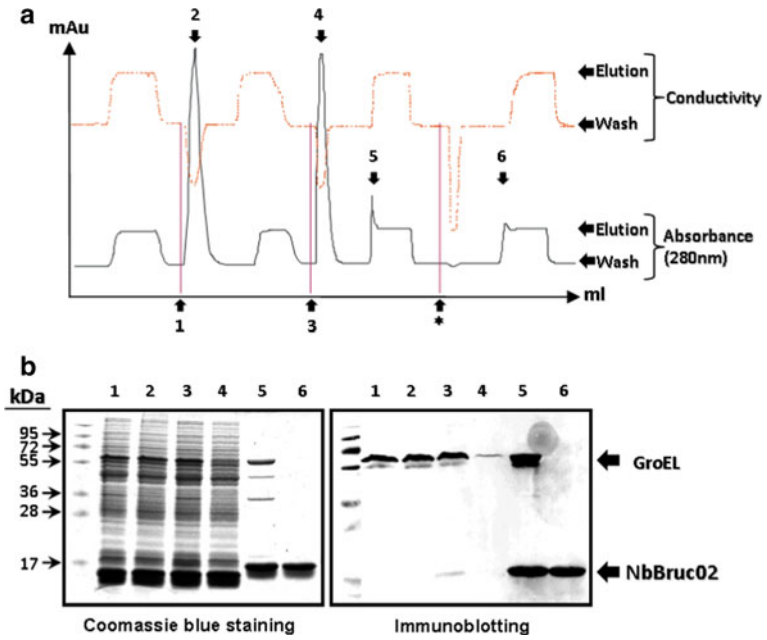

Fig. 1 Immunochromatography of *Brucella* GroEL by NbBruc02. **a** GroEL purification diagram by NbBruc02 affinity chromatography and using Ni–NTA purification column installed on AKTAprime FPLC system. The column was initialized with wash and elution buffers to receive three consecutive injections of samples (*vertical bars*). 1 mg protein of *Brucella* lysate was injected after being incubated in the absence (*1*) or in the presence of 50 µg of NbBruc02 (*3*), and the flow-through for each condition was collected (*2* and *4,* respectively). After wash and elution, purification pick (*5*) was only observed (and collected) in the presence of the Nanobody. Pure NbBruc02 (50 µg) was also injected (*) and resulted in no flow-through put in a purification pick (*6*). **b** Protein samples from the previous purification were separated into SDS–PAGE (10 %) and then detected directly either by coomassie blue staining (left) or by Immunoblotting with NbBruc02 (1/500) followed by monoclonal anti-6 × His tag antibody (*right*). The location of GroEL and NbBruc02 in the gel is indicated and defined as 60 and 16 kDa, respectively. The protein molecular weight ladder in the first lane

proteins (Figs. 2a, b). As expected, NbBruc02 was able to detect the blotted pure *Brucella* rGroEL but not other home-made *Brucella* immunodominant antigen such as rSP41 protein (Castaneda-Roldan et al. 2006). Furthermore, this Nanobody was able to detect, to the same extent, the *Yersinia* rGroEL (Fig. 2a, NbBruc02). This interaction between NbBruc02 and the rGroELs was specific since no immunodetection could be obtained when NbBruc02 was replaced by another *Brucella*-specific Nanobody (Fig. 2a, Nb) or by a polyclonal rabbit anti-SP41 antibody, which was clearly able to detect specifically its antigen (Fig. 2a, a-SP41).

In ELISA assay, NbBruc02, but not another *Brucella*-specific Nanobody, was able to detect the immobilized rGroEL. However, detection signal was more important for rGroEL of *Brucella* than that of *Yersinia* (Fig. 2b). To evaluate the interaction between NbBruc02 and its antigen, serial dilutions of NbBruc02 (1 mg/ml) were tested in the absence or presence of *Brucella* rGroEL (Fig. 2b, inset). In agreement with previous data from similar ELISA tests but using *Brucella* lysate instead of rGroEL, NbBruc02 was able to detect its antigen at a very high dilutions (up to 1:30,000 v:v), demonstrating the significance of this interaction.

SPR analysis of NbBruc02/GroEL interaction

The ability of NbBruc02 to interact specifically and strongly with its antigen was further supported using double-channels SPR biosensor. SPR enables the analysis of direct biomolecular interactions with several advantages including: fast-speed, no-labeling, real-time and micro-sample requiring, over traditional methods, for example, ELISA (Chuang et al. 2009). NbBruc02 was covalently immobilized on activated matrix of one sensor channel by an amine coupling method, and the second channel was left empty to obtain a constant reference value. SPR signals from both channels were recorded in a sensorgram of refractive index units (RIU) as a function of time (Fig. 3). In this experiment, immobilized NbBruc02 was able to bind and fix its target antigen from the injected flow of *Brucella* rGroEL. Such binding between NbBruc02 and its antigen seems to be so stable since new level of SPR signal was reached even after passing the washing buffer. Moreover, since the SPR signals are affected by the amount and the type of molecules attached to the sensor chip, sensorgram showed that immobilized NbBruc02 was able to fix increasing amounts of its antigen from a consecutive injects of *Brucella* rGroEL (Fig. 3). After an analysis, to obtain the next set of data, the

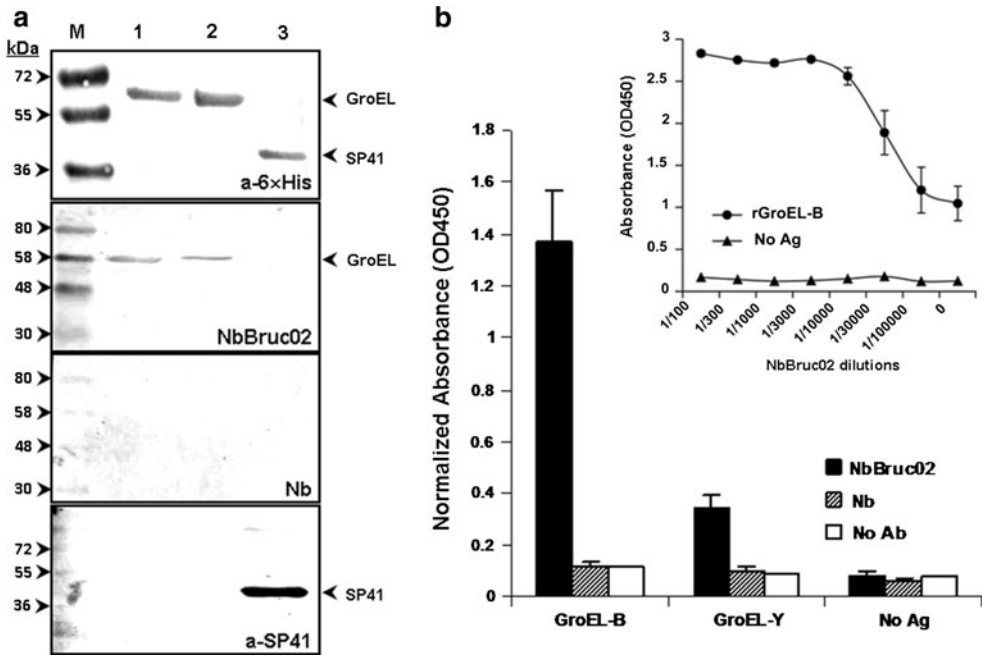

Fig. 2 Immunodetection of the rGroEL by NbBruc02. **a** Equal quantities (0.2 μg) of three different purified proteins; *Brucella* rGroEL (*lane1*), *Yersinia* rGroEL (*lane2*) and *Brucella* rSP41 (*lane3*), were loaded in SDS-PAGE (10 %). After transfer, membranes were incubated with mouse anti-6 × His tag (1:1,000), NbBruc02 (1:500), other Nanobody (Nb) (1:100) or rabbit anti-SP41 (1:1,000) antibodies. The locations of rGroEL and rSP41 in the blots are indicated by the arrows, and protein molecular weight ladder is shown in the first lane (*M*). **b** Pure rGroEL (0.2 μg/well) from *Brucella* and *Yersinia* were immobilized and detected by ELISA using NbBruc02 (1/500), other Nanobody (Nb) (1/100) or in the absence of antibodies (No Ab). *Inset* serial dilutions of NbBruc02(v:v) were ELISA-tested in the absence (No Ag) or presence of *Brucella* rGroEL (0.2 μg/well)

Nanobody-modified sensor surface was regenerated using high salt (6 M guanidine HCl) or low pH (0.1 M HCl or 0.1 M Glycine) buffers. After several regeneration cycles, the binding capacity of the immobilized NbBruc02 was not affected and it was still able to bind the antigen without a significant decrease (data not shown).

Since the ability of NbBruc02 to distinguish between *Brucella* and *Yersinia* rGroEL is related somehow to the conformational changes, the capacity of the immobilized NbBruc02 to detect the antigen in flows of rGroELs (1.6 μM) or in a total *Brucella* extract (1 mg/ml) prepared under native conditions was tested (Fig. 4a). As expected, only antigens from *Brucella* origins induced signal shifting. Yersinia rGroEL totally failed in binding the immobilized NbBruc02, confirming the specificity of NbBruc02 toward *Brucella* antigen under native conditions.

The SPR can also be used for the quantitative and kinetic analyses of NbBruc02/GroEL interactions without the need for labeling, which can influence the binding. From a plot of the resonance signal vs time (sensorgram) and using increasing concentrations of the antigen injected (from 0 to 1,600 nM) (Fig. 4b), the association ($k_{on} = 1.2 \times 10^4$ M^{-1} S^{-1}) and dissociation ($k_{off} = 3.72 \times 10^{-4}$ S^{-1}) rate constants for the interaction were determined and the affinity ($KD = 31.2$ nM) of the binding was then calculated (Fig. 4c).

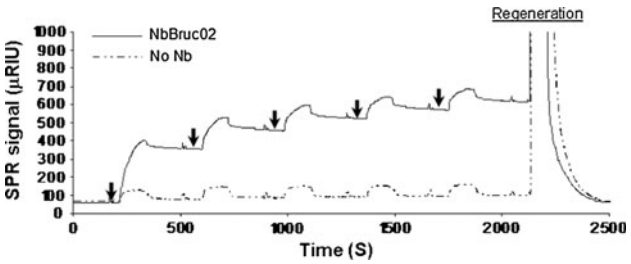

Fig. 3 Double-channels SPR sensorgram of NbBruc02/rGroEL binding. Signals from five consecutive injections (*arrows*) with *Brucella* rGroEL 1.6 μM on NbBruc02-coated (*continuous line*) and control channel (*dashed line*). Surface regeneration was performed with a flow of 6 M guanidin-HCl buffer

Discussion

The primary goal of Nanobody production is to obtain unlimited amounts of a high affinity and highly specific recognition unit for use in research, diagnostic tests or therapy. NbBruc02 is a recombinant antigen-binding antibody fragment that can be produced without limits (tens of mg/l of bacterial culture). In this work, the binding capacity of NbBruc02 was firstly shown by immunochromatography, where a single antigen was successfully purified from crude *Brucella* lysate. This is supported by a previously published data about the ability of NbBruc02 to precipitate

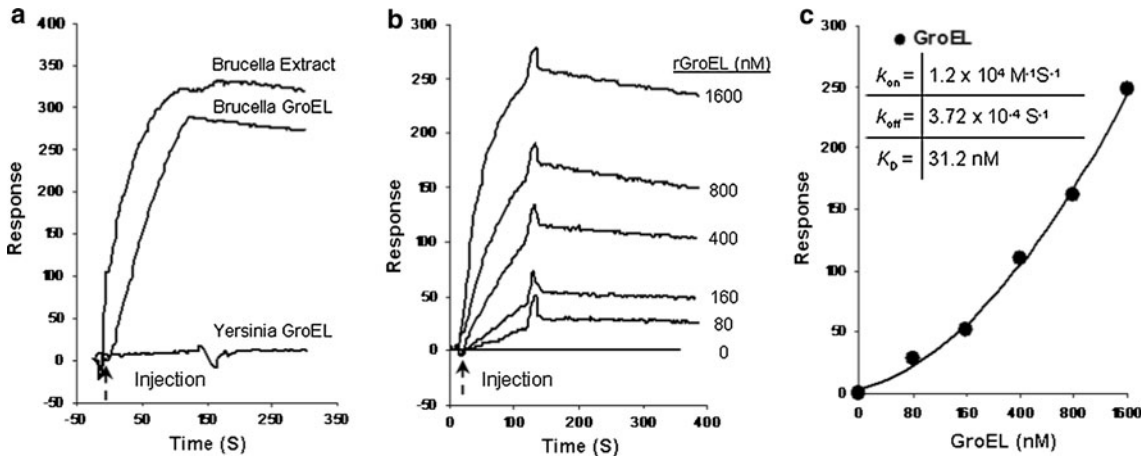

Fig. 4 SPR analysis of the NbBruc02 specificity to *Brucella* rGroEL. **a** Normalized SPR sensorgram of *B. melitensis* total lysate (1 mg/ml), *Brucella* rGroEL (1.6 μM) and *Yersinia* rGroEL (1.6 μM) binding to immobilized NbBruc02. **b** SPR sensorgram and fit, of subtractive signal of binding of several injected concentrations of *Brucella* rGroEL, from 80 to 1,600 nM to NbBruc02 immobilized on the sensor chip. **c** Dose–response binding, from graph **b**, for bound *Brucella* rGroEL used at nanomolar concentrations. Calculated kinetic constants (k_{on}, k_{off} and K_D) of the binding are shown. All SPR values were corrected for binding to the uncoated control flow cell

its antigen from a crude *Brucella* lysate by immunoprecipitation method (Abbady et al. 2012). A disadvantage of this strategy is the requirement of a great amount of the slow and risky *Brucella* cultures to obtain sufficient quantity of this pure antigen in order to study its direct interaction with NbBruc02. Furthermore, the identity of this antigen as GroEL was presumed from its molecular weight and from our previous work using Matrix Assisted Lazer Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF–MS) analysis on the immunoprecipitated antigen. Furthermore, NbBruc02 showed a strange behavior toward its antigens in *Brucella* and *Yersinia* extracts according to their folding state (Abbady et al. 2012). In this respect, studying the interaction between NbBruc02 and the recombinant *Brucella* and *Yersinia* rGroEL was the perfect confirmation of these findings. The immunochromatography results clearly demonstrated the capacity of NbBruc02 to bind the native as well as the denatured form of its specific antigen within *Brucella* extract.

A high-level expression system of C-terminal 6 × His-tagged-soluble recombinant GroEL from *Brucella* and *Yersinia* using pHEN6 plasmid was tested. Despite their reputation as cytoplasmic proteins, rGroELs were successfully expressed in the periplasm of *E. coli* via the N-terminal pelB leader signal encoded by pHEN6. Interestingly, no aggregations or precipitations were observed in the soluble periplasmic extracts or after protein purification of both rGroELs, which reflects their high solubility (data not shown). However, this finding does not necessarily confirm the correct folding of rGroELs and whether they are in their native states. In order to confirm their nativity, it would be of a great interest to proceed with functional in vitro studies of rGroEL as foldase enzyme after obtaining rGroES using the same expression system. The unmodified N-terminal of rGroEL should be functional as the reactive domain and the binding sites for ATP and GroES are all assured by this extremity (Lin and Rye 2006).

Similarly, NbBruc02 was able to recognize the purified rGroEL in its native (ELISA) and denatured (WB) forms. Despite targeting a highly conserved protein, the GroEL, NbBruc02 was able to recognize several strains, references and locals, belonging to the most important species of *Brucella* in Syria; *B. abortus* and *B. melitensis* (Abbady et al. 2012). In addition, this Nanobody was able to discriminate *Brucella* from many other tested strains of bacteria including *Y. enterocolitica O9* that shares many antigens with *Brucella*, mainly LPS O-ring epitope (Pappas et al. 2005). However, this discrimination was lost when the GroEL was in a denatured state as the case of immunoblotting detection, which was also confirmed using *Brucella* and *Yersinia* rGroEL. In ELISA tests, NbBruc02 could detect *Brucella* and to a lesser extent *Yersinia* GroELs. These findings imply that NbBruc02 detected a small portion of the *Yersinia* rGroEL, which has lost confirmation because of the adsorption effect on the plastic surface of the microtiter plate. However, when the NbBruc02 was covalently immobilized on SPR sensor chip, a significant interaction could be observed with a flow of *Brucella*, but not *Yersinia*, GroEL. This provided an extra evidence about the specificity of NbBruc02 toward *Brucella* antigen under native conditions (Abbady et al. 2012).

Molecular chaperones play a major role in protein folding and degradation by means of mechanisms

involving a reversible conformational change in their structure (Lund 2009). Interestingly, our results showed that NbBruc02 is able to sense *Yersinia* GroEL folded state. The susceptibility of Nanobodies for properly folded antigens is not surprising since Nanobodies distinguishing subtle changes in the structure or conformation of their target antigens have been reported already (Saerens et al. 2004; Habicht et al. 2007; Hendrickx et al. 2011). However, the opposite dependence of the NbBruc02 recognition of the folded or unfolded GroEL from *Yersinia* was totally unexpected. Only after treating the antigen under harsh conditions of denaturation, for example, during immunoblotting sample preparation, NbBruc02 is able to recognize its GroEL target from *Yersinia*. Although it remains puzzling, it could be explained since chaperones are highly conserved throughout evolution and if the epitope recognized on the *Brucella* GroEL, native structure is possibly hidden in the GroEL of other bacteria types, for example, *Yersinia*. We conclude that NbBruc02 binds an epitope that adopted easily an alternative conformation upon different experimental conditions. More investigations are required to fully conceive the picture of this astounding interaction between NbBruc02 and the different folding states of GroEL using the SPR biosensor system (Kuroda et al. 2006).

The determination of the exact affinity (KD, and also the kinetic binding rates) between NbBruc02 and its cognate antigen, the GroEL, is essential to characterize possible effects of conformational changes (of the antigen) or defining the diagnostic or therapeutic potential of NbBruc02. A high k_{on} rate is necessary for a fast detection (diagnosis) in biosensors, a slow k_{off} rate is important for inducing an effect and leading to therapy. The affinity determined by surface plasmon resonance analysis of this interaction was relatively high (about 10^{-8} M) explaining the high reported titer (1:30,000) of this Nanobody against its recombinant antigen as well as the total *Brucella* extracts in previous studies (Abbady et al. in press). This result demonstrated that NbBruc02 can bind tightly to GroEL with affinity constant similar to those found for Nanobodies directed against other protein antigens with potent inhibition effects (Omidfar et al. 2004; Zarebski et al. 2005; Stijlemans et al. 2011). However, the targeted epitope by NbBruc02 and its antigenic importance, as well as the capacity of NbBruc02, by binding this epitope, to neutralize the physiological role of GroEL as foldase enzyme, all these remain interesting questions need to be answered.

Since *Brucella* establishes a long-term residence within the host macrophages, it is subjected to extensive harsh conditions including extremes of pH and oxidative and nutritional stress. Under such conditions, the expression of heat shock proteins is vital for its survival in this inhospitable environment (Teixeira-Gomes et al. 1997a). *Brucella* GroEL might be an important protective molecule since it is implicated in antigen-specific T-lymphocyte-mediated activation of macrophages usually leading to the elimination of intracellular pathogens (Oliveira et al. 1996). Therefore, several attempts were done to evaluate the potential roles of GroEL as vaccine or as antigen for brucellosis serological tests (Bae et al. 2002; Oliveira et al. 1996; Bae and Toth 2000; Baloglu et al. 2000; Stevens et al. 1997; Lin et al. 1992). Furthermore, *B. abortus* GroEL was reported to be involved in bacterial internalization and intracellular signal transduction (Watarai et al. 2003).

Owing to their high stability and excellent folding capacity under different environmental conditions, Nanobodies have also been targeted successfully to the cytosol by removing the leader peptide and to the intracellular vesicles, such as the endoplasmic reticulum, by genetic fusion to suitable targeting sequences (Rothbauer et al. 2008; Serruys et al. 2009; Blanco-Toribio et al. 2010). Inside the living cell environment, Nanobodies have been found to be able to inhibit the physiological function of their target antigens (Kirchhofer et al. 2010). In this context, *Brucella* entry through lipid rafts is specifically mediated by the binding of surface-exposed Hsp60 to the cellular prion protein PrP of macrophages (Watarai et al. 2003). The possibility to interfere with *Brucella* adhesion and invasion, and to disrupt the cellular entry system of this pathogen, by an in vivo expressed NbBruc02, inspires an interesting perspective to explore as a new treatment approach against Brucellosis.

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