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Research Article

The Availability of a Recombinant Anti-SNAP Antibody in VHH Format Amplifies the Application Flexibility of SNAP-Tagged Proteins

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Antibodies are indispensable reagents in basic research, and those raised against tags constitute a useful tool for the evaluation of the biochemistry and biology of novel proteins. In this paper, we describe the isolation and characterization of a single-domain recombinant antibody (VHH) specific for the SNAP-tag, using Twist2 as a test-protein. The antibody was efficient in western blot, immunoprecipitation, immunopurification, and immunofluorescence. The sequence corresponding to the anti-SNAP has been subcloned for large-scale expression in vectors that allow its fusion to either a 6xHis-tag or the Fc domain of rabbit IgG2 taking advantage of a new plasmid that was specifically designed for VHH antibodies. The two different fusion antibodies were compared in immunopurification and immunofluorescence experiments, and the recombinant protein SNAP-Twist2 was accurately identified by the anti-SNAP Fc-VHH construct in the nuclear/nucleolar subcellular compartment. Furthermore, such localization was confirmed by direct Twist2 identification by means of anti-Twisit2 VHH antibodies recovered after panning of the same naïve phage display library used to isolate the anti-SNAP binders. Our successful localization of Twist2 protein using the SNAP-tag-based approach and the anti-Twist2-specific recombinant single-domain antibodies opens new research possibilities in this field.

1. Introduction

Several reasons contributed to the remarkable success of the technology based on the SNAP-stag among other methods conceived for protein labeling [1, 2]. The SNAP-tag is derived from the human *O*6-alkylguanine-DNA alkyltransferase involved in DNA repair and allows, both *in vitro* and *in vivo*, an efficient and rapid covalent labeling of proteins fused to SNAP-tag with any molecule possessing an *O*(6)-benzylguanine (BG) group. In contrast to labeling strategies based on reactive amino groups or cysteines, this reaction generates labeling at a defined site and with 1 : 1 stoichiometry. A large variety of common reagents used in biology and biotechnology applications are already available, and both proteins and antibodies have been successfully covalently conjugated with fluorochromes, PEG, biotin, drugs, and other suitable molecules [1–14].

However, despite its reliability, the method has been designed for exclusive single tagging and is not suitable for applications, such as correlative microscopy, in which double labeling is mandatory. Furthermore, it may be useful to pair other applications, such as staining with pull-down or PEG-binding and *in vivo* imaging. A specific antibody raised against SNAP would allow a second and independent labeling of polypeptides fused to SNAP-tag. However, only polyclonal anti-SNAP antibodies are available so far, although these reagents do not guarantee data reproducibility when different batches are used. Furthermore, full-size antibodies may be too bulky for some applications such as *in vivo* tissue penetration [15]. Therefore, we decided to

select and characterize anti-SNAP monoclonal recombinant antibodies in VHH format by panning the llama naïve library previously described in [16]. Isolated antibodies were expressed using vectors that enabled the fusion to different tags and the efficiency of these constructs was analyzed in the conventional immunotechniques.

2. Material and Methods

2.1. Panning, Identification, and Production of Anti-SNAP and Anti-Twist2 VHH Antibodies, and Protein-Protein Interaction Assays. The phage repertoire was panned using Maxisorp immunotubes (Nunc) coated with GST, GST-SNAP, and GST-Twist2 according to the protocol described in [16]. Twist2 was chosen based on the paucity of reliable antibodies for investigating the biology of this nuclear transcription factor [17]. A preliminary depletion panning step was performed in the presence of GST to eliminate the binders with specificity for the fusion carrier.

For each antigen, ninety-six single colonies from both the second and third panning steps were grown 4 hours at 37°C in 2xTY supplemented with 0.1 mg/mL ampicillin, 0.1% glucose, induced with 1 mM IPTG, and incubated overnight at 30°C. Cultures were harvested; the periplasmic fractions containing the soluble HA-tagged VHHs were diluted 1 : 3 and incubated with mouse supernatant anti-HA (10 μg/mL) for use in ELISA. Maxisorp 96-well plates (Nunc) were coated at the concentration of 100 ng/well with either GST alone or the fusion constructs overnight in 50 mM sodium carbonate buffer at 4°C. After standard blocking and washing steps, the color reaction was developed by the addition of ABTS, and the absorbance at 405 nm was measured after 30 min. Clones having an absorbance value at least four times higher than the background and showing no affinity for the fusion tag GST were considered positive and sequenced to identify unique binders. Such clones were subcloned into the pHEN6 vector [16] and transformed into XL1Blue competent cells to obtain His-tagged recombinant VHH binders. Bacteria were grown at 28°C in Terrific Broth supplemented with 0.1% glucose, 0.1 mg/mL ampicillin, and 0.01 mg/mL tetracycline till the optical density at 600 nm reached the value of 0.6. Recombinant expression was induced with 1 mM IPTG, and the bacteria were harvested after 16 hours. The periplasmic fraction [16] was purified by affinity chromatography using a HiTrap HP chelating column and an ÄKTA FPLC Explorer (GE Healthcare).

For GST pull-down experiments, SNAP-Twist2 was *in vitro* translated (IVT) with [^{35}S]-methionine (Perkin Elmer) using the TNT System (Promega). IVT-Twist2 was incubated together with 3 μg of recombinant GST-Twist1, GST-Twist2, or GST in binding buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, and 0.1% Nonidet-P40) plus 30 μL of Glutathione-Sepharose Resin (GE Healthcare). The resin was incubated for 2 hours at 4°C and then extensively washed. Bound proteins were separated by SDS-PAGE and the gels were stained with Coomassie Brilliant Blue, dried, and exposed to X-ray films (Kodak) to identify the radioactive SNAP-Twist2 fusion protein.

2.2. Preparation of the pFuseVHH Vectors and VHH-Fc Antibody Production. The pFUSE-xFc2-adapt-scFv plasmids [18] were modified to allow the direct inframe cloning of VHH sequences from pHEN4. The vectors were digested with NcoI and BglII, and the PCR product was obtained using the primers 5′ATCGG**CCATGG**CTGAGGTGCAGC-TG3′ (Fw, NcoI recognition sequence in bold), and 5′GGA-GG**AGATCTGCGGCCGC**TGGAGA3′ (Rev, BgIII and NotI sequences in bold), and the sequence of the 2C1 VHH as a template was first digested with the same restriction enzymes and finally ligated. The original VHH sequence was cut out using the restriction sites NcoI-NotI and substituted with a staffer (GST-sequence). Its presence was used to evaluate the quality of the vector digestion and simplify the discrimination between self-ligated clones and clones in which VHH sequences were correctly subcloned from other vectors. The resulting constructs were fusion sequences of VHHs and Fc domains of human, mouse, or rabbit origin.

HEK293T cells at 90% confluence were transfected with 20 μg/plate of the modified vectors using calcium phosphate protocol and grown in Dulbecco's Modified Eagle Medium (DMEM) plus 10% FBS, 1% L-glutamine, 1% PS-5 antibiotic, 1% Na pyruvate 1% nonessential amino acids. The medium was renewed 24 hours after transfection and substituted 48 hours after transfection by serum-free medium. It was finally collected 96 hours after transfection, and Fc-VHH antibodies were affinity purified using a protein A column (GE Healthcare).

2.3. Western Blot and Immunoprecipitation. Fractions corresponding to purified recombinant GST-SNAP (10–100 ng) or GST-SNAP overexpressed in bacterial total lysate (10 μL) were separated in 15% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked with 5% skimmed milk in PBS and incubated with VHH periplasmic extract diluted 1 : 3 and 10 μg/mL of anti-HA monoclonal antibody. The membranes were finally washed in PBS plus 0.1% Tween (PBST), and the specific signal was detected by incubation with HRP-conjugated anti mouse.

Biotinylated VHH anti-SNAP antibodies (0.5–1 μg) were incubated in the presence of recombinant GST-SNAP (0.1–0.5 μg), and the immune complex was precipitated after 1 hour incubation with 50 μL of avidin beads (Pierce). Beads were washed four times with PBST and boiled in 2x Laemmli sample buffer before SDS-PAGE.

The anti-SNAP VHH 3G1B-Fc(rabbit) was incubated for 2 hours at room temperature with the lysates recovered from SNAP-Twist2-transfected and control HeLa cells. Protein A beads (50 μL) were added to the immune complex. After 1 hour incubation, beads were washed four times with PBST and boiled in 2x Laemmli sample buffer before SDS-PAGE.

2.4. Immunopurification. Three different constructs were compared: VHH 3G1B-Fc(rabbit), VHH 3G1B-6xHis, and llama polyclonal antibodies. One- and-half mg of affinity-purified antibodies against SNAP was used to cross-link 1 mL of AminoLink coupling gel according to the manufacturer's instructions (Pierce). Antibody coupling to the resin was

checked by analyzing few microliter of each sample by SDS-PAGE, and the three samples were loaded onto empty columns to drain out the buffer. Resins were washed with 20 mL of 1 M NaCl before equilibrating the columns with 20 mL of PBS. Each column was loaded with 15 mL of lysate recovered from bacteria over expressing the SNAP-Twist2 construct, incubated 1 hour by gentle end-over-end rocking and washed with 30 mL of PBS. Protein samples were eluted by applying 4 mL of glycine buffer (100 mM, pH 3.0) and immediately neutralized by the addition of 100 μL of 1 M Tris-HCl, pH 8.5.

2.5. Immunofluorescence. 10^6 HeLa cells were seeded in 15 cm^2 plates before being transfected with 20 μg of plasmid DNA coding for the SNAP-Twist2 construct, using the calcium phosphate transfection method. Transfected HeLa cells were seeded on coverslips coated with 0.5 mL of 0.1% gelatin solution and the day after labeled using SNAP-Cell TMR-Star (NEB) according to manufacturer's instructions.

Slides were rinsed three times with PBS, and cells were fixed with 4% formaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 for 5 min, washed three times, and blocked with 2% BSA. Successively, cells were incubated for 1 hour at 37°C in the presence of 5 μg/mL of purified anti-SNAP VHH 3G1B-Fc(rabbit), washed three times and incubated with FITC-conjugated (1 : 50) secondary anti rabbit antibodies. Finally, slides were incubated 3 min with DAPI, mounted, and assessed by fluorescence microscopy at the DAPI, GFP, and Cy3 channels.

When the HA-tagged VHH anti-Twist2 antibodies were used, the cells were incubated for 1 hour at 37°C in the presence of the periplasmic VHH fraction and of the mouse monoclonal anti-HA antibody, washed three times, and incubated with a secondary FITC-conjugated (1 : 50) anti mouse antibody.

3. Results

Purified recombinant proteins GST-SNAP and GST-Twist2 have been used to isolate single-domain antibodies by panning against a large llama naïve library according to the previously described protocol in [16]. The specificity of the isolated clones was evaluated by ELISA, and the most reactive antibodies were considered for further characterization. Sequencing results indicated the presence of two independent anti-SNAP (3C4B and 3G1B) and three different anti-Twist2 (2C12, 2D11, 3E1) clones. The latter were intended primarily for immunofluorescence applications and, therefore, were produced as HA-tagged VHHs. For simplifying their large-scale purification, the anti-SNAP VHHs were initially subcloned to obtain 6xHis-tagged recombinant antibodies and their reliability was tested in the standard immunotechniques.

VHH antibodies preferentially recognize epitopes such as cavities and grooves that can be lost when the antigen is unfolded. For this reason, they are not the reagents of choice for WB. However, both 3C4B and 3G1B clearly recognized 10 ng of purified GST-SNAP protein (Figure 1(a)) and the same recombinant protein was present in a total bacterial lysate (Figure 1(b)). These preliminary results indicated that the selected anti-SNAP VHHs could be used for identifying by WB targets that are overexpressed recombinant proteins.

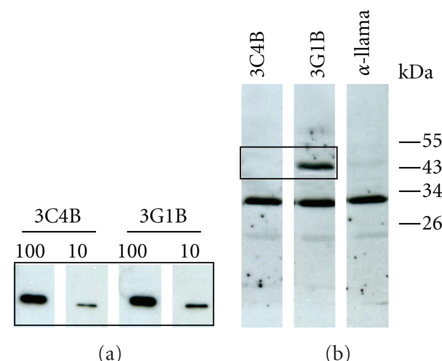


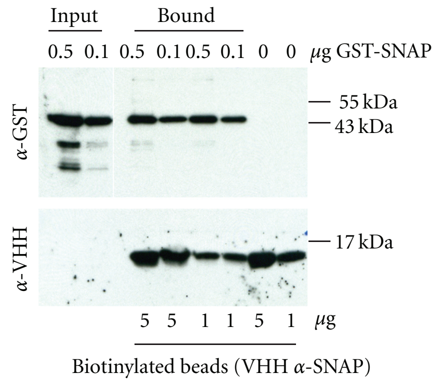
Figure 1: Application of anti-SNAP antibodies in western blot experiments. (a) Detection of 100 and 10 ng of blotted purified recombinant GST-SNAP protein by means of the VHH antibodies 3C4B and 3G1B. (b) Detection of overexpressed GST-SNAP in bacterial total lysates using the same VHH antibodies. The secondary antibody anti-llama immunoglobulins used for detection recognized nonspecifically a further band of 32 kDa.

The secondary anti-llama polyclonal antibodies recognized a bacterial protein nonspecifically (Figure 1(b)). Therefore, we decided to exploit the opportunity of manipulating recombinant material for designing a new expression vector that would allow overcoming this drawback. Consequently, a new plasmid (pFuseVHH) was developed that enables the fusion of the VHH sequence to a Fc domain (see Figure S1 in supplementary material available confine at doi:10.1155/2010/658954). It is based on a plasmid originally conceived for the efficient production of scFvs [18] and that provides a Fc domain for affinity purification with yields comparable to those of the His-tag. However, Fc domain is a significantly better target for secondary antibodies (and labeled Protein A) and increases the antibody avidity by dimerization.

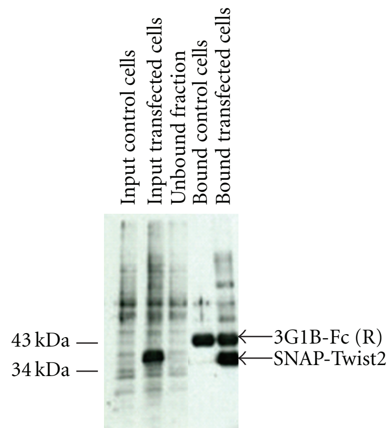
The anti-SNAP VHH that gave the highest signal in WB (3G1B) was subcloned in the pFuseVHH plasmid (with rabbit Fc), and both His-tagged and Fc-tagged 3G1B antibodies were tested for immunoprecipitation experiments.

The immunoprecipitation feasibility was first confirmed using purified GST-SNAP protein and biotinylated purified His-tagged 3G1B (Figure 2(a)). Successively, the 3G1B-Fc(rabbit) construct was used in combination with total lysate from mammalian cells transfected with SNAP-Twist2. The recombinant antibody was able to completely deplete the target protein from the solution, as clearly showed in Figure 2(b).

We next compared the potentiality of the two 3G1B constructs (His- and Fc-tagged) and that of a polyclonal antibody for immunopurifying recombinant SNAP-Twist2-protein from large volumes. The antibodies were covalently bound to sepharose beads and packed into columns. The effective binding between antibodies and beads was verified



(a)



(b)

Figure 2: Application of anti-SNAP antibodies in immunopurification experiments. (a) Purified GST-SNAP (0.5 or 0.1 μg) were incubated in the presence of the biotinylated anti-SNAP VHH 3G1B (5 or 1 μg), and the antigen-antibody complex was precipitated using avidin beads. The proteins were separated by SDS-PAGE, blotted, and finally detected using either anti-GST (upper membrane strip) or anti-VHH specific antibodies (lower membrane strip). Biotinylated VHHs were incubated in the presence of avidin beads and in the absence of GST-SNAP as a control of the pull-down efficiency. (b) Lysates from mammalian HEK293T cells transiently transfected with 20 μg/plate plasmid for expressing the SNAP-Twist2 fusion construct were incubated with the anti-SNAP 3G1B-Fc(R) antibody, and the antigen-antibody complex was precipitated by means of Protein A.

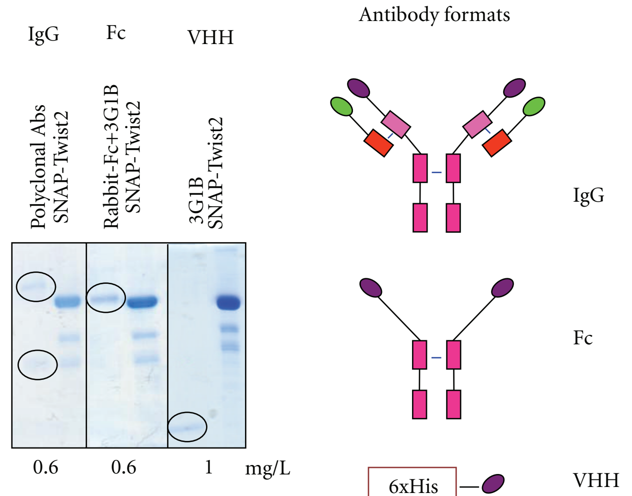


Figure 3: Comparison of the protein yields obtained by immunopurification using different antibody formats. The protein SNAP-Twist2 was immunopurified from total lysate using sepharose columns to which the following antibodies were covalently linked: llama IgG anti-SNAP polyclonal antibodies; 6xHis tagged llama recombinant antibody 3G1B in VHH format; the same 3G1B antibody fused to the rabbit Fc domain.

by analyzing the washing fractions and by boiling a small bead sample for each experimental condition (Figure 3). The target protein was successfully purified from the total lysate by using all three configurations, although a certain yield difference was observed (Figure 3).

Finally, we designed an immunofluorescence experiment to show that anti-SNAP antibodies could be used for double tagging of a target protein fused to SNAP-tag. Cultured HeLa cells were transfected with a plasmid for the expression of SNAP-Twist2 and the recombinant protein was identified either by using the membrane-permeable SNAP-Cell TMR Star fluorochrome that directly reacts with the tag or using the 3G1B-Fc(rabbit) recombinant antibody in combination with an anti-rabbit FITC secondary antibody. The attempt of using His-tagged 3G1B in immunofluorescence was abandoned due to the poor quality of anti-His secondary antibodies available for this technique. The nuclear/nucleolar subcellular localization of both SNAP signals, namely the one identified by the fluorochrome covalently bound to the active SNAP site and the other due to the anti-SNAP antibody recognition, is clearly showed in Figures 4(a)–4(d). No significant background was detected in nontransfected control cells, excluding the possibility that native *O*6-alkylguanine-DNA alkyltransferase may interfere with the signal of recombinantly expressed SNAP-fused proteins (Figure 4(i)). Similarly, the anti-SNAP 3GB1 did not recognize endogenous *O*6-alkylguanine-DNA alkyltransferase in CHO (hamster) and NIH (mouse) cells (data not shown). GST pull-down experiments using the SNAP-Twist2 IVT protein demonstrate that the SNAP-tag does not affect the ability of Twist2 to form homo-(not shown) or heterodimers with GST-Twist1 (Figure S2). No nonspecific binding was detected in the presence of GST-only recombinant protein.

The nuclear localization inferred by the two SNAP detection methods is in agreement with the reports concerning the physiological role of Twist2 [19, 20] and was further confirmed by the direct protein labeling by means of the specific anti-Twist2 VHH antibody 2C12 (Figures 4(e)–4(h)). Interestingly, although the anti-Twist2 antibody 2C12 does not detect endogenous Twist2 in the cell nucleus (Figure 4(j)), it seems that another anti-Twist2 candidate (3E1) can identify it (Figure 4(k) and Figure S3). Epsin 8 is a protein interacting with actin [21], and the construct SNAP-Epsin8 correctly accumulated in the cytoplasm (Figure 4(l)). This control experiment rules out a SNAP-dependent nuclear localization.

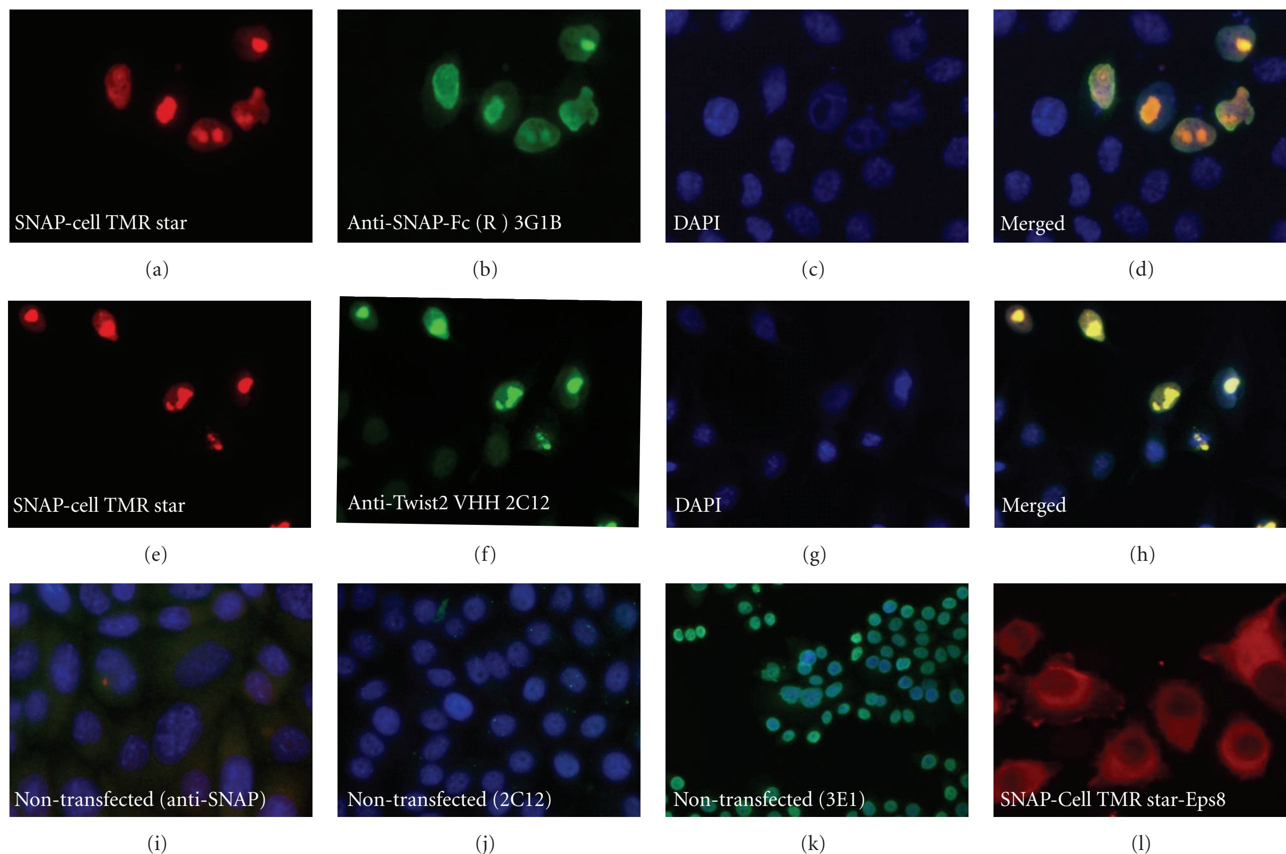


Figure 4: Double staining of SNAP-tagged Twist2 obtained by coupling specific antibodies and benzylguanine-fluorochromes. HeLa cells were transiently transfected using 20 μg/plate of plasmid for the expression of the SNAP-Twist2 protein. Its subcellular localization was identified: (a) and (e) by the *in vivo* addition of the permeable SNAP-Cell TMR Star fluorochrome (dilution 1 : 200); (b) by means of the purified recombinant anti-SNAP antibody 3G1B fused to the rabbit Fc (5 μg/mL); (f) by means of the cell culture supernatant containing the recombinant anti-Twist2 VHH antibody 2C12 (dilution 1 : 2); (d) and (h) are the merged images of (a) + (b) and (e) + (f), respectively; (c) and (g) correspond to the nuclear DAPI staining; (i), (j), and (k) are nontransfected cells (controls) treated with the indicated antibodies. HeLa cells transfected with SNAP-Epsin8 were stained with SNAP-Cell TMR Star fluorochrome (dilution 1 : 200) to rule out a SNAP-dependent nuclear staining (l).

4. Discussion

High quality reagents are crucial for obtaining reliable results in basic research and diagnostics. Monoclonal antibodies are preferred since their reproducibility does not depend on individual response, as in the case of polyclonal antibodies, and their availability assures the possibility of comparing data from different laboratories. This is, for instance, the case of antibodies directed against tags since these can be linked to a large variety of proteins and matrixes under different experimental conditions [22]. The SNAP-tag not only enables the covalent binding of any molecule with a BG group, but its further detection also by means of an antibody would allow a double tagging of the SNAP and its fusion partners, a favorable condition, for instance, for correlative microscopy applications.

We chose the selection of a monoclonal recombinant antibody in VHH format because of the high stability, elevated yields, and small dimension of this antibody class as well as the possibility to manipulate the construct using standard molecular biology techniques [15]. It was, therefore, possible to evaluate three different tags fused to VHHs (6xHis, HA, and Fc). His-tag allows the straightforward affinity purification of the linked antibody, and, probably because of its low molecular mass, the His-VHH was the most suitable format for preparing affinity column for large-scale immunopurification (Figure 3). However, it is common experience that His-tag constitutes a poor target for secondary antibodies and for this reason, it is not normally utilized in immunofuorescence applications. Therefore, we designed a new vector for Fc-fusion of the VHH antibodies. Fc-domain is compatible for Protein A-based affinity purification, and since it induces dimerization, it has a higher avidity with respect to monomeric constructs. We first showed the reliability of the Fc-VHH construct in immunoprecipitation assay. It should be underlined that any single-domain antibody has the advantage over the conventional IgGs of giving a single signal (heavy chain) instead of two (heavy and light chains) in blot detection (Figure 2(b)). Furthermore, VHHs can be fused to three

different Fc domains (human, mouse, and rabbit) that can be chosen accurately to avoid background signals caused, for instance by using mouse monoclonal antibodies on murine cells. As an example, we successfully used the rabbit Fc-VHH immunofluorescence analysis in human cells (Figure 4).

More specifically, we defined the SNAP-Twist2 subcellular localization using three different labeling approaches. The recombinant protein was very clearly identified in nucleus/nucleoli using: (a) a permeable fluorochrome specific for SNAP; (b) the anti-SNAP Fc-VHH; (c) an anti-Twist2 VHH antibody. The data obtained with transfected cells seem to be confirmed by staining wild-type cells with another anti-Twist2 VHH antibody that apparently recognizes the endogenous protein. This achievement is remarkable since so far there was only a single report of Twist2 immunofluorescence staining using an anti-Twist2 antibody on transfected cells [20]. However, the signal was quite blurred and the manufacturer (Abcam) does not indicate in the attached data sheet that the commercial antibody is suitable for immunofluorescence. It would be interesting to understand if the lack of the light chain confers to VHHs a broader recognition capacity among slightly modified structures of the same antigens, such as the Twist2 expressed in human cells and as a recombinant protein in a prokaryotic system.

5. Conclusions

An anti-SNAP VHH recombinant antibody was isolated, extensively characterized according to the most recent recommendations [23], and it was subcloned in a new vector that enables the production of Fc-fusions. The antibody was suitable for all the standard immunotechniques and, alone or in combination with direct SNAP labeling, allowed the immunofluorescence localization of Twist2. The nuclear/nucleolar localization of this protein was further confirmed by using specific anti-Twist2 VHH antibodies. This is in agreement with the only previous immunofluorescence report, but the resolution obtained with the VHHs and SNAP system was significantly higher.

Finally, the availability of a reliable anti-SNAP antibody that can be fused to any suitable tag introduces the possibility of double tagging the proteins fused to SNAP since the residue for covalent binding of this tag remains available for reacting with any BG-conjugated molecule. This option can be used, for instance, to couple immunodetection to pull-down experiments, but we expect that it will be particularly beneficial in correlative microscopy, a technique in which both fluorescent and gold probes may be detected. The small dimension of the VHH antibodies should confer a further advantage in terms of image resolution.

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