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Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor 1alpha

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Hypoxia triggers the transcription of genes responsible for cell survival via the key player transcription factor hypoxia-inducible factor 1alpha (HIF-1α). Overexpression of this protein has been implicated in cardiovascular disorders, carcinogenesis and cancer progression. For functional and diagnostic studies on the HIF-1α protein, we have identified single-domain antibody fragments directed against this protein by using a llama-derived nonimmune phage display library. This library displays the variable domains of the heavy-chain antibody subclass, found in these animals. Phage display selection with six recombinant HIF-1α proteins yielded five different antibody fragments. By epitope-mapping, we show that all five antibody fragments bind within the functionally important oxygen-dependent degradation domain of the HIF-1α protein. Two of these antibody fragments were engineered into bivalent antibodies that were able to detect human HIF-1α by immunohistochemistry, Western blotting and immunoprecipitation, and mouse HIF-1α by immunofluorescence and immunoprecipitation. These are the first single-domain antibody fragments that may be used in exploration of HIF-1α as a possible therapeutic target through molecular applications.
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The half-life of hypoxia-inducible factor-1alpha protein (HIF-1α) is regulated by a sophisticated system of degradation, which involves binding to the von Hippel–Lindau tumor suppressor protein (pVHL), controlled by prolyl hydroxylases. During hypoxia, HIF-1α forms a complex with HIF-1β, resulting in an active HIF-1 transcription activation complex. Genes that contain a specific hypoxia-responsive element (HRE) in their promoter sequences are activated during hypoxia in an HIF-1α-dependent way. In order to secure cell survival, these hypoxia-responsive target genes are triggered to regulate metabolic adaptation to compensate for the reduced oxygen levels.[1] This metabolic adaptation allows tumors, that reach a certain size and therefore outgrow their blood supply, to continue thriving with subsequent tumor progression and unfavorable outcome for the patient.[2,3] High HIF-1α expression plays a role in ischemic heart disease and different types of cancer,[4,5] where HIF-1α is considered to be a potential target for therapy.[6–10] We have shown the involvement of HIF-1α in breast carcinogenesis[11] and in drug resistance.[12] Furthermore, we have shown that high HIF-1α expression,[13] especially of the perinecrotic type[14] correlates with bad prognosis. This latter finding has recently been confirmed by Dales *et al*[15] in 745 breast cancer tissue samples. Studies on the role of HIF-1α are, however, hampered by the small range of available antibodies. In this study, we have identified a set of llama heavy-chain antibody-derived, variable single-domain antibody fragments, also referred to as VHH,[16–18] against both human and mouse HIF-1α. These VHH are the smallest naturally occurring intact soluble antigen-binding units with a molecular weight of about ~15 kDa.[16] VHH are stable at high temperatures,[19,20] can bind antigen in high salt

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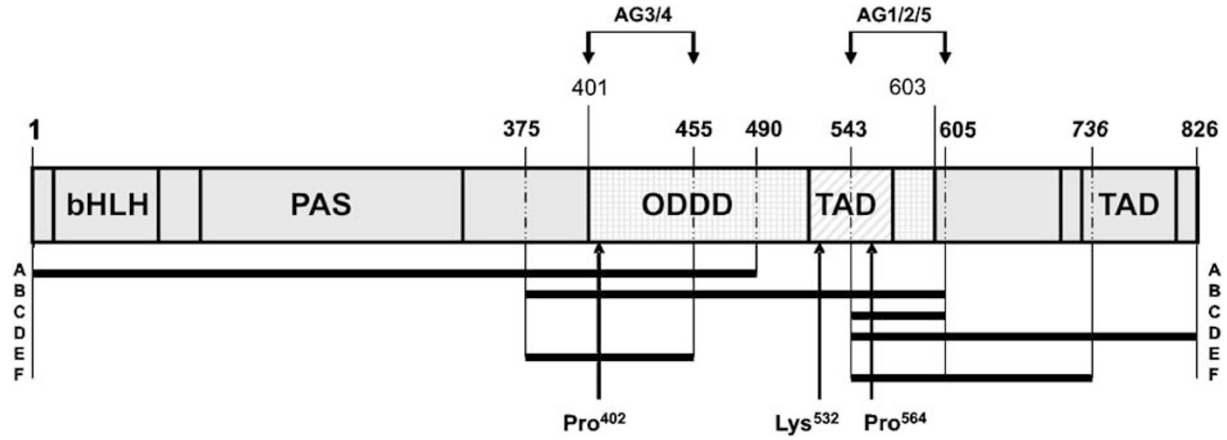

Figure 1 Schematic representation of the recombinant HIF-1α fragments, used for selection of VHH. From top to bottom, A aa 1–490, B aa 375–605, C aa543–605, D aa543–826, E aa 375–455 and F aa 543–736, in correspondence to the amino-acid sequence of the human HIF-1α protein. Pro^{402}, Lys^{532} and Pro^{584} in the ODDD (aa402–603) are the regulatory target residues. Approximate positioning of VHH AG1–5 recognition sites within the HIF-1α protein are shown at the top of the figure.

concentrations,[21,22] cripple intracellular viral replication,[23] block enzymatic activity,[24] are able to pass the blood–brain barrier,[25] and can be used for various immunological applications like classical antibodies. We have selected a nonimmune VHH phage display library against six different recombinant HIF-1α protein fragments. These fragments span across the human full-length HIF-1α protein (Figure 1). One of the fragments partially represents a described alternative, transcriptionally active, splicing product of HIF-1α: HIF-1α isoform 2.[26] Here, we report the identification of five novel monoclonal anti-HIF-1α VHH. These VHH were mapped to epitopes within the oxygen-dependent degradation domain (ODDD),[1,27] that contains both target sites of prolyl hydroxylation,[28,29] essential for pVHL-dependent proteasomal degradation of the HIF-1α protein (Figure 1). These anti-HIF-1α VHH were engineered into higher affinity bivalent VHH,[30,31] and validated for their use in several molecular techniques and histological studies.

Materials and methods

Construction of Procaryotic Protein Production Vector pETλHIS

For VHH selection strategies, a new procaryotic protein production vector was engineered. This vector allows the production of recombinant proteins with an N-terminal T7 tag and a C-terminal HIS6 tag. The pET-28a vector (Novagen, Madison, WI, USA) was modified for this purpose by removing the N-terminal HIS6 sequence. The rest of the original reading frame of pET-28a was unmodified and the optional C-terminal HIS6 tag was used for protein purification.

Synthesis of the Recombinant HIF-1α Fragments

PCR with primers from Sigma-Genosys Ltd (Cambridge, UK), using pCEP4/HIF-1alpha[32] plasmid as template resulted in the following products: fragment A coding for amino acids 1–490 of the human HIF-1α protein was amplified using forward primer 5′-**CGGGATCC**ATGGAGGGCGCCGGCGGCGCGAA-3′ and reverse primer 5′-**GGAGCGGCCGC**GGTAAAA GAAAGTTCCAGTGAC-3′. All restriction enzymes and buffers were purchased from Roche (Basel, Switzerland). After digestion with *Bam*HI and *Not*I, this fragment was ligated into the *Bam*HI- and *Not*I-digested pETλHIS vector. Fragment B coding for amino acids 375–605 and fragment E coding for amino acids 375–455 of the human HIF-1α protein were amplified with forward primer 5′-**CGGGATCC** CAGCTATTCACCAAAGTTGAATC-3′ in combination with reverse primers 5′-**GGAGCGGCCG C**GTTAACTTGATCCAAAGCTCTG-3′ and 5′-**GGG AAGCTT**GGTGGGTAATGGAGACATTGC-3′, respectively. Fragment C encoding for amino acids 543–605 and fragment D for 543–826 of the human HIF-1α protein were amplified with primer combinations forward 5′-**CGGGATCC**GACACAGAAG CAAAGAACCC-3′ and with reverse primers 5′-**GGG AAGCTT**AGTCTGCTGGAATACTGTAAC-3′ and 5′-**GGGAAGCTT**GTTAACTTGATCCAAAGCTCTG-3′, respectively. The fragments were digested with *Bam*HI and *Hind*III and cloned into a *Bam*HI- and *Hind*III-digested pETλHIS vector. Fragment F codes for amino acids 543–736 of the HIF-1α isoform 2[30] and was amplified by PCR with the latter forward primer and reverse primer: 5′-**GGGAAGCTT**T CAAATAATTCCTACTGCTTGAAAAAG-3′ The constructs were transformed into BL21(DE3)-RIL CodonPlus Competent cells (Stratagene, La Jolla, CA, USA) for protein expression. After induction with 1 mM of isopropyl-β-D-thiogalactopyranoside

(Sigma-Aldrich Co., St Louis, MO, USA), cells were lysed with Bacterial Protein Extraction Reagent (Pierce Co, Rockford, IL, USA). Recombinant fragments were purified with immobilized metal ion affinity chromatography (IMAC). Fragments that were insoluble were recovered using buffers with high urea contents. The produced recombinant HIF-1α fragments were dialyzed against PBS. Concentrations of the purified fragments were calculated from a BSA standard range using the BCA™ Protein Assay Kit (Pierce Co., Rockford, IL, USA). The fragments were checked by Western blot and Coomassie staining for the presence of tags and their purity. A construct with *Bam*HI restrictions sites flanking full-length HIF-1α was kindly provided by Professor G Simos[33] and cloned into pET*λ*HIS. This full-length recombinant HIF-1α was also purified using IMAC and used in Western blot experiments. An expression construct coding for amino acids 401–603 (ODDD) of the HIF-1α protein fused to the yellow fluorescence protein (YFP) was a kind gift of Dr T Brummelkamp and was used for immunoprecipitation-based epitope mapping.

Selection of VHH

The library used for VHH selections is a llama-derived nonimmune VHH library (Hermans *et al*, in preparation), which was kindly provided for this study by Unilever Research, Vlaardingen, the Netherlands. This phage display library was generated essentially as described before.[34] Peripheral blood lymphocytes collected from the blood of eight nonimmunized llamas were used for mRNA extraction. cDNA was synthesized and VHH genes were PCR amplified introducing appropriate restriction sites for cloning. Phagemids carrying the VHH genes were then transformed into TG1 *Escherichia coli* and plated on selective plates. This resulting nonimmune library with a clonal diversity of 5×10^9 was used for phage preparations. VHH selections were performed according to an optimized selection protocol as described by Verheesen *et al*, (submitted for publication).[35] This protocol utilizes different modes of antigen presentation to the phage antibody library that favors isolation of a diverse group of VHH against the antigen. HIF-1α-specific VHH were selected in two successive rounds of selection utilizing T7-tagged recombinant-produced HIF-1α fragments. A selection round with presentation of the antigen in solution by capturing with a T7-tag-directed monoclonal antibody was followed by a second selection round were direct panning of the recombinant fragments was used. The excess of noncaptured protein was removed by washing, and the coated antigens were incubated with the phage-VHH library. For capturing selections, 10% normal mouse serum (NMS) was added to the phage incubation to reduce binding of phage-VHH to the capturing agent. After removal of nonbinding phages by extensive washing, bound phages were eluted and rescued. At the end of selection procedures, enrichment for the six recombinant HIF-1α fragments was observed. Ninty-six wells masterplates were picked from the corresponding second round phage outputs.[36]

Screen for VHH

For each masterplate, monoclonal VHH fragments were screened for their recombinant HIF-1α-binding ability in ELISA. To assess genetic diversity between positive clones, they were subjected to DNA fingerprint analysis[36] and those with different digestion patterns were sequenced on an ABI3100 (Applied Biosystems Inc., Foster City, CA, USA).

VHH Production

VHH clones of interest were cultured, IPTG induced, and VHH were purified from the periplasm by using IMAC.[36] Purified fractions were pooled and dialyzed to PBS. Concentrations of the purified VHH were calculated from a BSA standard range using the BCA™ Protein Assay Kit (Pierce Co, Rockford, IL, USA). The purified monoclonal VHH were stored at −20°C for further use.

Western Blot on HeLa Total Cell Lysate with Monovalent VHH

Desferrioxamine (DFO) (Sigma) is an iron chelator that mimics the effect of hypoxia and stabilizes the HIF-1α protein.[37] All cell culture necessities were purchased from Gibco BRL (Paisley, UK). HeLa cells were grown to near confluency in 225 cm^2 cell culture flasks and stimulated for 12 h with 0.1 mM DFO or left untreated before lysis in 2 ml of Laemmli loading dye. Both samples were sheared with a common insulin syringe and 50 μl was loaded onto a 6% SDS-PAGE gel for separation of the proteins. Proteins were transferred to a PVDF membrane (Millipore Co., Bedford, MA, USA) and blots were blocked with 5% MARVEL (dried skimmed milk, Premier International Foods, Coolock, UK) in PBS/0.025% Tween-20 (MPBST) at room temperature for 1 h and incubated overnight with 1 μg/ml VHH in 15 ml of 0.1% MPBS at 4°C. Next, the membranes were washed in PBS and incubated with a HRP-conjugated mouse anti-6xHIS monoclonal antibody (BD Biosciences, San Diego, CA, USA) in 0.1% MPBS. ECL (Amersham Biosciences, Buckinghamshire, UK) was used for visualization as described by the manufacturer.

VHH AG1 and AG2 Solid-Phase Competition Assay

NUNC MAXISORP (NUNC, Roskilde, Denmark) plates were absorbed o/n at 4°C with VHH AG1 (0.48 μM) in 100 μl per well in PBS. Wells were

blocked with 200 μl 1% BSA in PBS/0.1%Tween-20 (BSAT) for 1 h at 25°C at 300 rpm in a TERMOstar incubator (BMG LABTECH, Offenburg, Germany). All following incubation steps were performed under these conditions in this incubator. A non-related VHH (VHH-R2), which was kindly provided by Dr E Dolk,[38] was used as a negative control in this experiment. Recombinant HIF-1α fragment C (4 μM) was incubated with immobilized VHH AG1 in the absence or presence of increasing concentrations of VHH AG1 or AG2 (0–17 μM) in BSAT in a volume of 100 μl 25°C. After three rapid washes with PBST, bound recombinant HIF-1α fragment C was detected by incubation with HRP-conjugated mouse anti-T7 tag antibody in a volume of 100 μl (Novagen) (1:5000 in BSAT) for 20 min, followed by staining using the ImmunoPure TMB Substrate kit (Pierce Co) according to fabricant instructions. Data were corrected for binding to empty microtiter wells, which was less than 5% relative to binding to wells containing immobilized VHH AG1. The signal intensities were measured using an ELISA reader (BioRad) at a wavelength of 450 nm.

Immunoprecipitation with Monovalent VHH

HeLa cells grown to near confluence in 225 cm^2 cell culture flasks were stimulated for 12 h with 0.1 mM DFO, or left untreated. The cells were scraped and collected in 2 ml immunoprecipitation buffer (IPB): 40 mM Tris pH 8.0, 1% Triton, 10% Glycerol, 280 mM NaCl supplemented with a protease inhibitor cocktail (Roche). Collected cell lysates underwent three freeze–thaw cycles and were subsequently spinned down at 14 000 rpm for 10 min at 4°C. In all, 200 μl supernatant of both lysates was incubated with 1 μg of VHH. Simultaneously, 1 μg mouse anti-6xHIS monoclonal was bound (BD Biosciences, San Diego, CA, USA) to 7.5 μl protein A/G plus-agarose beads (Santa Cruz Biotech, Santa Cruz, CA, USA) in 200 μl IPB at 4°C head-over-head for 1 h. Antibody-coated protein A/G beads were blocked with 1% BSA in IPB at 4°C for 15 min and washed once with IPB buffer. Next, the preincubated VHH containing lysates were added to the beads and were incubated, head-over-head, overnight at 4°C. Beads were washed 10 times with IPB and resolved in 50 μl Laemmli loading dye. Samples were loaded onto a 6% SDS-PAGE gel for separation of the proteins and analyzed with mouse anti-human HIF-1α (clone 54, BD Transduction Laboratories, San Diego, CA, USA) and HRP-conjugated goat anti-mouse IgG + IgM (Biosource, Camarillo, CA, USA) by Western blot. All five VHH were tested to check whether binding sites were confined to the ODDD of the HIF-1α protein. An expression construct coding for the ODDD of the HIF-1α protein fused to YFP was transfected into HeLa cells with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were collected in IPB and immunoprecipitation protocol was continued as described above, replacing the 6xHIS for a mouse anti-*Myc* antibody (Invitrogen). For detection of the immunoprecipitated ODDD-YFP, a rabbit polyclonal anti-green fluorescent protein (anti-GFP, crossreacts with YFP, Santa Cruz Biotech, Santa Cruz, CA, USA) and HRP-conjugated goat anti-rabbit IgG (H + L) (BioRad, Hercules, CA) were used.

Western Blot with Monovalent VHH against Recombinant HIF-1α Proteins

In total, 100 ng of purified full-length recombinant HIF-1α and recombinant HIF-1α fragment B containing the ODDD domain of HIF-1α were loaded onto a 6 and 12%, respectively, SDS-PAGE gel by using a one-slot comb. Separated proteins were transferred to a PVDF membrane. Blots were blocked with 5% MPBST/0.025% and incubated with 1.5 μg/ml VHH in 0.1% MPBST at 4°C. Next, the membranes were washed in PBST and incubated with a mouse-anti-*c-Myc* monoclonal antibody (kindly provided by PW Hermans, Biotechnology Application Centre BV, Bussum, The Netherlands) and HRP-conjugated goat anti-mouse IgG + IgM. As a positive control, an HRP-conjugated mouse anti-6xHIS monoclonal antibody was used. VHH-R2 was used as a negative control in this experiment.

Determination of the VHH Binding Affinity to HIF-1α

NUNC MAXISORP plates were absorbed o/n at 4°C with 100 μl per well recombinant HIF-1α fragment B at a final concentration of 5 μg/ml in PBS. Wells were blocked with 1% BSA in 200 μl PBS/0.1% Tween-20 (BSAT) for 1 h at 25°C at 300 rpm in a TERMOstar incubator (BMG LABTECH). All the following incubation steps were performed under these conditions in this incubator. VHH were incubated at various concentrations (0–18 μM) with immobilized HIF-1α fragment B in a volume of 100 μl in BSAT for 1 h at 25°C. After three rapid washes with PBST, bound VHH were detected by HRP-conjugated mouse anti-*Myc* antibody (Invitrogen) (1:5000 in BSAT) for 15 min at 25°C. The signal intensities were measured using an ELISA reader (BioRad) at a wavelength of 450 nm. The apparent affinities of the interaction between VHH and HIF-1α were approximated from the determined concentration yielding half-maximum binding to HIF-1α fragment B.

Engineering of Mono- and Hetero-Bivalent VHH

X22 is a VHH production vector for expression of c-*Myc* and HIS6-tagged bivalent VHH. The encoded bivalent VHH are fusion proteins of two VHH in tandem. The vector is like the described pKC6[30] and

shares the same plasmid backbone. In the X22 vector, the *Nco*I site is replaced by an *Sfi*I site and the *Bst*EII and *Not*I sites are interchanged. Also, the X22 has an additional c-*Myc* tag. A two-step cloning procedure was performed to create these bivalent VHH. The first step involves cloning of the C-terminal VHH in the *Pst*I and *Bst*EII sites of X22, followed by an *Sfi*I and *Not*I cloning for introduction of the N-terminal VHH. To introduce a necessary *Pst*I site, VHH were amplified by PCR with forward primers 5′-**GGGCTGCAG**ATGGCC CAGGTAAAGCTGGA-3′ 5′-**GGGCTGCAG**ATGGCC GATGTGCAGCTGGT-3′, in combination with reverse primer 5′-CGCTTGCGGCCGCTGAGGA GACGGTGACCTG-3′ by using the corresponding library phagemid vectors as template (clones AG1 and AG2). Reading frames were confirmed by sequencing. Monovalent VHH AG1 and AG2 were fused bivalent with their analog hinge and a 10 amino acid linker, AAAQVQLQMA, instead of the described[30] 'long hinge' linker. Purification was performed as described in *VHH production*. The result was a hetero-bivalent VHH AG-1N2C-MYC that was also recloned into the X22-VSV: a bivalent VHH production vector in which the c-*Myc* tag was replaced for a VSV-G tag (AG-1N2C-VSV).

Recombinant HIF-1α Fragment Competition Assay

Protein A/G plus-agarose beads (7 μl) were coated overnight with 20 μg of purified hetero-bivalent VHH AG-1N2C-MYC in 200 μl IPB. Antibody-coated beads were blocked with 1% BSA-IPB at 4°C for 15 min. DFO-stimulated HeLa lysates together with quantities of recombinant HIF-1α protein fragments C and E were added to the coated beads, and the protocol was continued as described above in Immunoprecipitation with monovalent VHH. The presence of recombinant proteins in DFO-stimulated HeLa lysates was confirmed by Western blotting with the HRP-conjugated mouse anti-6xHIS monoclonal after separation of the total lysates on a 12% SDS-PAGE gel.

Immunoprecipitation with Bivalent VHH AG-1N2C-VSV

Purified hetero-bivalent VHH AG-1N2C-VSV (7 μg) was incubated at 4°C head-over-head overnight with 7 μl protein A/G plus-agarose beads in 200 μl IPB. Antibody-coated beads were blocked with 1% BSA-IPB at 4°C for 15 min and washed once with IPB buffer before adding lysates of HeLa cells or NIH 3T3 cells and protocol was continued as described above in Immunoprecipitation with monovalent VHH. For detection of the mouse HIF-1α protein, we used the rabbit anti-mouse HIF-1α polyclonal (Novus Biologicals Inc., Littleton, CO, USA) and goat anti-rabbit IgG (H + L) HRP-conjugated (BioRad).

Immunohistochemistry

Paraffin-embedded material from renal cell cancer was obtained from the archives of the Department of Pathology, UMC, Utrecht. Immunohistochemistry was performed on 4-μm thick sections. All sections were dewaxed and rehydrated. For the detection of HIF-1α with the hetero-bivalent VHH AG-1N2C-VSV, antigen retrieval was performed in sodium citrate buffer (pH 6.0) at 96°C for 20 min. After a period of cooling down, the sections were incubated with the primary antibody (AG-1N2C-VSV; 40ng/ml) for 60 min at 20°C. This was followed by the incubation with mouse-anti-VSV monoclonal (dilution 1/400, Roche) for 60 min at 20°C. The PowerVision histostaining kit (Poly-HRP-goat-anti-mouse/rabbit/rat IgG, ready to use, ImmunoVision Technologies, Daly City, CA, USA) was used for detection. HIF-1α staining was performed as described previously.[11] Antigen retrieval was performed for 45 min at 96°C in target retrieval solution (DAKO, Glostrup, Denmark). The primary mouse antibody (anti-HIF-1α; 1/500 dilution, clone 54, BD Transduction Laboratories) was incubated for 30 min at 20°C. The Catalyzed Signal Amplification System (DAKO) was used for detection. All sections were developed using diaminobenzidine, and subsequently counterstained with hematoxylin. Before the slides were mounted, all sections were dehydrated in alcohol and xylene.

Immunofluorescence Microscopy

NIH 3T3 mouse fibroblast cells were cultured on glass slides by using Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin–streptomycin and 100 U/ml L-Glutamine at 5% CO_2 and 37°C. For the induction of HIF-1α, cells were stimulated with 0.1 mM DFO for 12 h. Slides were rinsed with PBS and cells were fixed with 3.7% formaldehyde–PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 for 10 min and incubated with 50 mM glycine–PBS for 10 min and blocked with 1% BSA–PBS for 30 min. Subsequently, the cells were incubated with different concentrations (2–100ng/μl) of hetero-bivalent VHH AG-1N2C-MYC at RT for 1 h, followed by incubation with 0.44 ng/ml mouse-anti-c-*Myc* monoclonal and 1 μg/μl DAPI in 1% BSA–PBS at RT for 1 h. FITC-conjugated polyclonal goat anti-mouse (DAKO, dilution 1/75) in 1% BSA–PBS was used for the detection. VectaShield (Vector Laboratories Inc., Burlinghame, CA, USA)-mounted microscope slides were assessed with fluorescence microscopy (LEICA DMR, Mannheim, Leica Geosystems, Germany).

Results

Generation of Recombinant HIF-1α Proteins

Recombinant proteins produced from HIF-1α-specific constructs that span across the full-length HIF-1α

protein (Figure 1) resulted in six HIF-1α fragments of expected molecular weight (data not shown). Fragments A, B, C, D and E were expressed with an N-terminal T7 and a C-terminal HIS6 tag. Fragment F was expressed from the unmodified pET28a vector, which resulted in a recombinant protein with an N-terminal T7 and HIS6 tag. This fragment ends with an extra Isoleucine to distinguish from HIF-1α isoform 2. Recombinant proteins B, C and E appeared in the soluble protein fraction; A, D and F appeared to be insoluble and were purified under denaturing conditions. The integrity and molecular weight of these fragments were determined with Coommasie blue staining and on Western blot using tag-specific antibodies. Homogeneity was >95% (data not shown). These purified recombinant HIF-1α fragments were used for the selection of VHH from the phage display library.

Selections and Screening for Anti-HIF-1α VHH

Significant enrichments in phage outputs were observed after two rounds of selection against different HIF-1α fragments, except for fragment E. In total, 170 ELISA-positive clones for fragments A, B, C, D and F from selected VHH subpopulation were identified. Fingerprint analyses followed by sequence analyses of the VHH cDNA reading frames revealed that an identical VHH clone was selected against fragments B, D and F. These fragments contain an overlapping part of HIF-1α. This VHH was coded AG1. Surprisingly, from the selected phage subpopulation from the recombinant fragment C, a VHH with *identical* coding complementarity determining regions (CDR), but with *different* coding framework regions (FR), was identified as AG2 (Table 1).[39] cDNA sequences between AG1 and AG2 differed even more (data not shown). Identification of AG2 validates the use of different fragments in our selection procedure. Had we used only fragment B for selection, we would never have identified VHH AG2 representing a completely different frame works as compared to AG1 (Table 1). We are the first to report selection of VHH with identical CDR and different FR from a nonimmune VHH phage display library.

Competition of VHH AG1 and AG2 for Binding to HIF-1α

We used a solid-phase competition assay to address the question whether or not VHH AG1 and AG2 can compete for binding to HIF-1α. As demonstrated in Figure 2, both monovalent VHH compete for binding to the recombinant fragment C. These data imply that VHH AG1 and AG2 bind a similar region within HIF-1α.

Screening of Anti-HIF-1α VHH by Immunoprecipitation

We used immunoprecipitation analysis to select for additional VHH that could recognize the native HIF-1α protein. This approach revealed three additional VHH that could recognize the native HIF-1α protein: AG3, AG4 and AG5. VHH AG5 could also, like AG1, be identified from phage subpopulations corresponding to recombinant HIF-1α fragments B, D and F (Table 2). In addition, ELISA and Western blot crossreactivity experiments between these VHH and the recombinant HIF-1α fragments showed that all five novel anti-HIF-1α VHH could bind to their overlapping recombinant HIF-1α fragments, as expected (Table 2). VHH AG1 and AG2 detected

Table 1 Selected anti-HIF-1α VHH with FR1–4 and CDR1–3

	FR1	**CDR1**	**FR2**	**CDR2**
Name				
VHH				52A
	1 22	31	36	50|
AG1	QVKLEESGGGLVQPGGSLRLSCAASGSIS	INAMG	WYRQAPGKQREFVA	AIT TSGSTRYANSAKG
AG2	DVQLVESGGGLVQPGGSLRLSCAASGSIS	INAMG	WYRQAPGKQREFVA	AIT TSGSTRYANSAKG
AG3	AVQLVDSGGGLVHPGGSLRLSCAASGSIF	INTMG	WFRQAPGKEREFVA	AISGSNGKTYYADSVLG
AG4	QVQLVESGGGLVQPGGSLRLSCAASGSIF	INAMA	WYRQAPGKQRELVA	RIN RDGSTYADSVKG
AG5	EVQLVESGGGLVHPGGSLRLSCAASESIA	ASALG	WYRQIPG GRELVA	GIS SSGHTRYVDSVKG

	FR3	**CDR3**	**FR4**
Name			
VHH	82ABC	100ABCDEFGH	
	66 |	95 |	103
AG1	RFTISRDNAKNTVYLQMDSLIPEDTAVYYCMS	RAFDRTEPWR DY	WGQGTQVTVSS
AG2	RFTISRDNAKNTVYLQMNSLRPEDTAVYYCMS	RAFDRTEPWR DY	WGQGTQVTVSS
AG3	RSTISRDNAKSTVYLQMNSLKPEDTAVYYCAA	SGPYGSVWLTGRSYDY	WGQGTRVTVSS
AG4	RFTISRDNAKRSVYLQMNSLKPEDTAVYYCNA	RQIISHT EY	WGQGNQVTVSS
AG5	RFTISRDNTENTVYLQMNSLKPEDTAVYYCRY	IGRPN NV	WGQGTQVTVSS

Amino-acid sequence alignment of framework 1–4 and complementarity detemining region 1–3 for the five anti-HIF-1α VHH; sequence numbering according to Kabat *et al*.[39] Shaded: different amino acids in framework 1 and 3 of VHH AG1 and AG2.

DFO-induced HIF-1α at 120 kDa on Western blot (Figure 3a and data not shown).

All identified VHH could immunoprecipitate the human native HIF-1α and could detect recombinant HIF-1α fragment B as well as recombinant full-length HIF-1α, using Western blot analysis (Figure 3b and c). The ODDD-YFP fusion protein was used to show that all described VHH bind specifically to an epitope in the ODDD of HIF-1α. All of the VHH were able to immunoprecipitate this fusion protein from HeLa cells, indicating that each one of those VHH recognizes epitopes located in this domain (Figure 4). Well-defined nonrelated VHH were used as a negative control in all of these experiments (VHH-R2).[38]

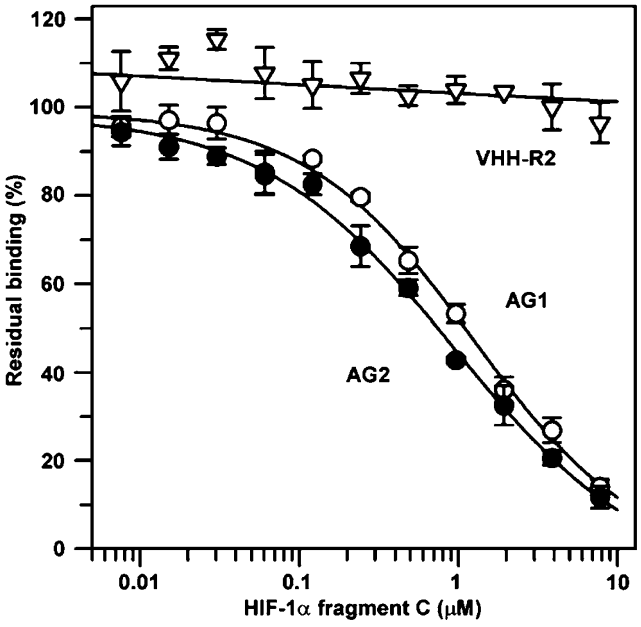

Figure 2 VHH AG1 and AG2 compete for binding to HIF-1α. Recombinant HIF-1α fragment C was incubated with immobilized VHH AG1 in the absence or presence of increasing concentrations of VHH AG1 and AG2. Residual binding is expressed as percentage of binding in the absence of competitor and is corrected for nonspecific binding. VHH-R2 was used as a negative control. Data represent the mean ± s.d. of three experiments.

Engineering of Bivalent VHH

An additional advantage of the used phage display technique is that the coding cDNA sequence of the selected VHH is known. This feature makes these VHH easy to clone for other applications. VHH antibodies AG1 and AG2 showed a stronger signal on Western blot than the other selected VHH (Figure 3b and c) and had the highest affinity (Figure 5). For this reason, we selected those two VHH to be cloned as bivalent VHH. VHH were cloned in tandem, either with two identical VHH as mono-bivalent VHH or as hetero-bivalent VHH with combinations of different VHH. The four combinations we cloned were: AG1–AG1, AG2–AG2, AG1–AG2 and AG2–AG1. All bivalent VHH combinations were expressed in *E. coli*. Differences in expression levels of bivalent VHH, when isolated from the periplasm, were considerable. The bivalent VHH combinations with an N-terminal AG2 produced very poor. A combination of AG1 produced fair, but a combination of an N-terminal AG1 and a C-terminal AG2 produced exceptionally well. Both of these monovalent VHH recognize an identical epitope in the HIF-1α region spanning amino acids 543–605, represented in fragment C, and this combination resulted in a hetero-bivalent VHH called AG-1N2C-MYC.

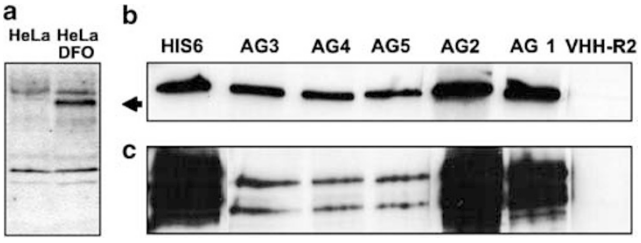

Figure 3 Detection of HIF-1α by Western blot. (**a**) Western blot with monovalent VHH AG2 on non/DFO-stimulated HeLa cell lysates. An induced band representing the endogenous HIF-1α protein is indicated by an arrow. Remaining bands are endogenous HeLa proteins recognized by the anti-6xHIS antibody. Western blot with the different anti-HIF-1α monovalent VHH AG1–5, negative and positive control VHH-R2/HIS6 for (**b**) recombinant HIF-1α fragment B (aa 375–605), and (**c**) recombinant full-length HIF-1α (aa 1–826).

Table 2 VHH properties

VHH found in pool selected against recombinant fragment	*Detection of recombinant HIF-1α fragments A–F in ELISA/Western blot*					
	A	*B*	C	*D*	*E*	*F*
Name VHH						
AG1 BDF	−	+	+	+	−	+
AG2 C	−	+	+	+	−	+
AG3 A	+	+	−	−	+	−
AG4 A	+	+	−	−	+	−
AG5 BDF	−	+	+	+	−	+

VHH antibodies found in VHH subpopulations. Recombinant HIF-1α fragment recognition of the five VHH in ELISA and Western blot experiments: cross-reactivity +/− between VHH and recombinant HIF-1α fragments.

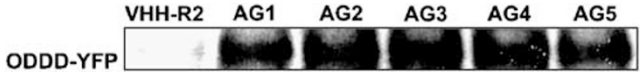

Figure 4 All selected VHH bind within the ODDD of the HIF-1α protein. Immunoprecipitation from HeLa cells of an ODDD-YFP fusion protein by the different anti-HIF-1α monovalent VHH AG1–5. As a negative control, VHH-R2 was used. All five VHH recognize specific epitopes within the ODDD of the HIF-1α protein.

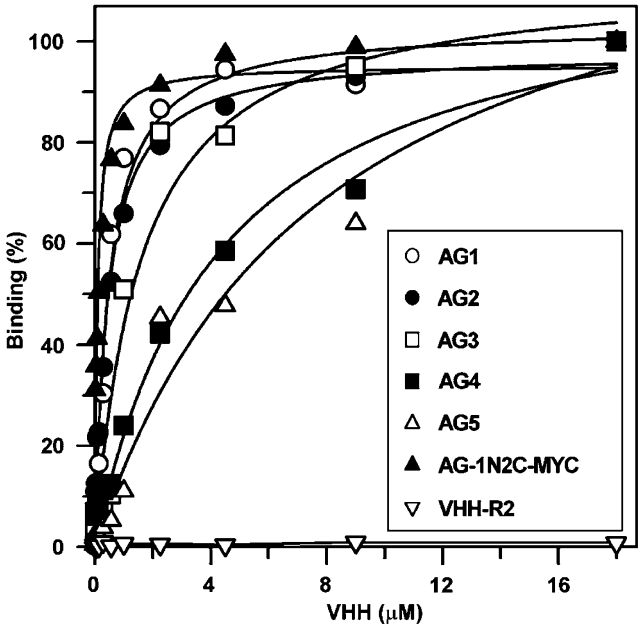

Figure 5 Apparent affinities for binding of VHH to HIF-1α. VHH were incubated with immobilized recombinant HIF-1α fragment B. Binding is expressed as percentage of maximum binding for each VHH to recombinant HIF-1α fragment B. The apparent affinity is approximated from the determined concentration yielding half-maximum binding to HIF-1α. VHH-R2 is used as a negative control. AG1 ≈ 0.48 μM, AG2 ≈ 0.45 μM, AG3 ≈ 1.82 μM, AG4 ≈ 4.26 μM, AG5 ≈ 7.75 μM and AG-1N2C-MYC ≈ 0.09 μM. Data represent the mean of duplicate experiments.

For this bivalent, VHH yields up to 20 mg/l were obtained. To show the specificity of hetero-bivalent VHH AG-1N2C-MYC for native HIF-1α protein, a competition experiment was performed to verify once again specificity for native HIF-1α after engineering of the bivalent VHH. DFO-stimulated HeLa lysates, with native HIF-1α, were incubated with two different recombinant fragments in an immunoprecipitation experiment. The fragments used were fragment E (aa375–455) and fragment C (aa543–605) without homology to and nonoverlapping with fragment E. Fragment C could compete with the native HIF-1α protein for binding to the bivalent VHH, as expected, because monovalent VHH used for cloning of the bivalent VHH preferentially bind to this fragment as has been shown in previous experiments (Table 2). This experiment indicates the specific binding of native HIF-1α protein with hetero-bivalent VHH AG-1N2C-MYC (Figure 6).

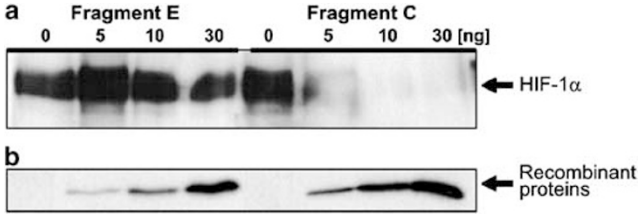

Figure 6 Bivalent VHH AG-1N2C-MYC is specific for native HIF-1α. Recombinant HIF-1α fragment C competes with native HIF-1α for binding with hetero-bivalent VHH AG-1N2C-MYC in immunoprecipitation of HIF-1α protein from DFO-stimulated HeLa cell lysates, indicating specific binding to the native HIF-1α protein. Recombinant HIF-1α fragment E, nonspecific for bivalent AG-1N2C-MYC VHH did not compete at all with the native HIF-1α protein. ng: range/amount of the recombinant HIF-1α protein added. (**a**) Western blot for HIF-1α detection. (**b**) Western blot for recombinant proteins E and C to illustrate how much of the recombinant protein was added to the HeLa cell lysates. Combined panels (**a** and **b**) show that the more fragment C is added, the less native HIF-1α protein can be immunoprecipitated, as addition of fragment E to the lysate has no effect on the immunoprecipitation.

Determination of the VHH Binding Affinity to HIF-1α

To study the binding capacity of the VHH to HIF-1α, the apparent affinity was determined by calculation of half-maximum binding concentrations of VHH (Figure 5). The observed apparent affinities were ≈0.48 μM for AG1, 0.45 μM for AG2, 1.82 μM for AG3, 4.26 μM for AG4 and 7.75 μM for AG5. The hetero-bivalent VHH AG-1N2C-MYC, a fusion of AG1 and AG2, had an apparent affinity of 0.09 μM. Consistent with the Western blot analysis (Figure 3b and c), the highest apparent affinities are observed for VHH AG1 and AG2. In addition, the bivalent VHH AG-1N2C-MYC shows a five-fold increase of apparent affinity for binding to HIF-1α as compared with the corresponding monovalent VHH.

Optimization of Human and Mouse HIF-1α Protein Detection with VSV-Tagged Bivalent VHH

The bivalent VHH AG-1N2C-MYC produced well in our bacterial expression system and is more efficient in the interaction with HIF-1α as compared to the monovalent VHH. For increase of detection variety and avoidance of background signals, we chose to replace the c-*Myc* tag of the bivalent VHH AG-1N2C-MYC by a VSV-G tag, which resulted in a hetero-bivalent VHH called AG-1N2C-VSV. This antibody could immunoprecipitate native human as well as mouse HIF-1α protein from DFO-treated cell lysates (Figure 7).

Endogenous Human HIF-1α Detection by Immunohistochemistry

Paraffin-embedded tumour sections of renal cell cancers that express mutant VHL protein and consequently high HIF-1α protein levels due to

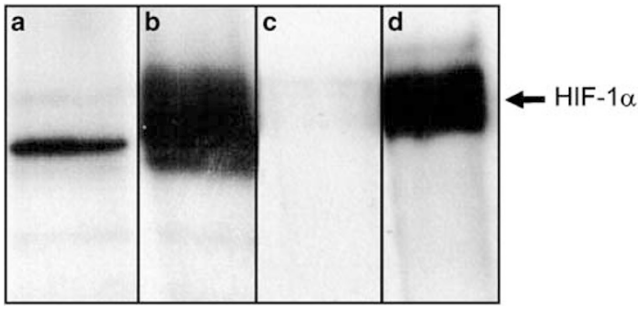

Figure 7 Hetero-bivalent VHH AG-1N2C-VSV is specific for the human and mouse HIF-1α protein. Immunoprecipitation of the native HIF-1α protein from human and mouse cell lysates with hetero-bivalent VHH AG-1N2C-VSV. In lane **a**, untreated NIH 3T3 lysate and lane **b**, DFO-stimulated NIH 3T3 lysates. In lane **c**, untreated HeLa lysates and lane (**d**), DFO-stimulated HeLa lysates. The band detected in lanes **b** (mouse) and **d** (human) corresponds to the induced endogenous HIF-1α protein.

impaired proteasomal degradation were used to test the usefulness of hetero-bivalent VHH AG-1N2C-VSV in immunohistochemistry. This bivalent VHH could detect specific HIF-1α nuclear staining in these tumors similar to a positive control antibody (clone 54, BD Transduction Laboratories) (Figure 8a and b).

Endogenous Mouse HIF-1α Detection with Anti-HIF-1α VHH by Immunofluorescence Microscopy

We investigated whether anti-HIF-1α VHH was able to recognize endogenous mouse HIF-1α in cultured cells with fluorescence microscopy. Upon DFO stimulation, stabilized HIF-1α translocates to the nucleus and induces transcription of target genes.[34] Indeed, we could visualize translocation of the

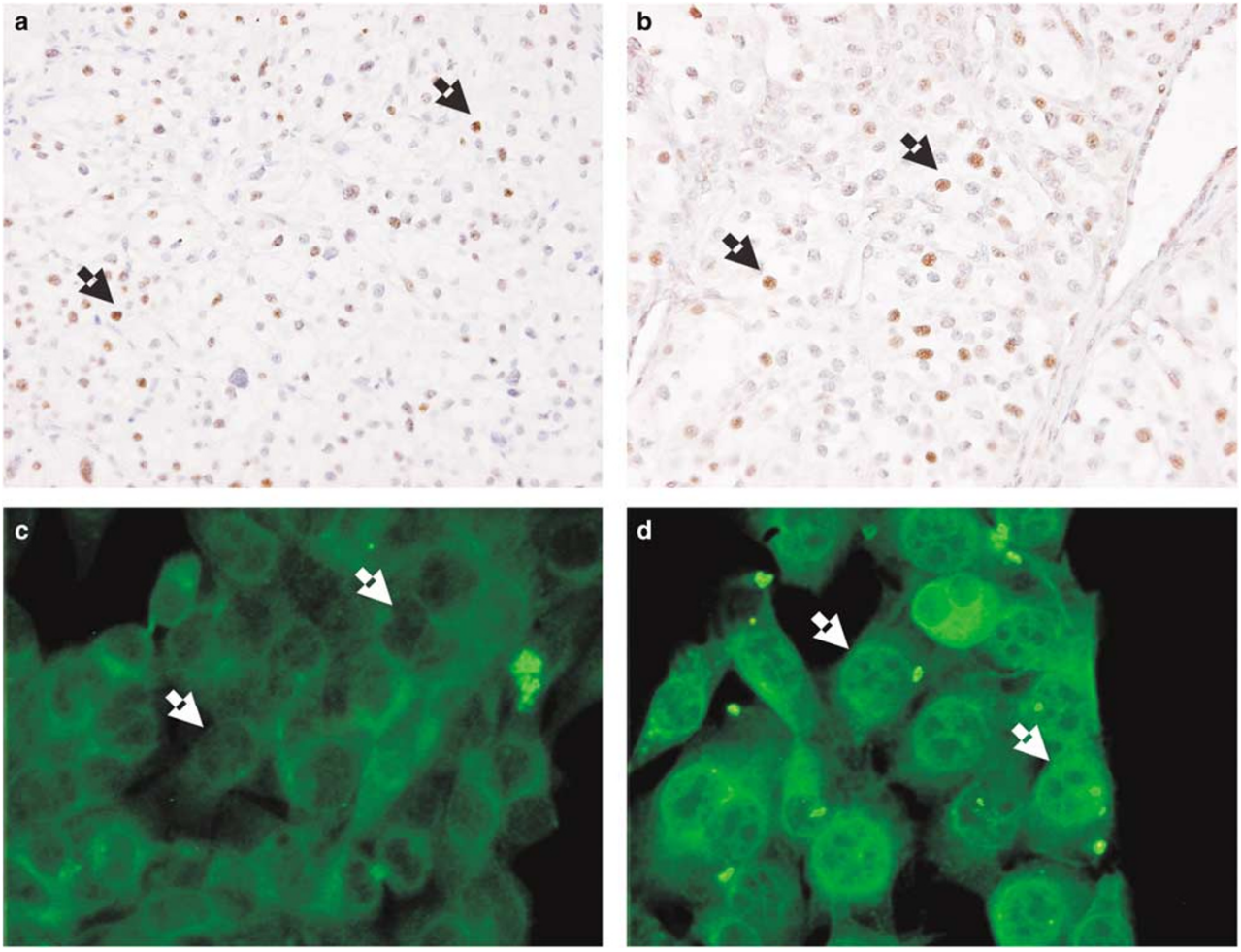

Figure 8 Hetero-bivalent VHH AG-1N2C-VSV detects specific nuclear signal in renal cell cancer specimens. Immunohistochemistry on paraffin-embedded slides with (**a**) commercial mouse monoclonal (BD Transduction Laboratories); (**b**) hetero-bivalent VHH AG-1N2C-VSV. Both antibodies recognize identical nuclear HIF-1α staining patterns. HIF-1α-negative nuclei stain blue and HIF-1α-overexpressing nuclei stain brown, as indicated by the arrowheads. Bivalent VHH AG-1N2C-MYC detects specific nuclear signal in NIH 3T3 cells after DFO stimulation, representing endogenous HIF-1α protein. Immunofluorescence microscopy on cultured NIH 3T3 mouse fibroblasts. Cells were stained with 12.5 ng/μl hetero-bivalent VHH AG-1N2C-MYC. (**c**) Untreated NIH 3T3 cells with negative nuclei indicated by the arrow heads. (**d**) DFO-stimulated NIH 3T3 cells with positively stained nuclei indicated by the arrow heads.

mouse HIF-1α protein into the nucleus in DFO-stimulated mouse NIH 3T3 fibroblasts (Figure 8c and d). Both the monovalent VHH AG1 and AG2 could detect a similar nuclear signal (data not shown). In agreement with the observed higher affinity for HIF-1α (Figure 5), hetero-bivalent VHH AG-1N2C-MYC could be used at a lower concentration as compared with monovalent VHH.

Discussion

Now that the human genome has been largely mapped, the next challenge will be to uncover the proteome. To this end, a wide range of specific antibodies to each protein is needed to detect the level, localization, splice variants and various post translational modifications that are crucial to protein function. As HIF-1α is almost exclusively post-transcriptionally regulated, antibodies are of particular interest to study its function. In this manuscript, we describe the generation of llama-derived antibodies for diagnostics and molecular research on the HIF-1α protein. Currently, available antibodies and their performance in certain immunological applications are mostly limited.

Selection of VHH against HIF-1α by phage display, an important target in cardiovascular disease and human cancer, proved to be very successful. We identified five monovalent VHH able to recognize HIF-1α within the ODDD (Figures 1 and 4). Some of these isolated VHH recognize epitopes more N-terminal on the HIF-1α protein than the commercial mouse monoclonal (clone 54, BD Transduction Laboratories, San Diego, CA, USA) that was used. Two of the monovalent VHH (AG1 and AG2) harbor identical CDR topped on different FR (Table 1) and competed for binding to HIF-1α (Figure 2). This strongly suggests that both VHH recognize the same region within the ODDD. Fusion of AG1 and AG2 into a hetero-bivalent VHH markedly increased the binding affinity for HIF-1α (Figure 5). We proved specificity of the engineered hetero-bivalent VHH AG-1N2C-MYC for the native human HIF-1α protein by competition with recombinant fragment C (aa543–605) (Figure 6). This bivalent antibody detected mouse endogenous HIF-1α protein in NIH 3T3 fibroblasts after DFO stimulation (Figure 8c and d). Furthermore, engineered hetero-bivalent VHH AG-1N2C-VSV was useful in immunohistochemistry of human renal cell cancer (Figure 8a and b) and could immunoprecipitate native human and mouse HIF-1α protein (Figure 7). This is one of the first engineered antibodies recognizing mouse HIF-1α that may be used in multiple molecular applications in human as well as mouse HIF-1α model studies.

The significant advantage over conventional antibody generation is that phage display allows setting defined parameters, during selection, for the desired type of antibody. Selection strategies with altered presentation of the used recombinant HIF-1α antigen proteins in VHH selection rounds in combination with the used nonimmune library showed to be very effective for isolating HIF-1α-specific monovalent VHH against the HIF-1α protein. Furthermore, it generates infinite sources of monoclonal antibody.

The newly identified anti-HIF-1α VHH were crossvalidated with a widely employed and accepted anti-HIF-1α mouse monoclonal. This latter monoclonal antibody was raised against amino acids 610–727 of the human HIF-1α protein (clone 54, BD Transduction Laboratories, San Diego, CA, USA). Therefore, this commercial antibody would not recognize all splice products or homologs of the HIF-1α protein.[27] We might have limited ourselves, in the identified number of HIF-1α binding VHH, by using this antibody in our validation screen by immunoprecipitation. Further investigation of the selected pool of a 170 VHH from the present study may lead to the identification of VHH that can recognize these homologs and different splice variants of the HIF-1α protein.

We attempted to isolate a VHH that could discriminate a one amino-acid difference (Ile^{736}) between isoform 2[26] and the full-length HIF-1α protein. However, we did not succeed due to the fact that recombinant fragment F (543–736), which represents isoform 2, also contained an epitope that appeared to be very immunogenic to the used phage display library. This epitope is located in the overlap of recombinant fragments B, C, D and F (Figure 1). This is underlined by the fact that most of the available antibodies recognize the same confined region within the HIF-1α protein. However, the use of phage display may allow selecting VHH to less-immunogenic epitopes. This could in theory be established by preclearing the VHH phage display library with recombinant fragment D (aa543–826), or by blocking the immunogenic epitope in recombinant fragment F (aa543–736), with identified anti-HIF-1α VHH AG1, AG2 and AG5 during selections.[40,41]

In our selection procedures, we used six recombinant HIF-1α fragments, taking into account important amino-acid residues within the HIF-1α protein, especially the residues in the ODDD spanning amino-acid sequence aa 401–603[1,8,27] (Figure 1). Fragments A (aa 1–490) and E (aa 375–455) contain amino acid residue Pro^{402} and fragments C, D and F amino-acid residue Pro^{564}. Fragment B (aa 375–605) contains both of these, important for hydroxylation targeted residues, necessary in regulation of HIF-1α degradation.[28,29] This fragment also contains amino-acid residue Lys^{532}, a target for acetylation, also important in the regulation of the stability of HIF-1α protein.[42] We show that all selected VHH bind within the ODDD (Figures 1 and Figure 4). Therefore, binding of these VHH to HIF-1α may block crucial functions of protein and may contribute to the elucidation of the functions of the HIF-1α protein. If indeed interference with the functions of the HIF-1α occurs, it may be worthwhile to

explore the possible therapeutic potential of the identified anti-HIF-1α VHH. Currently, we are investigating the effect on HIF-1α stability and transcriptional activity by expressing these VHH intracellularly as intrabodies.[23,43]

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