Phage display selection of Affibody molecules with specific binding to the extracellular domain of the epidermal growth factor receptor

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Affibody molecules specific for the epidermal growth factor receptor (EGFR) have been selected by phage display technology from a combinatorial protein library based on the 58-residue, protein A-derived Z domain. EGFR is overexpressed in various malignancies and is frequently associated with poor patient prognosis, and the information provided by targeting this receptor could facilitate both patient diagnostics and treatment. Three selected Affibody variants were shown to selectively bind to the extracellular domain of EGFR (EGFR-ECD). Kinetic biosensor analysis revealed that three monomeric Affibody molecules bound with similar affinity, ranging from 130 to 185 nM. Head-to-tail dimers of the Affibody molecules were compared for their binding to recombinant EGFR-ECD in biosensor analysis and in human epithelial cancer A431 cells. Although the dimeric Affibody variants were found to bind in a range of 25–50 nM affinities in biosensor analysis, they were found to be low nanomolar binders in the cellular assays. Competition assays using radiolabeled Affibody dimers confirmed specific EGFR-binding and demonstrated that the three Affibody molecules competed for the same epitope. Immunofluorescence microscopy demonstrated that the selected Affibody dimers were initially binding to EGFR at the cell surface of A431, and confocal microscopy analysis showed that the Affibody dimers could thereafter be internalized. The potential use of the described Affibody molecules as targeting agents for radionuclide based imaging applications in various carcinomas is discussed.

Keywords: Affibody/EGFR/phage display/seletion/targeting

Introduction

Targeting molecular markers overexpressed in tumors will allow for tumor visualization. Agents targeting these markers, or their natural ligands, can be used for radionuclide based tumor detection, to guide therapy by demonstrating the presence of appropriate molecular targets, to assess response after therapy and to evaluate new therapeutic compounds (Britz-Cunningham et al., 2003).

Antibodies have been used as tumor targeting agents for the last two decades and presently several antibodies with good affinity and specificity to tumor receptors are in clinical use. However, antibodies are disadvantageous in radionuclide based diagnostic settings, since their large size will lead to a slow clearance and thereby increased background signal. Therefore, there is an increasing interest in smaller molecules, like antibody derivatives, e.g. Fab, single-chain Fv (scFvs), diabody, minibody and their fusions (Holliger et al., 2005), and there are a number of reports on the use of these in vivo imaging (Tahtis et al., 2001; Sundaresan et al., 2003; Olafsen et al., 2005; Adams et al., 2006). Further size reductions of these affinity-based proteins could potentially improve extravasation, increase tissue penetration and speed up blood clearance (Michel et al., 1999), thereby increasing imaging contrast. Peptides have also been considered as targeting agents, but it has been proven difficult to generate peptides with high affinity for their targets (Landon et al., 2003). Alternative binding molecules, so-called single-scaffold proteins, including designed ankyrin repeat proteins (Binz et al., 2004), Src homology domains 2 and 3 (Malabarba et al., 2001), PDZ domains (Schneider et al., 1999), lipocalins (Beste et al., 1999), fibronectins (Koide et al., 1998) and protein A-derived Affibody molecules (Nord et al., 1997) have rather recently entered this field. These have relatively small size and several have been selected with good affinity to various targets.

Affibody molecules (Nord et al., 1995) are based on the 58 amino acid residue protein domain derived from the IgG-binding domains of staphylococcal protein A. This three-helix bundle domain has been used as scaffold for construction of combinatorial phagemid libraries, by allowing randomization of 13 surface residues, from which Affibody variants binding desired target proteins can be selected using phage display technology (Nord et al., 1997; Rönnmark et al., 2002). The simple, robust structure of Affibody molecules in combination with their low molecular weight (~6 kDa), make them suitable for a wide variety of applications, like their use as detection reagents (Andersson et al., 2003; Renberg et al., 2005), and to inhibit receptor interaction (Sandström et al., 2003). Recently, the first example of the selection of an Affibody binding to a cancer marker, human epidermal growth factor receptor type 2 (HER2), was described (Wikman et al., 2004; Steffen et al., 2005) and a second generation variant of this Affibody exhibited promising results in the visualization of xenografted HER2 expressing tumors in mice (Orlova et al., 2006).

Overexpression of EGFR is frequently associated with various malignancies and poor patient prognosis. When overexpressed, these receptors can dimerize, to thereby activate the intracellular tyrosine kinase domain, leading to changes...
in proliferation, migration, apoptosis, differentiation or other cellular processes (Wells, 1999; Yarden et al., 2001; Vermeer et al., 2003). Overexpression of EGFR is common in glioblastoma multi-forme (GBM) (Libermann et al., 1985; Kleihues et al., 1999, 2000), head and neck squamous cell carcinomas (HNSCC) (Santini et al., 1991; Rikimaru et al., 1992), urinary bladder carcinoma (Neal et al., 1990; Harney et al., 1991; Sauter et al., 1994; Gardmark et al., 2005) and several other malignancies of epithelial origin such as lung and breast carcinomas (Salomon et al., 1995).

Since EGFR is overexpressed in many cancer types, it is an interesting target and two mAbs targeting EGFR have been extensively studied in patients: Cetuximab (Erbitux) and Panitumumab (Vectibix) which was approved in 2004 (Calvo and Rowinsky, 2005; Horak et al., 1990), a DNA fragment encoding a hexahistidine (His6) tag and a multiple cloning site, together with a gene conferring resistance to kanamycin. Plasmid pAY430 contains in addition a unique cysteine in the C-terminus for direct labeling. The E. coli strain BL21(DE3) (Novagen, Madison, WI, USA) was used for protein production from the expression vectors.

Phage selection procedures
A ~100 kDa recombinant EGFR-ECD comprising 623 amino acids, corresponding to nucleotides 259-2127, was used as target protein during selections (Horak et al., 2005). The EGFR-ECD was biotinylated in vitro using EZ-Link™-Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA). A 20-fold molar excess of biotin was added to EGFR-ECD in phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, pH 7.2), and the mixture was incubated at room temperature (RT) for 1 h followed by extensive dialysis against PBS overnight (ON) at 4°C to remove the surplus of biotin.

Preparation of phage stocks from the library (a portion of Zlib2002) and between selections was performed according to previously described procedures (Nord et al., 1997; Hansson et al., 1999) using the helper phage M13K07 (New England Biolabs, Beverly, MA, USA). PEG/NaCl precipitation yielded phage titers of about 10^{13} phage-forming units (pfu) per millilitre. The selection was performed in solution and the bound phages were captured on streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway). To avoid unpecific binders all tubes were pre-treated with PBST (0.1% Tween-20 in PBS) supplemented with 0.1% gelatin (PBST-gelatin). To further avoid binders against the streptavidin present on the streptavidin-coated paramagnetic beads, ~1 ml of the phage stock in PBST-gelatin was pre-incubated (30 min, end-over-end rotation) with 0.2 mg of the beads for the first two rounds of selection.

Four rounds of biopanning, with four slightly different protocols for selection, starting at target concentrations 100 and 10 nM were performed as follows. In round 1, an aliquot of the Zlib2002 library containing ~10^{12} pfu was incubated in 1.2 ml of 100 and 10 nM, respectively, of biotinylated EGFR-ECD in PBST-gelatin for 1 h and 45 min at RT. For round 2, 100, 50, 10 and 7 nM, respectively, and for rounds 3 and 4, 100, 20, 10 and 2 nM, respectively, of biotinylated EGFR-ECD in 0.5 ml PBST-gelatin was incubated (1 h and 45 min, RT) with a portion of the phage stock from previous round. The bound phages were captured by incubation with streptavidin-coated M-280 Dynabeads for 15 min (RT, continuous end-over-end rotation). The amount of beads was added allowing an immobilization of ~2 μg of the target protein per milligram of beads, as previously determined by SDS-PAGE analysis (data not shown). To further increase the selection stringency, increasing washing steps were performed: the beads were washed twice in round 1, five times in round 2, seven times in round 3 and 10 times in round 4, with PBST-gelatin (three first washes) and PBST (subsequent washes). The phages were eluted with 500 μl of 50 mM glycine–HCl (pH 2.1) for 10 min, followed by immediate neutralization by adding 50 μl of 1 M Tris–HCl, pH 8.0 and 450 μl PBS. The eluted phages were used to infect log phase RRIΔM15 cells for 30 min at 37°C. The infected cell suspensions were spread on TYE agar plates (15 g/l agar, 3 g/l NaCl, 10 g/l tryptone and 5 g/l yeast extract), supplemented...
with 2% glucose and 100 mg/l ampicillin, and followed by ON incubation at 37°C. The grown colonies were collected by resuspension in tryptic soy broth (TSB, 30 g/l; Merck, Darmstadt, Germany), supplemented with 5 g/l yeast extract, 2% glucose and 100 mg/l ampicillin, and a fraction (~500 times excess of cells compared with the phage titer after elution) was used for inoculation, leading to the next generation of phage stock. Phagemid particles were rescued from infected cells using helper phage M13K07, purified and concentrated with PEG precipitation. The selection process was monitored by titrating the phage stocks before each selection and after elution. A serial dilution of phages was allowed to infect log phase RRI ΔM15 cells for 5 min at RT, followed by plating on TYE agar plates, supplemented with 2% glucose and 100 mg/l ampicillin and ON incubation at 37°C.

ELISA

After four rounds of biopanning, an ELISA was performed on 372 randomly picked colonies, from all four-selection strategies, to exclude phagemid (pAffi1) inserts with background binding capacity to streptavidin-coated paramagnetic beads. Cell lysates from the randomly picked colonies were incubated in pre-blocked (PBST supplemented with 2% dry milk) 96 well streptavidin coated plate (Nunc, Roskilde, Denmark) for 1.5 h at RT. A primary rabbit IgG anti-Affibody antibody (1.5 h, RT, continuous shaking) and a secondary rabbit immunoglobulin-HRP antibody (PO448 DAKO Cytomation, Glostrup, Denmark; 1 h in RT, continuous shaking) were used. The A405 nm was measured with a Tecan Sunrise spectrophotometer after addition of the substrate solution (Immunopure TMB; Pierce Biotechnology, Rockford, IL, USA).

DNA sequencing and sequence clustering

DNA sequencing of phagemid (pAffi1) inserts was performed on non-streptavidin binding clones from the fourth round of panning. 64 clones each from selection strategy 1 and 2, and 32 each from selection 3 and 4. Specific primers and Big Dye terminator (Amersham Biosciences, Uppsala, Sweden) were used and the Sanger fragments analyzed on a DNA sequencer ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Subcloned DNA fragments were verified by the same procedure. The sequences of the Affibody molecules were clustered using the so-called average-link hierarchical clustering method described in more detail by Orlova et al. (2006).

DNA constructions

DNA fragments encoding different variants of EGFR-binding Affibody molecules (ZEGFR) were subcloned into the expression vectors pAY442 and pAY430. The fragments were amplified from the pAffi1 vector with specific primers introducing an AccI overhang both 3' and 5', and ligated into the pAY442 and pAY430 vectors, previously restricted with the same enzyme and dephosphorylated using calf intestine alkaline phosphatase (CIAP; Fermentas, Ontario, Canada). The amplified DNA fragments were purified with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and hybridized prior to ligation with T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA). The ligation results in expression vectors encoding, under the control of the T7 promoter, the different Affibody molecules fused to an N-terminal His6 tag, allowing purification by immobilized metal ion affinity chromatography (IMAC). Affibody constructs expressed from the pAY430 vector had a unique cysteine introduced at their C-termini for direct labeling. Dimer constructs of the EGFR-binding Affibody molecules from both vectors were constructed, where a second Affibody gene fragment was introduced head-to-tail, giving rise to His6-(ZEGFR)2 and His6-(ZEGFR)2-cys Affibody molecules. All plasmid preparations were, after cultivation of transformed E. coli cells ON, performed using QIAprep Spin Miniprep Kit (Qiagen GmbH) according to the manufacturer’s instructions.

Protein expression and purification

Selected Affibody variants were expressed as His6-tagged fusion proteins from the pAY442 and pAY430 plasmids in E. coli strain BL21(DE3). Cells were inoculated in 10 ml of TSB medium (30 g/l TSB) containing 50 mg/l kanamycin and grown ON at 37°C at ~120 rpm in shake flasks. Fresh TSB (500 ml) supplemented with 5 g/l yeast and 50 mg/l kanamycin was inoculated with 5 ml of ON culture and grown ~2.5 h at 37°C in shake flasks, when gene expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG; Apollo Scientific Ltd, Bradbury, UK) to a final concentration of 1 mM. After 3–4 h cultivation, the cells were harvested by centrifugation (2200g, 8 min, 4°C). The cell pellets were resuspended in denaturing buffer (6 M guanidinium chloride, 47 mM Na2HPO4, 2.65 mM NaH2PO4, 10 mM Tris–HCl, 104 mM NaCl, pH 8.0) and disrupted by sonication. After centrifugation (35 000g, 20 min), soluble denatured protein from the supernatant was denatured in denaturing buffer, filtered (0.45 μm; Pall Life Sciences, Cornwall, UK), and applied to a TALON™ metal-affinity resin column (BD Biosciences Clontech, Palo Alto, CA, USA). The bound protein was eluted with urea buffer (8 M urea, 104 mM NaCl, 30 mM HAc, 70 mM NaAc, 50 mM Na2HPO4, pH 5.0). The monomeric motifs are hereafter referred to as ZEGFR:no (pAY442 vector) and ZEGFR:no-cys (pAY430 vector) and the dimeric motifs referred to as (ZEGFR:no)2 (pAY442 vector) and (ZEGFR:no-cys)2 (pAY430 vector). Protein concentrations were calculated from absorbance measurements at 280 nm, using the appropriate extinction coefficient for each protein. Protein concentrations for selected ZEGFR variants were also determined by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden). The purified proteins were further analyzed by SDS–PAGE on Phastgel™ Homogenous 20% gels using a Phast system (Amersham Biosciences, Uppsala, Sweden).

Biosensor analyses

A Biacore® 2000 instrument (Biacore AB, Uppsala, Sweden) was used for real-time biospecific interaction (BIA) between selected Affibody molecules and the target protein. EGFR-ECD (diluted in 10 mM NaAc, pH 4.5) was immobilized (~2600 RU) on the carboxylated dextran layer of one flow-cell surface of a CM5 sensor chip (Biacore) by amine coupling, according to the manufacturer’s instructions. Another flow-cell surface was activated and deactivated to be used as a reference surface and HER2-ECD (Horak et al., 2005) and human IgG (Amersham Biosciences, Uppsala, Sweden) were immobilized on separate flow-cell surfaces on the CM5 sensor chip, to serve as negative controls.
All Affibody samples were diluted in the running buffer HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) and filtrated (0.45 μm; Millipore, Billerica, MA, USA) before binding analysis was performed at 25 °C. In a first experiment, ~1 μM of each Affibody (diluted in HBS) was injected over all surfaces with a flow rate of 20 μl/min. An anti-HER2 Affibody (previously described by Wikman et al., 2004; Orlova et al., 2006) as negative control, and the natural ligand hEFG (Chemicon International, Temecula, CA, USA) as a monoclonal antibody in clinical use, Cetuximab (MERCK, Darmstadt, Germany) as positive controls, were also injected. After each injection, the flow cells were regenerated by the injection of 10 μl of 10 mM HCl.

In a second experiment, the monomeric ZEGFR and dimeric (ZEGFR)2 Affibody variants were subjected to kinetic analysis, in which the proteins were injected over an EGFR-ECM surface at concentrations ranging from 6.25 nM to 6.4 μM (monomer) and from 6.25 nM to 3.2 μM (dimer) with a flow rate of 30 μl/min. The dissociation equilibrium constant (KD), the association rate constant (ka) and the dissociation rate constant (kd) were calculated using BLAevaluation 3.2 software (Biacore), assuming a one-to-one binding. The samples were run in duplicates in random order, and after each injection the flow cells were regenerated by the injection of 10 μl of 10 mM HCl.

Cell culture

The EGFR-rich squamous carcinoma cell line A431 was obtained from European Collection of Cell Cultures (ECACC; Wiltshire, UK; flow cytometry and confocal microscopy studies) and from American Type Culture Collection (ATCC; Rockville, MD, USA; immunofluorescence microscopy studies and studies on radiolabeled Affibody molecules). The human neuroblastoma SH-SY5Y cell line, used as negative control, was obtained from ATCC. The A431 cells were cultured in MEM-Earles medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids and 1% antibiotic-antimycotic (called complete medium; Gibco, Invitrogen, Paisley, Scotland, UK), or Ham’s F-10 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids and 1% antibiotic-antimycotic (called complete medium; Biochrom Kg, Berlin, Germany). The cells were grown at 37 °C in an incubator with humidified air equilibrated with 5% CO2.

Fluorophore labeling

The (ZEGFR:942)-cys, (ZEGFR:948)-cys and (ZEGFR:955)-cys Affibody molecules expressed from pAY430 vector were labeled directly to the unique cysteine (at C-terminus) with Oregon Green 488-maleimide (Amersham Biosciences, Uppsala, Sweden) equilibrated with PBS. The iodine was coupled to the (ZEGFR:942) Affibody for ~30 min on ice. After centrifugation and wash with PBS+1% BSA, the cell pellet was resuspended in 300 μl PBS+1% BSA and subjected to flow cytometric analysis. A Oregon Green 488 labeled negative control Affibody, (Zbeta:1170)-cys, was analyzed the same way.

Radiolabeling

The Affibody molecules were indirectly labeled to 125I (Amersham Biosciences, Uppsala, Sweden) via N-succinimidyl groups as previously described (Steffen et al., 2005). Acetic acid (2 μl, 0.1% acetic acid in milli-Q, MERCK Darmstadt, Germany) and N-succinimidyl-4-[tri-methylstannyl] benzoate (5 μl, 5% acetic acid in methanol) was added to the 125I (15 MBq). The iodine was coupled to the N-succinimidyl-4-[tri-methylstannyl] benzoate by adding chloramine-T (10 μl, 4 mg/ml in water; Sigma, St. Louis, MO, USA), The solution was then resuspended for 30 s and further incubated in RT for 5 min. To stop the reaction 15 μl Sodium metabisulphite (15 μl, 4 mg/ml in water; Aldrich, Steinheim, Germany) was added. The Affibody molecules were diluted in borate-buffer (0.07 M) and added to the iodine solution, and additional borate-buffer was added to a total volume of 150 μl and the solution was then incubated for 30 min. To separate labeled Affibody molecules from low molecular weight compounds, a NAP-5 column (Sephadex G-25; Amersham Biosciences, Uppsala, Sweden) equilibrated with PBS was used.

Specificity test

A431 cells were cultured in 24-well plates (Nunc Nunclon; Nalgene Nunc International, Roskilde, Denmark). The cells were kept on ice and washed once with cold serum free Ham’s F-10 medium. The three Affibody molecules were labeled with 125I and added to the cells with a molar excess of ~5:1 in relation to the number of available receptors (Fabricant et al., 1977). They were incubated for 4 h, with gentle shaking, on ice in an environment where air from an incubator was trapped within a plastic bag together with the cell plate. In some wells, unlabeled Affibody molecules were added together with 125I labeled Affibody molecules at a...
molar excess of ~500:1, to determine the unspecific binding to and study whether the three Affibody molecules had cross-reacting epitopes. The cells were then washed six times with serum free Ham’s F-10 medium and detached by adding 0.5 ml Trypsin-EDTA (0.25% Trypsin/0.02% EDTA, Biochrom Kg) and incubated at 37°C for 30 min or until the cells were detached. One milliliter of Ham’s F-10 complete medium was added, and the cells were resuspended. In some wells, 0.5 ml suspension was used to count the cells in a cell counter (Beckman Coulter Z2, Fullerton, CA, USA). The radioactivity was measured with a gamma counter (1480 Wizard, Wallac Oy, Turku, Finland).

Affinity measurements on cells

The affinity of dimeric EGFR-binding Affibody molecules to native EGFR on A431 cells was studied. To determine the affinity constant, a saturation curve analysis was performed using radiolabeled Affibody molecules. The EGFR-rich cell line A431 was cultured in 24-well plates. Cells were kept on ice and washed once in cold serum free Ham’s F-10 medium. A dilution series of the 125I radiolabeled Affibody molecules was prepared and added to the cells. The cells were incubated for 4 h, with slow movement, on ice in a sealed bag maintaining the CO2 level from the incubator. In order to determine specific binding control groups were blocked with unlabeled Affibody molecules at a molar excess of ~300:1. The cells were then washed six times in cold Ham’s F10 serum free medium and detached by adding 0.5 ml Trypsin-EDTA (0.25%/0.02%) and incubated at 37°C for 30 min or until the cells were detached. One milliliter of Ham’s F-10 complete medium was added and the cells were resuspended. In some wells, 0.5 ml suspension was used to count the cells. The radioactivity (1.5 ml or 1 ml, respectively, for the cells that were counted) was measured with a gamma counter. After reaching saturation, the data were analyzed by non-linear regression using GraphPad Prism 4 (GraphPad Software San Diego, CA, USA) and affinity constants calculated.

Immunofluorescence microscopy

Immunofluorescence was studied for dimeric (ZEGFR:955)2 binding to A431 cells in two separate experiments. In a first study, cell-binding was assayed with light microscopy. Subconfluent cells were detached with a Trypsin/0.53 mM EDTA solution (Invitrogen, Carlsbad, CA, USA), washed once with PBS and resuspended in complete growth medium. Approximately 10 000 cells in 20 μl of medium were added per well to an eight well, multi-well slide (Histolab, Göteborg, Sweden), and the slides were incubated ON at 37°C in 5% CO2. On the following morning, the cells were fixed on to the multi-well slides with 3% formaldehyde in PBS for 15 min, washed once with PBS and then stained for EGFR expression using the (ZEGFR:955)2-cys Affibody molecule (2 μg/ml, 20 μl/well). The slides were incubated with the Affibody molecule for 45 min and thereafter washed twice in PBS. A mixture of goat anti-Affibody molecule IgG (5 μg/ml) and Alexa488-labeled chicken anti-goat antibody (5 μg/ml, Molecular Probes, Invitrogen) were used as second step reagents with an incubation time of 30 min. The slides were washed repeatedly in PBS, counterstained with 20 μl DAPI (Molecular Probes, Invitrogen) at a concentration of 1 μg/ml for 10–20 s, washed again and dried for 1 h. A negative control Affibody, (Zabeta:1170)2, was analyzed the same way. The (ZEGFR:955)2-cys Affibody molecule and the negative control Affibody were also analyzed on SH-SY5Y cells as described above. The slides were mounted with anti-fading reagent (Vector Laboratories Inc, CA, USA), and membrane fluorescence was analyzed using a DM-LA microscope, equipped with a Leica DC camera (Leica Microsystems, Darmstadt, Germany). Images of EGFR staining on A431 and SH-SY5Y cells were acquired and saved as overlays using the IM1000 software (Leica Microsystems).

A second immunofluorescence experiment was performed studying cell binding and internalization using confocal microscopy. Approximately 300 000 A431 cells were seeded per 35 mm Petri dish (Greiner Bio-One GmbH, Frickenhausen, Germany) the day before the experiment. The Oregon Green 488 labeled (ZEGFR:942)2, (ZEGFR:948)2 and (ZEGFR:953)2 Affibody variants were diluted to ~10 μg/ml in serum-free MEM-Earles medium, added to separate Petri dishes and incubated in the dark for 1 h on ice. The three Affibody variants were also diluted as above in complete MEM-Earles medium, added to separate Petri dishes and incubated in the dark for 2 h at 37°C. Following the incubation, the cells were washed once with serum-free medium, and ~1 ml medium was added for live cell imaging in the confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany). Consecutive scans were performed to cover the thickness of the cell, and a scan representing the center of the cell was chosen. A negative control Affibody, (Zabeta:1170)2, similarly labeled was analyzed the same way.

Results

Phage selection and post selection analysis of EGFR-binding Affibody molecules

Phage display in vitro selection technology was used to isolate novel Affibody molecules, binding to the EGFR-ECD, using an Affibody phagemid library in the biopanning procedure. The library is based on the 58 amino acid residue protein A-derived Z domain (Nilsson et al., 1987) and has been constructed by combinatorial substitution mutagenesis of 13 positions located at the surface originally responsible for Fc binding, as described earlier (Nord et al., 1995, 1997).

A total of 372 randomly picked colonies collected from the fourth round of selection (according to the four selection strategies) were subjected to an ELISA to exclude Affibody clones that bound the solid phase matrix, being streptavidin-coated paramagnetic beads (data not shown). Positive clones were discarded and 192 colonies from the fourth selection cycle were sequenced. Analysis of sequence clustering led to the identification of 15 unique sequences. After exclusion of sequences with amber stop codons and cysteine residues that could cause unwanted disulfide-bridged dimers, nine Affibody molecules remained for further studies (Fig. 1). Among these nine sequences, one clone was represented 11 times, ZEGFR:942, indicating convergence towards this clone in the selection (Fig. 1). An alignment of the 13 randomized positions of the nine unique variants is presented in Fig. 1. It was somewhat surprising, and unlike most previous Affibody selections (Nord et al., 1997, 2001; Gunneriusson et al.,
M.Friedman et al.

Fig. 1. Nine unique sequences from the selection against EGFR-ECD, aligned to the amino acid sequence of the wild-type Z domain to demonstrate amino acid sequence differences of the Affibody variants. The 13 randomized amino acid residues (Q9, Q10, N11, F13, Y14, L17, H18, E24, E25, R27, N28, Q32 and K35) are presented. Horizontal bars indicate amino acid identities with the wild-type Z domain. The three α-helices in the wild-type Z domain are boxed. Figures to the right represent the number of times each variant revealed upon DNA sequencing of 192 colonies.

Table 1. Amino acid sequences of the nine unique Affibody variants selected against EGFR-ECD (Fig. 1).

<table>
<thead>
<tr>
<th>Affibody Variant</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>Zwt</td>
<td>VDNKFNQEQNAPFYEILH</td>
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<tr>
<td>ZEGFR:940</td>
<td>-WSA-AS-SSG</td>
</tr>
<tr>
<td>ZEGFR:942</td>
<td>-MLI-ME-GS</td>
</tr>
<tr>
<td>ZEGFR:947</td>
<td>-TGA-MR-N</td>
</tr>
<tr>
<td>ZEGFR:948</td>
<td>-FYA-IT-TR</td>
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<tr>
<td>ZEGFR:949</td>
<td>-HAK-MW-GN</td>
</tr>
<tr>
<td>ZEGFR:951</td>
<td>-SLA-SV-SH</td>
</tr>
<tr>
<td>ZEGFR:955</td>
<td>-LEK-YN-RN</td>
</tr>
<tr>
<td>ZEGFR:956</td>
<td>-AAP-WT-VR</td>
</tr>
<tr>
<td>ZEGFR:957</td>
<td>-LIW-TS-VE</td>
</tr>
</tbody>
</table>

Helix 1 | Helix 2 | Helix 3
---|---|---
| VDNKFNQEQNAPFYEILH | ..... | LAF--V--V |
| -WSA-AS-SSG | -----K | G--EQ--L--W |
| -MLI-ME-GS | -----W | L--FF--V--V |
| -TGA-MR-N | -----N | W--MV--S--S |
| -FYA-IT-TR | -----G | L--VA--F--R |
| -HAK-MW-GN | -----L | S--CK--R--M |
| -SLA-SV-SH | -----G | W--MT--A--V |
| -LEK-YN-RN | -----W | G--KQ--V--H |
| -AAP-WT-VR | -----R | H--GV--R--L |
| -LIW-TS-VE | -----M | QAPK |

The nine purified candidate Affibody molecules were analyzed for EGFR-ECD binding with real-time BIA analysis using the Biacore biosensor instrument. They were injected over separate flow-cell surfaces containing the amine-coupled target protein EGFR-ECD and the control proteins HER2-ECD and IgG. Functionality of the immobilized target proteins was established by injection of the natural ligand hEGF and the clinical anti-EGFR mAb, Cetuximab. Out of the nine candidates, three Affibody molecules, ZEGFR:942, ZEGFR:948, and ZEGFR:955, demonstrated a significant binding capacity to immobilized EGFR-ECD (Fig. 2A), whereas the six other Affibody molecules showed only moderate or no binding (data not shown). As expected, no significant binding of the candidate Affibody molecules could be demonstrated to control proteins, IgG (data not shown) or HER2-ECD (Fig. 2B), although there exists a 44% sequence homology between EGFR-ECD and HER2-ECD (Earp et al., 1995). Some data suggest that ZEGFR:942, ZEGFR:948 and ZEGFR:955 bind selectively to the target protein EGFR-ECD.

Affinity determination and kinetic analyses of monomeric and dimeric Affibody constructs

In an effort to improve the affinity for subsequent studies, head-to-tail dimeric Affibody constructs were generated to increase the avidity as has been demonstrated before (Steffen et al., 2005). Head-to-tail dimeric constructs of ZEGFR:942, ZEGFR:948, and ZEGFR:955 Affibody molecules were subcloned, expressed and purified in accordance with the monomeric constructs and were denoted (ZEGFR:942)2, (ZEGFR:948)2, and (ZEGFR:955)2. The monomeric and dimeric proteins were analyzed by real-time BIA analysis using a Biacore biosensor instrument and subjected to kinetic analysis in order to determine the kinetic binding constants. The Affibody molecules were injected over an EGFR-ECD flow-cell surface at concentrations (determined by amino acid analysis) ranging from 6.25 nM to 6.4 μM for monomer and 3.2 μM for dimer constructs. Using a one-to-one model (BIAevaluation 3.2 software) the affinity for the monomeric and apparent affinity for the dimeric constructs was estimated. The association rate constants (kA) and dissociation rate constants (kD) were used to calculate their individual dissociation equilibrium constants (KD) (Table 1). As can be seen in Table 1, all three Affibody molecules showed affinity in the same order of magnitude and dimerization resulted in increased avidity contributions (ranging from 2.5- to 10-fold improvement). Although the affinities were similar, a rather clear difference could be observed in the dissociation rates (Table 1, Fig. 2C). Since ZEGFR:955 was found to have the slowest dissociation rate, it might be suggested that this Affibody could be expected to perform well in cellular binding assays.

The affinity of the dimeric Affibody molecules, (ZEGFR:955)2, (ZEGFR:948)2 and (ZEGFR:942)2, to native EGFR on cultured A431 cells was further studied. A saturation curve analysis was performed using a dilution series of 125I-labeled Affibody molecules on A431 cells (Fig. 3). After reaching saturation, the samples were measured in a gamma counter, the data were analyzed by non-linear regression using GraphPad Prism 4 (GraphPad Software San Diego, California, USA) and the affinity constants were calculated. The affinity constants (KD) were determined to be in the low nanomolar range for all three Affibody molecules (Fig. 3). It could be speculated that the higher affinity demonstrated on intact cells might be due to that the Affibody molecules recognize native EGFR better than EGFR-ECD immobilized by means of amine coupling on biosensor flow-cell surface.

Binding of fluorescently and radiolabeled Affibody molecules to native EGFR on cells

The (ZEGFR:942)2, (ZEGFR:948)2 and (ZEGFR:955)2 Affibody molecules were subjected to flow cytometric analyses in order to confirm their binding to native EGFR present on the...
cell surface. Prior to the flow cytometric analysis, the Affibody molecules were fluorescently labeled site-specifically with Oregon Green® 488 maleimide utilizing the unique C-terminal cysteine residue and then incubated with human epithelial cancer cell line A431 cells. All three Affibody molecules demonstrated binding to native EGFR on A431 cells (peak 1 in Fig. 4). No non-specific binding was observed, neither to human neuroblastoma SH-SY5Y cells (data not shown) nor for a negative control Affibody molecule to A431 cells (peak 1 in Fig. 4). This indicates that the anti-EGFR Affibody molecules bind specifically to EGFR. Since a significant effort was made to use equimolar amounts in the analysis, the FACS could be used to estimate how the different Affibody molecules would rank in their binding performance to native EGFR. With this experimental set-up, the Affibody molecules were found to rank accordingly: (ZEGFR:955)2 > (ZEGFR:948)2 > (ZEGFR:942)2 (Fig. 4).

The binding specificity to native EGFR was studied with radiolabeled Affibody molecules. They were indirectly radio-labeled with 125I by amine coupling, and the specificity was studied using A431 cells. The binding of radiolabeled (ZEGFR:955)2, (ZEGFR:948)2 and (ZEGFR:942)2 could be efficiently blocked by the addition of an excess of the same non-labeled Affibody (Fig. 5A2, B2 and C2), indicating a specific binding to native EGFR. Furthermore, the three EGFR-binding Affibody molecules were found to bind EGFR in a competitive manner, since they inhibited the binding of each other (Fig. 5A3–4, B3–4 and C3–4). This cellular binding assay suggests that they have the same, or at least compete for the same, epitope. In addition, since we made a significant effort to apply the same amount of Affibody molecule to each cell culture the results would suggest that ZEGFR:955 had a higher, ZEGFR:948 an intermediate and ZEGFR:942 the lowest binding to cells (Fig. 5A1, B1 and C1). This would in this case correlate with the results from the FACS (Fig. 4) and the dissociation rates in biosensor analysis (Fig. 2C).

**Immunofluorescence microscopy**

The binding of the Affibody molecules to native EGFR on cells was supported by results from the immunofluorescence microscopy study. Following incubation of fixed A431 cells with a (ZEGFR:955)2 Affibody and an indirectly fluorescently labeled Affibody-specific antibody, a distinct staining of cell membrane for all the three Affibody molecules was observed; the staining of (ZEGFR:955)2 is shown in Fig. 6. A control Affibody molecule demonstrated insignificant membrane binding to A431 cells and (ZEGFR:955)2 did not show any significant staining to EGFR-negative human neuroblastoma SH-SY5Y cells (Fig. 6).

Further studies of (ZEGFR:955)2 Affibody binding to A431 cells using confocal immunofluorescence microscopy confirmed the membrane staining pattern (Fig. 7, left figure) and demonstrated in addition intracellular staining after incubation for 2 h at physiological conditions (Fig. 7, right figure). These results further supported the selective binding of anti-EGFR Affibody molecule to native EGFR and suggested internalization of the Affibody molecule.

**Discussion**

Phage display technology was used to select novel Affibody molecules that selectively bind to the human EGFR often overexpressed in glioblastoma multi-forme, head and neck squamous cell carcinoma and urinary bladder carcinoma. Three Affibody molecules, ZEGFR:942, ZEGFR:948 and ZEGFR:955, demonstrated significant EGFR binding capacity whereas six other clones were selected that exhibit binding properties slightly above background. The three Affibody molecules, ZEGFR:942, ZEGFR:948 and ZEGFR:955, were clearly found to compete in their binding to the EGFR-expressing A431 cells, indicating overlapping binding sites. However,
since the selected amino acid residues (Fig. 1) show a very low degree of similarity, we cannot conclude whether they actually bind the identical epitope. Nevertheless, \( Z_{\text{EGFR}:948} \) and \( Z_{\text{EGFR}:955} \) seem to be somewhat more related having identical amino acids in positions 24, 25 and 27. Interestingly, \( Z_{\text{EGFR}:942} \) has the glycine and tryptophane residues in positions 24 and 25 in reverse order that potentially could suggest a different binding orientation. Only one of these sequences, \( Z_{\text{EGFR}:942} \), was found more than once (11 times) among the sequenced clones and could, because of this convergence, be expected to exhibit the best binding characteristic to the target protein EGFR-ECD. Previously, convergence in the output from the selection procedure has typically been strongly associated with the most promising binding characteristics (Nord et al., 1997, 2001; Gunneriusson et al., 1999; Wikman et al., 2004, 2006). However, in our study the Affibody molecule \( Z_{\text{EGFR}:955} \) appeared to be an equally good binder, and in some applications even to exhibit stronger binding than \( Z_{\text{EGFR}:942} \), although it was only found once in the sequencing assays. It could be speculated that the convergence in the selection towards \( Z_{\text{EGFR}:942} \) is not only as a result of stronger binding, but may also be related to slight differences in expression level. The better performance of \( Z_{\text{EGFR}:955} \) in the cell assays might be due to the \( \sim \)10-fold slower off-rate as observed in Biacore analysis and \( Z_{\text{EGFR}:955} \) therefore was selected as the preferred binder for future studies. It is however still not evident why this slow off-rate did not favor the selection of \( Z_{\text{EGFR}:942} \) in the panning procedure.

Affinity and size are two important properties of a tumor-targeting agent. A slow dissociation rate is related to durable retention on tumor cells, which is important in radionuclide based imaging applications. As has been previously demonstrated for Affibody molecules (Gunneriusson et al., 1999; 

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**Table I.** Monomeric and dimeric Affibody molecules were compared in a biosensor analysis for their EGFR binding and approximate kinetic parameters are presented.

<table>
<thead>
<tr>
<th>Monomer</th>
<th>( K_D ) (nM)</th>
<th>( k_a ) (M(^{-1}) s(^{-1}))</th>
<th>( k_d ) (s(^{-1}))</th>
<th>Dimer</th>
<th>( K_D ) (nM)</th>
<th>( k_a ) (M(^{-1}) s(^{-1}))</th>
<th>( k_d ) (s(^{-1}))</th>
</tr>
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<tr>
<td>( Z_{\text{EGFR}:942} )</td>
<td>130</td>
<td>( 3.0 \times 10^5 )</td>
<td>( 4.0 \times 10^{-2} )</td>
<td>( Z_{\text{EGFR}:942} )_2</td>
<td>25</td>
<td>( 6.0 \times 10^3 )</td>
<td>( 1.6 \times 10^{-2} )</td>
</tr>
<tr>
<td>( Z_{\text{EGFR}:948} )</td>
<td>185</td>
<td>( 4.2 \times 10^5 )</td>
<td>( 7.7 \times 10^{-2} )</td>
<td>( Z_{\text{EGFR}:948} )_2</td>
<td>45</td>
<td>( 1.9 \times 10^5 )</td>
<td>( 8.1 \times 10^{-3} )</td>
</tr>
<tr>
<td>( Z_{\text{EGFR}:955} )</td>
<td>185</td>
<td>( 6.2 \times 10^5 )</td>
<td>( 1.2 \times 10^{-2} )</td>
<td>( Z_{\text{EGFR}:955} )_2</td>
<td>50</td>
<td>( 4.8 \times 10^4 )</td>
<td>( 2.4 \times 10^{-3} )</td>
</tr>
</tbody>
</table>

\( ^a \)Dissociation equilibrium constant.  
\( ^b \)Association rate constant.  
\( ^c \)Dissociation rate constant.

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Fig. 3. Affinity measurements on A431 cells. Saturation studies of (A) \([^{125}\text{I}]Z_{\text{EGFR}:942} \)_2, (B) \([^{125}\text{I}]Z_{\text{EGFR}:948} \)_2 and (C) \([^{125}\text{I}]Z_{\text{EGFR}:955} \)_2. Different concentrations of radiolabeled Affibody molecules were added to A431 cells and incubated on ice for 4 h. After reaching saturation the samples were analyzed in a gamma counter. The data were analyzed by non-linear regression using GraphPad Prism 4. Mean values and standard deviations from three values are shown. \( B_{\text{max}} \) is the maximum specific binding to be fit.

Fig. 4. FACS analysis of the binding of fluorescently labeled EGFR-binding dimeric Affibody molecules to native EGFR on A431 cells. (1) Negative control Affibody, (2) \( Z_{\text{EGFR}:942} \)_2, (3) \( Z_{\text{EGFR}:948} \)_2 and (4) \( Z_{\text{EGFR}:955} \)_2. The Affibody molecules were labeled directly on a unique C-terminal cysteine with Oregon Green 488.
Fig. 5. Cell binding and specificity of EGFR binding Affibody molecules towards A431 cells. A431 cells were incubated on ice for 4 h with (A1) \((\text{ZEGFR:942})_2\), (B1) \((\text{ZEGFR:948})_2\), and (C1) \((\text{ZEGFR:955})_2\). The specificity was tested by adding, simultaneously, an excess (~500:1) of unlabeled Affibody molecule together with the same \(^{125}\text{I}\)-labeled Affibody molecule: (A2) \((\text{ZEGFR:942})_2 + (\text{ZEGFR:948})_2\), (B2) \((\text{ZEGFR:948})_2 + (\text{ZEGFR:955})_2\), and (C2) \((\text{ZEGFR:955})_2 + (\text{ZEGFR:942})_2\). In order to determine whether the Affibody molecules had the same binding site, unlabeled Affibody molecule, in excess (~500:1), were incubated with each \(^{125}\text{I}\)-labeled Affibody molecule: (A3) \((\text{ZEGFR:948})_2 + (\text{ZEGFR:955})_2\), (B3) \((\text{ZEGFR:942})_2 + (\text{ZEGFR:955})_2\), (B4) \((\text{ZEGFR:955})_2 + (\text{ZEGFR:942})_2\), (C3) \((\text{ZEGFR:948})_2 + (\text{ZEGFR:955})_2\), and (C4) \((\text{ZEGFR:955})_2 + (\text{ZEGFR:948})_2\). The samples were analyzed in a gamma counter and counts per minute (CPM) per 10^5 cells are presented. Mean values and standard deviations from three values are shown.

Steffen et al., 2005, an effective and convenient method to reduce the dissociation rate is to convert a monomeric binder into a head-to-tail dimeric molecule, to thereby take advantage of avidity effects. A dimeric molecule possesses an increased concentration of binding sites in close proximity to the target protein which can promote accelerated rebinding, resulting in an apparent reduction in off-rate. By comparing monomeric and dimeric EGFR-binding Affibody molecules, an improvement in apparent affinity was observed for the dimeric Affibody molecules (Table 1, Fig. 2C). Affinity was determined both from biosensor analysis on immobilized recombinant EGFR-ECD (Table 1) and from saturation curve assay on native EGFR on A431 cells (Fig. 3). The affinity constants were found to be ~10-fold better for the cell assay, which may indicate that the EGFR is negatively influenced by its coupling to the biosensor chip and/or that the flexibility in the cell membrane facilitates the avidity contribution. Moreover, temperature differences between the two forms of assays could somewhat influence the apparent affinity constant.

Affibody molecules are significantly smaller (20 times) than full-sized antibodies. The half-life of several unrelated Affibody molecules seems to be in rather suitable range for imaging applications, being in the range of 15 min in mice (data not shown). If one would consider therapeutic applications, and longer half-lives would be desired, Affibody molecules can be genetically fused in frame with a small albumin binding domain thus yielding half-lives similar to that of serum albumin (Tolmachev et al., submitted for publication).

The Affibody molecules were in this study produced both as His6-Z and His6-Z-cys fusion proteins, the latter having a C-terminal cysteine residue. This offers convenient strategies for immobilization or conjugation opportunities, e.g. for directed thiol-modified immobilization of Affibody molecules in biosensor applications or as capturing agents for purification purposes (Grönwall et al., 2007). Here, the cysteine residue was utilized for directed conjugation of the fluorophore Oregon Green. This enabled us to successfully study the binding of the Affibody molecules to native EGFR on cells in FACS and confocal microscopy, where we also could study internalization of the fluorophore-labeled Affibody molecules. This possibility of thiol-mediated directed coupling at a site distant from the binding surface of the Affibody molecule, offers also the potential coupling of other compounds of interest for therapeutic applications such as cytotoxic small molecules, e.g. maytansinoids (Widdison et al., 2006).

EGFR exists in different ectodomain conformations, tethered/compact or untethered/extended. Cetuximab binds domain III of EGFR in the tethered configuration (Li et al., 2005) and EGF binds domain I and III of EGFR thereby promoting a domain rearrangement to the untethered conformation (Ferguson et al., 2003). The well characterized mAb 806 binds to both a mutant EGFRvIII and a subset of the wild-type EGFR when overexpressed (Johns et al., 2004). Future studies will include attempts to map the
binding site of the Affibody molecules to EGFR extracellular subdomains by competition studies with Cetuximab and EGFR. Furthermore, a more detailed study of the internalization of dimeric $Z_{\text{EGFR,955}}$, that was observed in the confocal microscopy study, as these preliminary results suggest that this dimeric Affibody molecule may be an effective agent for tumor imaging. It would be informative to investigate the internalization process by monitoring the presence of dimeric $Z_{\text{EGFR,955}}$ at several time points. Furthermore, it would be of significant interest to investigate tumor targeting in mice carrying xenografted tumors expressing human EGFR. In addition, if future in vivo tumor targeting studies would not give strong enough contrast in imaging, an affinity maturation strategy (in accordance with Orlov et al., 2006) could be used to identify second-generation EGFR-binding Affibody molecules with increased binding strength and off-rate for improved performance in tumor imaging applications.

In conclusion, the novel dimeric EGFR-binding Affibody molecules demonstrated significant binding to the EGFR-EC. In cellular assays, fluorescently labeled Affibody molecules were bound and internalized into EGFR-overexpressing human epithelial cancer cell line A431 cells. Radioiodination of Affibody molecules did not impair their ability to bind to EGFR and the radiolabeled Affibody molecules demonstrated specific binding to A431 cells. The small size of Affibody molecules is expected to be advantageous in tumor imaging applications as they should provide both better tumor penetration and faster blood clearance. Taken together, we believe that the small robust EGFR-binding Affibody molecules have potential to be used as tumor imaging agents in various malignancies where EGFR is overexpressed.

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