Selection and characterization of Affibody® ligands to the transcription factor c-Jun

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c-Jun is a highly oncogenic transcription factor involved in the development of different types of cancer. In the present study we have generated c-Jun-binding-affinity proteins from a phage-displayed library of so-called 'Affibody® ligands', developed by combinatorial engineering of a non-immunoglobulin-based scaffold protein. Homodimeric c-Jun protein was recombinantly produced in *Escherichia coli* and, prior to selection, the quality of the target protein was investigated by binding analyses, which indicated specific binding to a double-stranded DNA hairpin construct containing a c-Jun response element, but not to a control sequence. Isolated Affibody® variants from the phage selection were expressed in *E. coli*, purified by affinity chromatography and their interaction with c-Jun was analysed. In biosensor analyses, one Affibody® ligand, denoted $Z_{cJun518}$, was shown to interact with immobilized c-Jun protein with an apparent dissociation constant of 5 μM. By constructing a head-to-tail homodimeric version of $Z_{cJun518}$, its apparent affinity for c-Jun could be increased threefold, suggesting co-operativity effects in the binding to the immobilized c-Jun protein. Further characterization of the $Z_{cJun518}$ Affibody® molecule demonstrated, in both affinity-capture and Western-blotting experiments, its ability to interact selectively with c-Jun, even when the c-Jun target was present in a complex protein background consisting of a bacterial cell lysate. $Z_{cJun518}$ could also be used to stain the c-Jun-overexpressing cell line C8161 visualized by confocal fluorescence microscopy. Results from competition experiments indicated that the binding epitope on c-Jun for the $Z_{cJun518}$ Affibody® molecule was separate from the binding sites of both a polyclonal antibody raised against the unstructured N-terminal domain and a double-stranded DNA hairpin containing a c-Jun response element. The potential intracellular use of Affibody® ligands directed against transcription factors and other oncogenic factors is discussed.

Introduction

The members of the AP-1 (activator protein-1) family of transcription factors are formed by homo- or hetero- dimerization of members from the Jun, Fos, Maf and ATF protein families and are involved in a multitude of cellular processes ranging from proliferation and survival to apoptosis [1,2]. c-Jun can form both homo- and hetero-dimers and is often included in AP-1 proteins involved in cell proliferation [3]. Its activity is tightly regulated post-translationally: (i) the DNA-binding ability is modulated by redox regulation of Cys^{252} in the DNA-binding domain [4]; (ii) its transcriptional activity is modulated by phosphorylation of Ser^{63} and Ser^{73} in the N-terminal domain by JNKs (Jun N-terminal kinases) [5]; and (iii) its cellular level is regulated by phosphorylation of Thr^{239}, which leads to ubiquitination and degradation [6]. Owing to its involvement in proliferation, c-Jun plays an important role in the development of several types of cancer. Its expression is sometimes increased, which has been shown in cases of pancreatic cancer, squamous-cell carcinoma and basal-cell carcinoma [7,8]. It can also be overactivated, which has been shown in cases of breast, prostate and liver tumours, leading to a higher proliferation rate of the tumour cells [9–11]. Several *in vitro* studies to elucidate the role of c-Jun in cancer development have also shown that overexpression can greatly enhance the tumorigenic properties of the cell by inducing elevated motility, unresponsiveness to oestrogen and tamoxifen as well as increased tumour formation in nude mice [12,13].

In the present study we investigated the possibility of generating Affibody®-based ligands interacting with c-Jun. Affibody® ligands are small and robust affinity reagents based on a non-immunoglobin scaffold derived from Protein A, which has an anti-parallel triple-helical fold [14]. The most common manner in which Affibody® ligands are generated is by several rounds of biopanning of a phage-displayed Affibody® library against a desired target. In this way, Affibody® molecules have successfully been selected to

Key words: Affibody® ligand, c-Jun, oncogene, phage display, protein engineering, transcription factor.

Abbreviations used: AP-1, activator protein 1; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; IB, inclusion body; His_6, hexahistidine; HRP, horseradish peroxidase; HSA, human serum albumin; IPTG, isopropyl β-D-thiogalactopyranoside; JNK, Jun N-terminal kinase; RU, resonance unit; scFv, single-chain variable fragment; sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate.

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bind to numerous target proteins [15–17]. They have subsequently been employed in a variety of different applications, including bioseparation [16] and cell–cell interaction analyses [18]. Affibody® molecules have also been functionalized by expression with different fusion partners or by incorporation of functional groups, giving reagents suitable for target protein detection in experiments based on ELISA [19] and protein microarrays [20]. In addition, they have been used to quantify the amounts of receptors on cells and to detect xenografted tumour cells by gamma-camera imaging [21–23].

In the present paper we describe the production of a recombinant full-length c-Jun homodimer in *Escherichia coli*, followed by characterization of its interaction with DNA. This protein was used as a target antigen during biopanning for Affibody® ligands. The isolated Affibody® ligands were characterized for their binding to c-Jun in biosensor studies, resulting in the identification of one Affibody® variant that showed specific binding to c-Jun. This Affibody® ligand was biochemically characterized and was employed as a capture reagent of c-Jun as well as a primary detection reagent in Western blotting. Finally, the Affibody® ligand was used to stain the c-Jun overexpressing melanoma cell line C8161.

Materials and methods

General

All DNA restriction and modifying enzymes were from Fermentas (Helsingborg, Sweden) or New England Biolabs (via In Vitro Sweden, Stockholm, Sweden). All DNA constructs were verified by DNA sequencing using an ABI Prism® 3700 Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Except where otherwise noted, *E. coli* strain RR1ΔM15 [24] was used throughout the present study.

Subcloning and production of c-Jun

The full-length c-Jun gene was subcloned from plasmid pOTB7, which was obtained from LGC Promochem (Borås, Sweden). The gene was PCR-amplified, using the oligonucleotides cJun-f (5′-CACCACCATATGACTGCAAAGATGGAAACGA-3′) and cJun-b (5′-GTGGTGAAGCTTTTAAAATGTTTGCAACTGCTGCGTTA-3′), using the following programme: 98 °C for 15 s; 52 °C for 60 s; 72 °C for 3 min (repeated 30 times); then 72 °C for 7 min. The resulting PCR product was restricted with NdeI and HindIII, followed by ligation with pET21a(+) (Novagen, Madison, WI, U.S.A.), which had been restricted with the same enzymes, yielding the expression plasmid pT7-cJun. This plasmid was transformed to *E. coli* strain BL21(DE3) (Novagen). The bacteria were grown at 37 °C in TSBY [Tryptic Soy Broth (Merck, Darmstadt, Germany) with 5g/l yeast extract (Sigma–Aldrich, Steinheim, Germany)] supplemented with 100 mg/l ampicillin. When the attenuance (D_{600}) of the culture reached 0.5–1.0, protein expression was induced by addition of IPTG (isopropyl β-D-thiogalactopyranoside) to a final concentration of 1 mM, and production was carried out for 3 h. The cells were harvested by centrifugation (6000 ***g*** for 10 min at 4 °C) and were resuspended in TEN buffer (20 mM Tris, pH 7.9, 1 mM EDTA and 100 mM NaCl) supplemented with 1 mM DTT (dithiothreitol). c-Jun was produced as IBs (inclusion bodies) and purification was performed essentially as described by Lively et al. [25]. The cells were lysed by sonication [450 W power; pulsed bursts (1 s on/1 s off) for 3 min] on a VC750 (Sonics & Materials, Newtown, CT, U.S.A.), followed by centrifugation (16 000 ***g*** for 15 min at 4 °C) to pellet insoluble material. The cleared supernatant was isolated. The IBs were washed with 5 mM DTT and were solubilized with TEN buffer supplemented with 5 mM DTT and 7 M urea. This was followed by cation-exchange chromatography on a 4 ml column containing S-Sepharose FF under fully denaturating conditions using TEN buffer supplemented with 5 mM DTT and 7 M urea as running buffer. The process was monitored on an ÄKTAexplorer™ (GE Healthcare, Uppsala, Sweden) chromatography system and elution of bound material was accomplished by a linear gradient of NaCl from 0.1 to 0.5 M. Fractions containing c-Jun were dialysed in three consecutive steps to refold the protein: (i) in the first step the urea concentration was lowered from 7 to 1 M and NaCl was increased from 0.1 to 1 M; (ii) in the second step the urea was removed; and (iii) in the third step the NaCl concentration was lowered from 1 to 0.1 M.

DNA interaction analysis of c-Jun

A 96-well ELISA plate (BD Biosciences, Stockholm, Sweden) was coated with 50 μl of streptavidin per well [8 ng/μl in PBS (100 mM Na_2HPO_4/NaH_2PO4 and 100 mM NaCl, pH 7.5)] and incubated for 1 h at 37 °C and rinsed twice in water. Biotinylated oligonucleotides (50 μl), namely Bio-AP1 (5′-Bio-GGAGATCCGGC**TGACTCA**TCACTAGGGTTTT-CCCTAGTGA**TGAGTCA**GCCGGATC-3′; 1 ng/μl in PBS) or Bio-NC (5′-Bio-GGACACTGCGGCTCCGG-CCCCGGGTTTTCCCGGGGCCGGAGCCGCAGTG-3′; 1 ng/μl in PBS), were added to the wells, followed by incubation overnight at 4 °C. Both oligonucleotides form double-stranded hairpins (the complementary part is underlined) and Bio-AP1 contains a general AP-1 binding site (in bold), whereas Bio-NC does not, and it therefore functions as a negative control. The rest of the procedure was carried out at room temperature (22 °C). The wells were washed twice with water and incubated with blocking solution containing 5% (w/v) dried skimmed milk in PBS for 1 h. The blocking solution was removed and c-Jun, diluted in PBS supplemented with 100 ng/μl herring sperm DNA, 5 mM DTT and 5% (w/v) dried skimmed milk, was added at different concentrations to the wells. Incubation

was continued for 1 h, followed by washing five times with water. A 50 μl volume of 1 μg/ml anti-c-Jun antibody [rabbit polyclonal IgG anti c-Jun (N); Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.] was added to each well and incubation was carried out for 1 h, followed by washing five times with water. A 50 μl volume of goat anti-rabbit IgG antibody conjugated with HRP (horseradish peroxidase; DakoCytomation, Glostrup, Denmark), diluted 1:1000 in PBS, was added to each well and the mixture incubated for 30 min, followed by washing five times with water. The signals were developed using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, U.S.A.) as indicated in the manufacturer's instructions. The reaction was stopped after 10 min with 50 μl of 2.0 M H_2SO_4 and the A_{450} was determined for each well.

For competition studies using the selected Affibody® ligands, the same ELISA protocol was used, but c-Jun was preincubated with a 1000-fold molar excess of Affibody® molecules ($Z_{cjun518}$, $Z_{cjun5124}$ or Z_{wt}) for 1 h at 22 °C before being added to the ELISA plate.

Selection of Affibody® ligands

c-Jun was biotinylated *in vitro* for 2 h on ice using a 5-fold molar excess of EZ-Link™ sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-(biotinamido)hexanoate; Pierce, Rockford, IL, U.S.A.], after which excess biotin was removed using a PD-10 column (GE Healthcare). The extent of biotinylation was determined using an HABA dye (4′-hydroxyazobenzene-2-carboxylic acid)–avidin assay (Pierce) according to the manufacturer's instructions. An Affibody® library displayed as fusions to protein III on M13 bacteriophage was obtained from Affibody® AB (library size 3×10^9). Fresh phage stocks used in the selections were produced by using previously described procedures [15,26]. Five rounds of biopanning were performed as follows. All tubes were pretreated with PBST (PBS supplemented with 0.1% Tween-20) and 0.1% gelatin. A preselection was performed before each round where 1 ml of the phage stock supplemented with 0.1% gelatin was incubated with 0.5 mg of M-280 paramagnetic beads (Dynal Biotech, Smestad, Norway), previously washed twice with PBST. The unbound phages were collected and incubated with biotinylated c-Jun for 2 h with slow agitation. The mixture was then added to 0.5 mg of pre-washed and blocked paramagnetic beads, and incubated for 15 min with slow agitation. To increase the stringency in the selection, the amount of c-Jun was lowered from 100 nM in cycles 1 and 2 and to 20 nM in cycles 3, 4 and 5. Washing of the beads with PBST was increased from twice in round 1, three times in round 2, six times in round 3, to 12 times in rounds 4 and 5. Bound phages were eluted using 500 μl of 0.05 M glycine/HCl, pH 2.2, for 10 min, followed by immediate neutralization with 500 μl of 1 M Tris/HCl, pH 7.9. The eluted phages were used to infect *E. coli* cells and were plated on agar plates supplemented with 2% (w/v) glucose and 100 μg/ml ampicillin. The colonies obtained were resuspended and collected in TSBY supplemented with 100 μg/ml ampicillin and 2% (w/v) glucose, of which a fraction (100 times higher than the number of eluted phages during the previous round of panning) was used for inoculation and production of new phage particles to enter the next round of panning. After five rounds of panning, 18 individual colonies were isolated and the DNA sequence of the encoding Affibody® ligands was determined.

Subcloning and production of Affibody® ligands

DNA fragments encoding the Affibody® ligands were subcloned into the expression vector pAY442. A variant of $Z_{cJun518}$, where the cysteine residue at position 24 was replaced with serine (denoted $Z_{cJun518\_C24S}$), was also generated and subcloned into pAY442. In addition, a dimeric variant of $Z_{cJun518}$ and $Z_{cJun518\_C24S}$ was also generated and subcloned into pAY442. The pAY442 vector has been described previously [23] and the Affibody® ligands are expressed with an N-terminal His_6 (hexahistidine) tag, under control of a T7 promoter.

The pAY442 expression plasmids were transformed to *E. coli* strain BL21(DE3) cells, and overnight cultures were grown. On the following morning, the overnight cultures were diluted 100-fold into 100 ml of fresh TSBY medium supplemented with 50 mg/l kanamycin and were incubated at 37 °C until the D_{600} was about 0.7–1.0, at which point protein production was induced by addition of IPTG to a final concentration of 1 mM. At 4 h after induction, the cell cultures were harvested by centrifugation (6000 ***g*** for 10 min at 4 °C) and the cell pellets were resuspended in 25 ml of lysis buffer (6 M guanidinium chloride, 47.4 mM Na_2HPO_4, 2.65 mM NaH_2PO_4, 5 mM Tris/HCl and 0.1 M NaCl, pH 8.0) and disrupted using a Sonics VC750 sonicator (450 W; pulsed bursts 1s/1s; 3 min). Cell debris was removed by centrifugation (6000 ***g*** for 10 min at 4 °C) and the supernatants were loaded on to Talon metal affinity resin (BD Biosciences) columns. After washing the columns with 25 ml of lysis buffer, the bound proteins were released with elution buffer (8 M urea, 0.1 M NaCl, 29.6 mM acetic acid, 70.4 mM sodium acetate and 50 mM sodium phosphate, pH 5.0). Thereafter the proteins were refolded and desalted to PBS by passage through NAP-10 desalting columns (GE healthcare) according to the manufacturer's instructions.

The purified proteins were analysed by SDS/PAGE on a NuPAGE Bis-Tris/4–12%-(w/v) polyacrylamide gel using a Novex system (Invitrogen, Carlsbad, CA, U.S.A.). Protein concentrations of the Affibody® ligands were determined by Bradford analysis, except for $Z_{cjun518}$, where the concentration was determined by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden).

Biosensor binding analyses

The real-time biospecific interaction between the Affibody® ligands and different target proteins was investigated using a BIAcore 2000 instrument (Biacore, Uppsala, Sweden). The target protein, c-Jun, and the negative control proteins, human IgG (Pharmacia, Uppsala, Sweden) and HSA (human serum albumin; Sigma–Aldrich), were immobilized in different flow cells on a CM5 chip using a standard amine coupling protocol with EDC [*N*-ethyl-*N*′-(dimethylaminopropyl)carbodi-imide] and NHS (*N*-hydroxysuccinimide) according to the manufacturer's recommendations at a flow rate of 20 μl/min. The fourth flow cell did not contain any immobilized protein and was used as a reference surface. The density of immobilized c-Jun was 1700 RU (resonance units) which would yield an R_{max} (maximum binding capacity of the Affibody® ligands) of $\sim$170 RU in kinetic experiments with Affibody® ligands. Initial binding analyses were performed by sequentially injecting all Affibody® ligands at a concentration of 3 μM over the four surfaces at 23 °C in HBS-EP buffer [5 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.005% P20 (a surfactant from Biacore), pH 7.4)]. For kinetic analysis, duplicate samples of the $Z_{cJun518}$ Affibody® ligand were injected over the surface with immobilized c-Jun and the blank surface at concentrations ranging from 0.68 to 5.43 μM in a random order. All experiments were performed at a flow rate of 30 μl/min.The surfaces were regenerated by injection of 100 μl of 10 mM DTT, followed by a stabilization time of 45 min. The binding rate constants were determined by global analysis of the sensograms obtained after subtraction of responses from the reference surface using BIAevaluation software 3.2 (Biacore). To deternmine the dissociation equilibrium constant (K_D), the association rate constant (k_a) and the dissociation rate constant (k_d), three different models for interaction were tested: one-to-one Langmuir binding, bivalent analyte and binding with mass transfer. For analysis under reducing conditions, $Z_{cJun518}$ was preincubated with HBS-EP supplemented with 1mM DTT prior to injection over the c-Jun and the blank surfaces. In this case the HBS-EP running buffer was also supplemented with 1 mM DTT. A competition experiment between $Z_{cJun518}$ and an anti-c-Jun antibody (rabbit polyclonal; Santa Cruz Biotechnology) was performed. The surface with immobilized c-Jun was saturated by repeated injections with 1 μM anti-c-Jun antibody, directly followed by an injection with 1 μM $Z_{cJun518}$.

CD analyses

CD spectra were recorded on a Jasco (Easton, MD, U.S.A.) J-810 spectropolarimeter. Solutions of $Z_{cJun518}$ and $Z_{cJun518\_C24S}$ were prepared in PBS, pH 7.2, at concentrations of 43.5 μM and 39.2 μM respectively. Cuvettes with 1 mm pathlength were used for all experiments. Thermal melting was monitored at the same concentrations by measuring the ellipticity (θ_{220}) while increasing the temperature.

Specificity analysis of $Z_{cJun518}$

The specificity of the Affibody® ligand $Z_{cJun518}$ was evaluated by employing biotinylated $Z_{cJun518}$ in a Western-blotting setting to detect a recombinant c-Jun homodimer. $Z_{cJun518}$ was biotinylated *in vitro* by incubation on ice for 2 h with a fivefold molar excess of EZ-Link™ sulfo-NHS-LC-biotin, followed by removal of excess biotin using a PD-10 desalting column (GE Healthcare).

The samples containing c-Jun were separated by gel electrophoresis on NuPAGE Bis-Tris/SDS/4–12%-PAGE gels using a Novex system (Invitrogen). Proteins were transferred to a PVDF membrane (Invitrogen), followed by blocking for 1 h with TBST (20 mM Tris/HCl, 160 mM NaCl and 0.1% Tween-20, pH 7.6), supplemented with 1% gelatin. The membrane was incubated with biotinylated $(Z_{cJun518})_2$ diluted in TBST to a final concentration of 20 μg/ml for 1.5 h, followed by washing with TBST. This was followed by incubation with streptavidin–HRP (DakoCytomation) diluted 1:3000 in TBST supplemented with 1% gelatin for 40 min and washing with TBST. The membrane was developed using SuperSignal West Dura Extended Duration Substrate.

Capture of c-Jun

The ability of the Affibody® ligands to capture soluble c-Jun from a complex background consisting of an *E. coli* cell lysate was evaluated as follows. The Affibody® ligands were immobilized on a plastic surface (in this case at the bottom of a well in an ELISA plate), by incubating 40 μl of 0.2 mg/ml Affibody® in PBS solution overnight at 4 °C. All subsequent steps were carried out at room temperature. The wells were washed with PBS and blocked with 5% dried skimmed milk in PBS for 1 h. A protein solution containing 0.1 mg/ml c-Jun and 3.9 mg/ml *E. coli* cell lysate in PBS was prepared and added to each well, followed by incubation for 1 h. The wells were washed with PBS, followed by the addition of an anti-c-Jun antibody (Santa Cruz), diluted to 5 μg/ml in PBS supplemented with 5% dried skimmed milk. After incubation for 1 h, the plates were washed again. This was followed by the addition of a polyclonal HRP-conjugated goat anti-rabbit antibody (DakoCytomation) at a concentration of 5 μg/ml in PBS supplemented with 5% dried skimmed milk. The wells were incubated for 1 h, followed by washing with PBS. Finally the wells were developed using SuperSignal West Dura Extended Duration Substrate. The reaction was stopped after 10 min with 50 μl of 2.0 M H_2SO_4 and the A_{450} was read.

Cell culture and fluorescence microscopy

The human malignant melanoma cell line C8161 [27] was grown at 37 °C in 5%-CO_2 environment in Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum

and an antibiotic/antimycotic solution (all from Invitrogen). $Z_{cJun518}$ and Z_{wt} were labelled with the amine-reactive dye Alexa 488 (Alexa Fluor® 488 carboxylic acid, succinimidyl ester *mixed isomers*; Invitrogen). Dye (1 mg) was dissolved in 50 μl of dimethylformamide and 5 μl was added to 500 μl of Affibody® solution at a concentration of 1 mg/ml. The mixture was incubated for 1 h under continuous agitation before excess dye was removed using a NAP-5 desalting column (GE healthcare).

Subconfluent cells were detached with a trypsin/0.53 mM EDTA solution (Invitrogen), washed once with PBS and resuspended in complete growth medium. Approx. 500 000 cells in 1 ml of growth medium were seeded on to polylysine-treated coverslips in a 24-well plate and allowed to attach for 4 h. The cells were washed twice with ice-cold PBS before fixation with 4% (w/v) paraformaldehyde in PBS, pH 7.2, for 15 min. The cells were permeabilized by incubation with PBS supplemented with 0.1% Triton X-100 for three periods of 5 min each. Alexa 488-conjugated $Z_{cjun518}$ and Z_{wt} were diluted to 5 μg/ml in PBS supplemented with 5% dried skimmed milk and were incubated with the cells overnight at 4°C. The cells were washed once in PBS before being counterstained with 50 μl of DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes) at a concentration of 300 nM for 5 min, followed by washing four times, for 10 min each time, with PBS. The coverslips were mounted in PBS containing 50% (v/v) glycerol. The green and blue fluorescence of the cells were recorded sequentially with a Zeiss LSM 510 Meta inverted confocal scanning laser microscope using a 63×-magnification 1.4-numerical-aperture objective. Green fluorescence was excited at 488 nm and detected with a Zeiss LP 505 emission filter. DAPI fluorescence was excited at 405 nm and read with a Zeiss LP 420 emission filter. Preparations using non-labelled Affibody® molecules were used as negative controls.

Results

Production and DNA binding analysis of c-Jun

c-Jun was produced as inclusion bodies in *E. coli* strain BL21(DE3), purified under denaturating conditions by cation-exchange chromatography and refolded using three sequential dialysis steps. The isolated protein was analysed by SDS/PAGE (Figure 1B, lane 1) and the major band observed had a molecular mass close to that of a c-Jun monomer (35 kDa). An additional band with a molecular mass of 70 kDa could also be identified in the gel. When analysing the isolated protein by Western blotting, using a polyclonal anti-c-Jun antibody, both the major and the minor band are stained (Figure 1B, lane 2). From this analysis we conclude that the major band corresponds to a c-Jun monomer. Since c-Jun spontaneously dimerizes in its native state, the minor band could correspond to a c-Jun homodimer, even though it had been incubated with an SDS-containing buffer followed by electrophoresis. This is supported by the observations (i) that it has the molecular mass of two monomers and (ii) that it is was recognized by the antibody. The binding of the purified protein to a consensus TRE site (5′-TGACTCA-3′) was analysed and compared with that to a control DNA sequence in the presence of irrelevant competitor DNA in 100-fold excess. The results (Figure 1A) show that c-Jun selectively binds to the target TRE site in a dose-dependent manner.

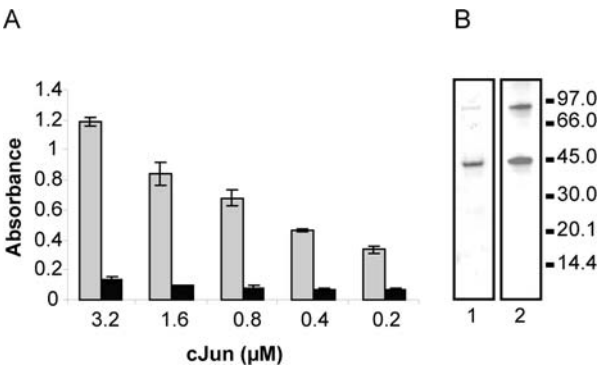

Figure 1 SDS/PAGE, Western-blotting and binding analysis of the c-Jun protein produced

(**B**) Lane 1, an SDS/PAGE gel, stained with Coomassie Brilliant Blue, where purified and refolded c-Jun was analysed; lane 2, Western blotting, using an anti-c-Jun specific antibody, of an SDS/PAGE gel where purified and refolded c-Jun had been separated. Values on the right indicate the molecular masses in kDa. (**A**) ELISA analysis showing c-Jun's specific interaction with a consensus AP-1 site (grey bars) and a control DNA sequence (black bars) at different concentrations of c-Jun. The *y*-axis corresponds to the absorbance measured at 450 nm and the *x*-axis shows the concentration of c-Jun. The experiments were performed in triplicate and the error bars correspond to 1 S.D.

Selection of Affibody® ligands

An *in vitro* phage-display selection was used to isolate Affibody® ligands binding to c-Jun from a combinatorial Affibody® library. The library is based on the 58-amino acid Z domain derived from staphylococcal Protein A and had been constructed by combinatorial substitution mutagenesis of 13 residues located at the surface responsible for IgG binding, as described previously [28]. The different Affibody® variants have been univalently displayed on protein III of M13 bacteriophage particles using a phagemid vector system [15,28]. Five rounds of phage-display selection were performed using a biotinylated recombinant c-Jun homodimer as target protein with an average of two biotin molecules incorporated per c-Jun monomer. During each round, the Affibody®-displaying phage particles were incubated with c-Jun, followed by capture on streptavidin-coated paramagnetic beads. The beads were washed, and

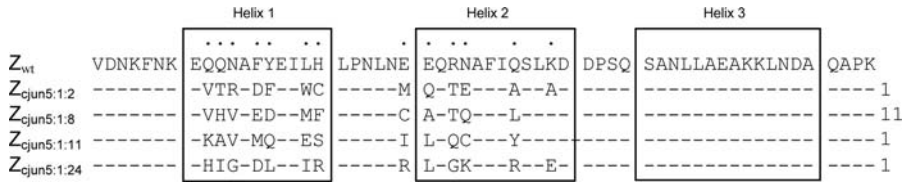

Figure 2 Amino acid sequence alignment of the wild-type Z domain and the selected Affibody® molecules

The three α-helices in the wild-type Z domain are boxed and the 13 randomized amino acid residues (positions 9, 10, 11, 13, 14, 17, 18, 24, 25, 27, 28, 32 and 35) are presented. Horizontal bars indicate amino acid identities. The values on the extreme right correspond to the number of times a particular clone was found.

captured phages were eluted by lowering the pH to 2.2. To increase the selection stringency, the concentration of target protein was lowered from 100 nM in cycles 1 and 2, to 20 nM in cycles 3–5. The washing stringency was also increased for each round of the selection. After the fifth round, the sequences of randomly picked colonies were determined. This gave 18 clones, of which 11 were identical (denoted '$Z_{cJun518}$') and the others were represented only once. $Z_{cJun518}$ was selected for further characterization, even though it contains a cysteine residue at position 24 which may cause unwanted covalent attachment to itself or to other molecules containing free SH groups). Three of the other seven Affibody® molecules were also selected for further characterization and were labelled '$Z_{cJun5124}$', '$Z_{cJun512}$' and '$Z_{cJun5111}$'. Their sequences are presented in Figure 2.

Production of Affibody® ligands

DNA fragments encoding the Affibody® ligands were subcloned and expressed with an N-terminal His_6 tag under control of the T7 promotor. During the subcloning procedure, site-directed mutagenesis was employed to generate a variant of $Z_{cJun518}$ where the cysteine at position 24 was replaced by serine, resulting in an Affibody® ligand denoted '$Z_{cJun518\_C24S}$'. Head-to-tail homodimeric gene fusions of $Z_{cJun518}$ and $Z_{cJun518\_C24S}$ were also constructed. The different Affibody®-based fusion proteins were expressed in *E. coli* and purified by immobilized metal affinity chromatography. Upon SDS/PAGE analysis under reducing conditions, the purified proteins were observed as single bands of expected molecular mass (7 kDa for the monomers and 14 kDa for the constructed dimers), indicating pure and stable Affibody® ligands (Figure 3A and results not shown). $Z_{cJun518}$ and $Z_{cJun518\_C24S}$ and their dimeric versions $(Z_{cJun518})_2$ and $(Z_{cJun518\_C24S})_2$ were also separated by SDS/PAGE under non-reducing conditions (Figure 3B). This analysis indicates that 70% of $Z_{cJun518}$ was present as a dimer, whereas 10% of $(Z_{cJun518})_2$ was present as a tetramer. By contrast, in the constructs with the C24S mutation, no multimers were observed. All Affibody® ligands demonstrated high expression levels, namely between 100 and 200 mg/litre of cell culture.

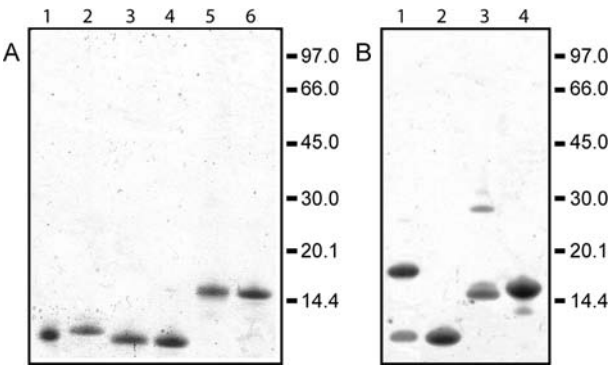

Figure 3 SDS/PAGE of purified Affibody® ligands

(**A**) SDS/PAGE analysis under reducing conditions of purified Affibody® ligands: Z_{wt} (lane 1) $Z_{cJun5124}$ (lane 2), $Z_{cJun518}$ (lane 3), $Z_{cJun518\_C24S}$ (lane 4), $(Z_{cJun518})_2$ (lane 5) and $(Z_{cJun518\_C24S})_2$ (lane 6). (**B**) SDS/PAGE analysis under non-reducing conditions of purified Affibody® ligands: $Z_{cJun518}$ (lane 1), $Z_{cJun518\_C24S}$ (lane 2), $(Z_{cJun518})_2$ (lane 3) and $(Z_{cJun518\_C24S})_2$ (lane 4). Marker molecular masses are shown in kDa on the right of each panel. Protein bands were visualized with Coomassie Brilliant Blue staining.

Biosensor binding analyses of the Affibody® ligands

Real-time biospecific interaction analysis was used to determine if the selected Affibody® variants demonstrated target-specific binding. The purified Affibody® ligands were injected at different concentrations over the flow cells on the sensor chip containing immobilized c-Jun and the control proteins human IgG and HSA, as well as a blank reference surface. The $Z_{cJun518}$ Affibody® ligand was shown to bind to c-Jun, but not to the control proteins (Figure 4A). By contrast, no binding to c-Jun was observed for the Affibody® ligands $Z_{cJun5124}$ (Figure 4B), $Z_{cJun518\_C24S}$, $Z_{cJun512}$ or $Z_{cJun5111}$ (results not shown). Also, for the control Affibody® ligand, Z_{wt}, no obvious binding to c-Jun could be observed (results not shown). The kinetics of $Z_{cJun518}$ binding to c-Jun were determined by injecting the Affibody® ligand at different concentrations (from 5.43 to 0.68 μM). Upon evaluation of the curves obtained (Figure 4C), different models describing the interaction were tested: one-to-one Langmuir binding, bivalent analyte and mass-transfer-limited interactions. The best fit was obtained using the simple Langmuir one-to-one model (χ^2 0.66), with which the dissociation equilibrium constant (K_D) was determined to be $\sim$5 μM (note that this

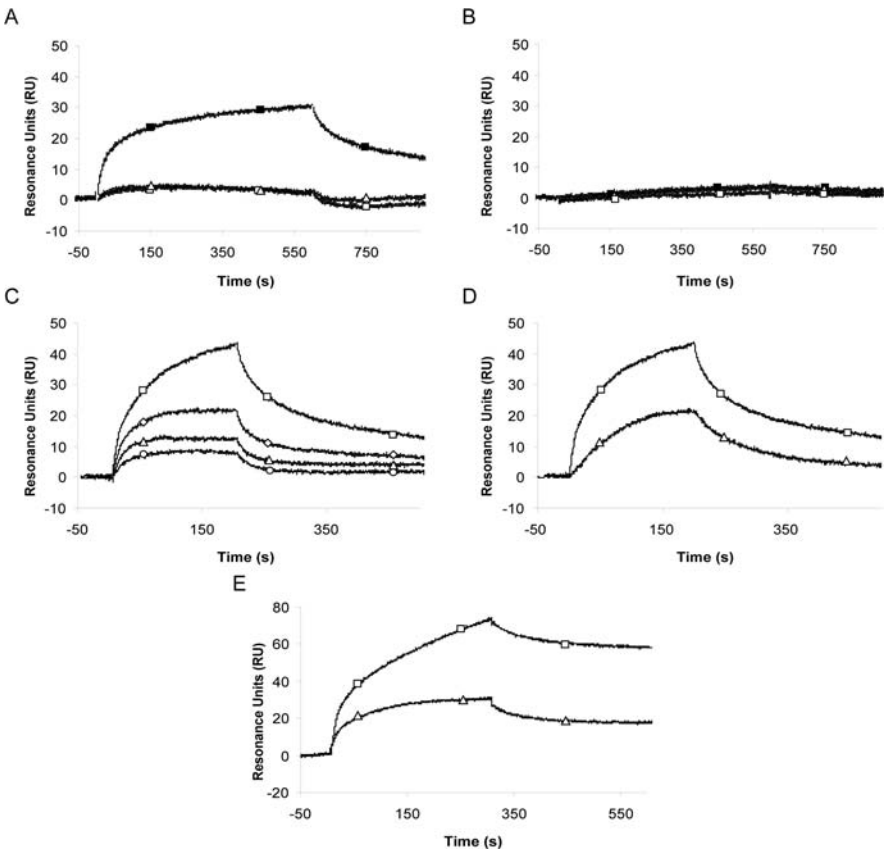

Figure 4 Biosensor binding analysis

(**A**) Sensorgram obtained after injection of 2.71 μM $Z_{cJun518}$ over surfaces containing immobilized c-Jun (■), HSA (Δ) and human IgG (□). (**B**) Sensorgram obtained after injection of $Z_{cJun5124}$ over the same surfaces as in (**A**). (**C**) Sensorgram obtained after injection of $Z_{cJun518}$ over a flow-cell surface containing c-Jun at selected concentrations: 5.43 μM (□), 2.71 μM (◊), 1.36 μM (Δ) and 0.68 μM (○). (**D**) Sensorgram obtained after injection of $Z_{cJun518}$ at a concentration of 5.43 μM in a non-reducing buffer (□) and in a reducing buffer (Δ) over the flow-cell surface containing immobilized c-Jun. (**E**) Sensorgram obtained after injection of 2.71 μM $Z_{cJun518}$ (Δ) and 2.71 μM $(Z_{cJun518})_2$ (□) over the surface with immobilized c-Jun.

is an apparent K_D reflecting the binding ability of $Z_{cJun518}$, which is partly dimerized). The binding kinetics were also measured under reducing conditions to estimate the effect of the dimerization of the ligand. In this experiment the recorded response from the flow cell with immobilized c-Jun became gradually weaker, probably because the chip surface could not withstand the reducing agent (1 mM DTT) during a long run. Therefore, a comparison between the injections of 5.43 μM $Z_{cJun518}$ under reducing and non-reducing conditions were performed (Figure 4D). Under reducing conditions, the association rate was lowered from $2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ to $0.5 \times 10^3 \cdot M^{-1}s^{-1}$ compared with non-reducing conditions. The dissociation rate was constant in both cases. From Figure 4(D) it can be concluded that $Z_{cJun518}$ in a monomeric state is able to interact with c-Jun, since a response *per se* is obtained under reducing conditions. Since a higher equilibrium response is obtained under non-reducing conditions, the dimeric version of $Z_{cJun518}$ is also able to interact with c-Jun. If the dimeric version of $Z_{cJun518}$ is unable to interact with c-Jun, a lower equilibrium response would be obtained under non-reducing conditions than under reducing conditions, since 70% of $Z_{cJun518}$ is present as a dimer and hence would not interact with c-Jun. The affinity of the constructed dimer $(Z_{cJun518})_2$ was also evaluated and compared with that of the monomer (Figure 4E). In this case the association rate was similar, whereas the dissociation rate was three times lower for the constructed dimer, resulting in an approx. 3-fold higher affinity.

Secondary-structure measurements of Affibody® ligands

In an attempt to gain insight into why the C24S mutation disrupts the interaction with c-Jun, the secondary-structure content and thermal stability of $Z_{cJun518}$ and $Z_{cJun518\_C24S}$ were determined by CD and compared. Both spectra in Figure 5(A) have a negative peak at θ_{220}, indicating a high level of α-helicity. Also, both spectra have a similar shape to

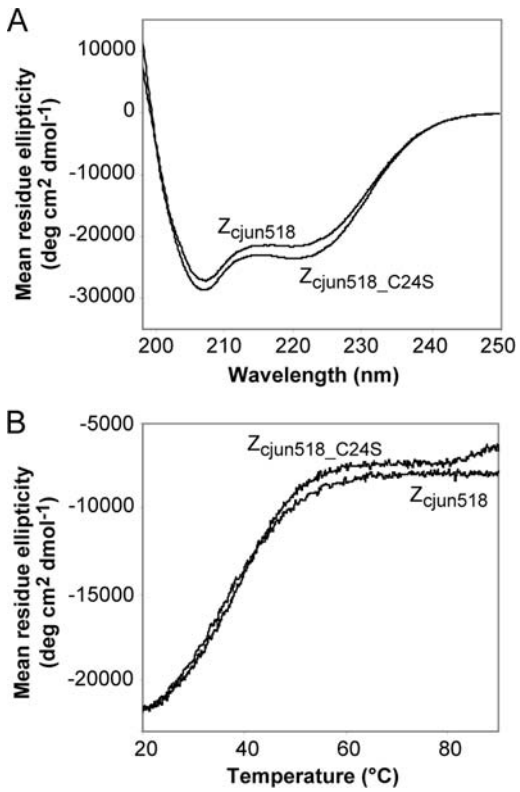

Figure 5 CD analysis of selected Affibody® ligands

(**A**) CD spectra of $Z_{cjun518}$ and $Z_{cjun518\_C24S}$ at concentrations of 43.5 and 39.2 μM respectively, recorded at 20 °C. (**B**) Thermal melting of $Z_{cjun518}$ and $Z_{cjun518\_C24S}$ at concentrations of 43.5 and 39.2 μM respectively, monitored by θ_{220}.

those previously published for other Affibody® molecules, as well as to that of Z_{wt} with an anti-parallel three-helical conformation [29,30]. Thermal melting profiles show that $Z_{cjun518}$ and $Z_{cjun518\_C24S}$ both have a melting temperature of 38 °C (Figure 5B). This is lower than the melting temperature (T_m) for Z_{wt} (75 °C) [31], but has sometimes been observed for other first-generation Affibody® molecules [30]. This result indicates that the C24S mutation does not distort the helices of the Affibody® ligand. Why the C24S mutant disrupts the c-Jun interaction cannot be explained using the present data.

Specificity analysis of $Z_{cJun518}$

The specificity in the interaction between $Z_{cJun518}$ and c-Jun was evaluated by Western blotting, where biotinylated $(Z_{cJun518})_2$ was employed as a primary detection probe of different amounts of recombinantly produced c-Jun mixed with whole-cell lysate from *E. coli* cells (Figure 6A). The bands appearing on the blot correspond in size to c-Jun in a monomeric (~35 kDa) and dimeric (~70 kDa) state with little background, indicating high specificity of the Affibody® ligand. The presence of both monomeric and dimeric c-Jun could also be observed in Western blots when staining with an anti-c-Jun antibody (Figure 1A). Since a conjugate of streptavidin and HRP is used in the detection procedure, any biotin-containing proteins could be visualized in the blot in Figure 6(A). Biotinylated carboxyl carrier protein, present in the *E. coli* cell lysate, was therefore also detected and appeared as a band at approx. 22 kDa of the same intensity in lanes 2–5 [32]. The same amounts of c-Jun mixed with whole-cell lysate were also separated on an SDS/PAGE gel run in parallel. All proteins on this gel were visualized by staining with Commassie Brilliant Blue (Figure 6B).

Capture of c-Jun by $Z_{cJun518}$

To analyse further the specificity of $Z_{cJun518}$, it was allowed to adsorb to a solid support and employed to capture recombinant soluble c-Jun mixed with a cell lysate from *E. coli*. The captured c-Jun was detected by an anti-c-Jun antibody followed by a secondary HRP-conjugated antibody. The results are displayed in Figure 6(C): a strong signal was observed when *E. coli* lysate mixed with c-Jun was incubated with $Z_{cJun518}$ (Figure 6C, I). By contrast, no signal is obtained

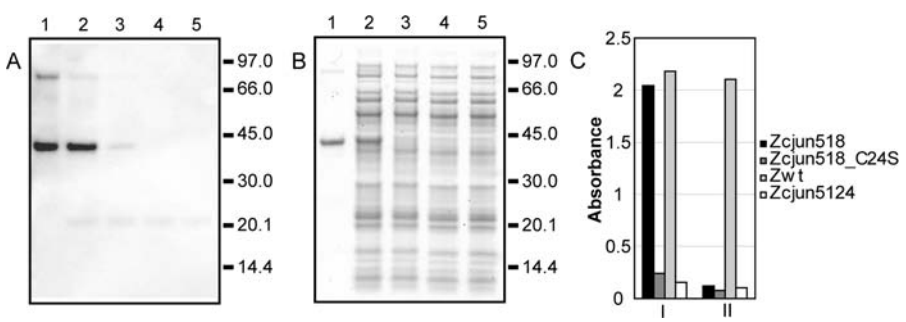

Figure 6 Western-blotting analysis and ELISA-based capture of c-Jun

(**A**) Western Blotting of an SDS/PAGE gel using biotinylated $(Z_{cJun518})_2$. Lane 1, 0.9 μg of purified c-Jun; lane 2, 0.9 μg of c-Jun mixed with 50 μg of *E. coli* lysate; lane 3, 90 ng of c-Jun mixed with 50 μg of *E. coli* lysate; lane 4, 9 ng of c-Jun mixed with 50 μg of *E. coli* lysate; lane 5, 50 μg of *E. coli* lysate. (**B**) SDS/PAGE gel stained with Coomassie Brilliant Blue. Lanes are the same as in (**A**). (**C**) Capturing of c-Jun from its mixture with an *E. coli* cell lysate. Affibody® ligands were adsorbed to an ELISA plate, followed by addition of the c-Jun/cell lysate mixture (I) or cell lysate (II).

after incubation of only *E. coli* lysate with $Z_{cJun518}$ (Figure 6C, II). This further demonstrates that $Z_{cJun518}$ does not interact non-specifically with any components in the complex mixture that an *E. coli* lysate comprises. Neither the mutant $Z_{cjun518\_C24S}$ nor the Affibody® ligand $Z_{cjun5124}$ was able to capture c-Jun (Figure 6C, I). This is in accordance with the biosensor results obtained for these Affibody® ligands (Figure 4B and results not shown). Z_{wt} was used as a positive control, and it binds directly to the anti-c-Jun antibody used for detection. Consequently, it gives a strong signal in both bar groups (I and II) in Figure 6(C).

Competition analyses

A competition study between $Z_{cJun518}$ and an anti-c-Jun antibody binding to the N-terminal transcriptional modulation domain was performed by biosensor analysis. The surface containing immobilized c-Jun was saturated with the antibody, followed by injection of $Z_{cJun518}$. The net response of the injection of $Z_{cJun518}$ was similar to that obtained when injecting $Z_{cJun518}$ without prior saturation of the surface with an antibody (results not shown). This indicates that $Z_{cjun518}$ does not bind to the same epitope as the antibody.

To determine whether $Z_{cjun518}$ could interfere with c-Jun's ability to bind the consensus TRE site (5′-TGACTCA-3′), c-Jun was pre-incubated with a 1000-fold molar excess of $Z_{cJun518}$ or the negative controls $Z_{cJun5124}$ or Z_{wt}, before it was allowed to bind to the DNA sequence in an ELISA set-up. The same binding of c-Jun to the TRE site was measured after preincubation with $Z_{cjun518}$ or with the negative controls as was measured without preincubation. This indicates that $Z_{cJun518}$ interacts with c-Jun at a site that is separate from the DNA-binding site.

Fluorescence-microscopic analyses

The human malignant melanoma cell line C8161 has previously been shown to have a high cellular level of c-Jun [27]. Fluorescently labeled $Z_{cJun518}$ was used as a primary probe to stain fixed and permeabilized C8161 cells, followed by visualization under a confocal fluorescence microscope. This resulted in strong intracellular staining dispersed throughout the cell (Figure 7A), whereas the negative control, Z_{wt}, showed no detectable staining of the cells (Figure 7B).

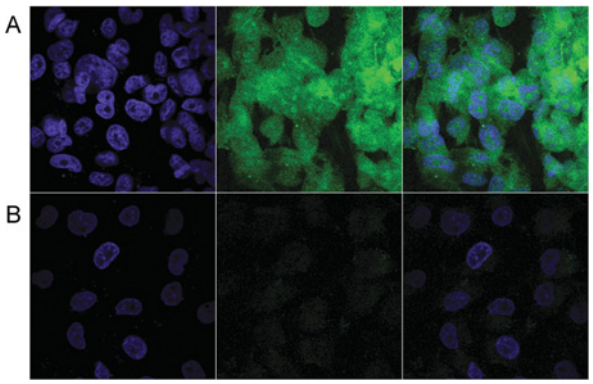

Figure 7 Confocal micrographs of C8161 human melanoma cells

The nuclear stain (DAPI) is shown in blue in the panels to the left, the Affibody® stain is shown in green in the central images, and an overlay of the two images is displayed to the right. (**A**) Staining of C8161 cells with Alexa 488-conjugated $Z_{cjun518}$. (**B**) Staining of c-Jun in C8161 cells with Alexa 488-conjugated Z_{wt} (negative control).

Discussion

c-Jun is a highly oncogenic transcription factor and its expression level and activity are often increased in transformed cells (see, e.g. [7,11]). To have good affinity reagents is therefore important for both diagnostic applications and to be able to study the complex biology of c-Jun and its involvement in cancer. The aim of the present study was to investigate the possibility of generating Affibody® ligands binding to c-Jun as a first step towards constructing such reagents. Prior to Affibody® selection, c-Jun was expressed as inclusion bodies in *E. coli*, followed by purification and refolding. Others have previously shown that c-Jun produced in this manner is capable of interacting with its natural partner, namely transcription factor IID [25]. To investigate if c-Jun produced in this manner was also capable of recognizing its DNA sequence, which would be a further indication of its correct folding, an ELISA experiment was performed where c-Jun was shown to specifically interact with its consensus DNA-binding sequence (5′-TGACTCA-3′). Detection in this experiment was done by a polyclonal antibody recognizing the N-terminal part of c-Jun, further suggesting that the refolded c-Jun was present in a biologically relevant form. This protein was used as a target antigen for selection of Affibody® ligands from a phage-displayed Affibody® library. After five rounds of biopanning, an Affibody® ligand could be isolated with a binding constant of approx. 5 μM. This ligand ($Z_{cJun518}$) contains a single cysteine residue situated in the loop between helix 1 and 2 and which results in partial dimerization, which in turn contributed to a faster on-rate and hence lowered the K_D value for this variant. It is possible that other variants in the library with a lower K_D value but not having the possibility of dimerizing, were outcompeted by $Z_{cJun518}$ and thereby lost during the selection procedure. One solution to minimize the advantage gained from being in a dimeric form could have been to incubate the Affibody®-expressing phages with a reducing agent prior to the biopanning.

Cysteine residues in a protein can form covalent attachment to other proteins and are therefore undesirable in some cases. Site-directed mutagenesis was therefore employed to replace the cysteine residue at position 24

with serine. This did not affect the secondary-structure content or thermal stability of the ligand determined by CD. However, no interaction between the mutant and c-Jun could be observed, indicating a loss of affinity into the millimolar range or higher. It cannot presently be explained why $Z_{cjun518\_C24S}$ does not bind to c-Jun, but the C24S mutation could involve, for example, a rearrangement of the Affibody® that cannot be detected by CD, such as a shift in the angle between the helices. In future studies the cysteine residue could be replaced by amino acids other than serine, either alone or in concert with spatially adjacent amino acids, which may result in functional binders. This can be achieved by affinity-maturation strategies [16,33], where part of the binder is re-randomized. Such strategies can potentially also produce ligands with higher affinity for c-Jun. A head-to-tail homodimeric version of $Z_{cJun518}$ was also constructed to investigate whether this strategy could be used to increase the affinity for c-Jun. This variant displayed a 3-fold higher affinity for c-Jun than the monomer.

Even though $Z_{cJun518}$ showed a rather moderate affinity for c-Jun of 5 μM, it could still recognize c-Jun in a complex background of an *E. coli*-derived cell lysate. It could recognize both a fully folded c-Jun in solution in the capture experiments as well as c-Jun that had been partially unfolded in a Western-blotting setting with high specificity. We were also able to show that the Affibody® ligand could be used to stain the c-Jun-overproducing melanoma cell line C8161.

Traditionally, polyclonal or monoclonal antibodies are used as affinity reagents in diagnostic and biological applications, and several reagents interacting with c-Jun have been described. One advantage with Affibody® molecules compared with antibodies is that they can be completely synthesized *in vitro* by standard peptide-synthesis techniques. This allows for direct incorporation of one or several functional groups in a site-specific manner [34]. Also, Affibody® ligands can be produced in the cytoplasm of mammalian cells, which was demonstrated when Affibody® molecules were included as a building block in adenovirus fibres [35]. In addition, they do not contain any disulphide bridges, an observation suggesting that they could fold in the reducing environment of the cell cytoplasm. This could allow for intracellular applications such as live-cell imaging using an Affibody® coupled to a fluorescent protein. The mapping experiments suggested that the Affibody® ligand generated interacts with c-Jun at a site differing from some of the most biologically important sites. This further suggests that $Z_{cJun518}$ could be adapted for live-cell imaging for study of c-Jun biology. Immunoglobulin-based affinity reagents are normally difficult to adapt for intracellular applications, partly because of a need for the formation of proper intramolecular disulphide bridges, which cannot form in the cytoplasm. In special cases, scFvs (single-chain variable fragments) of antibodies have been found that can fold properly without forming disulphide bridges [36]. Also, strategies where scFv fragments are selected inside mammalian or yeast cells, generating functional affinity reagents, have been described [37]. Still, two of the main sources of monoclonal affinity reagents are generated by hybridoma techniques or selection of scFvs displayed on phage. In both these formats, most of the isolated antibodies do not function inside cells.

Taken the findings together, an Affibody® ligand has been generated that shows specific interaction with the transcription factor c-Jun. The ligand was shown to interact specifically with c-Jun in capture and Western-blotting experiments. It could also be used to stain c-Jun-overexpressing cells in fluorescence-microscopic experiments.

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