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Identification and characterization of tumor antigens by using antibody phage display and intrabody strategies

Anne-Laure Goenaga^{a,b,c}, Yu Zhou^{b,c}, Christine Legay^a, Houcine Bougherara^a, Lan Huang^d, Bin Liu^b, Daryl C. Drummond^e, Dmitri B. Kirpotin^e, Christian Auclair^a, James D. Marks^b, and Marie-Alix Poul^{a,*}

^a Laboratoire de Biotechnologie et Pharmacologie Génétique Appliquée (LBPA), UMR CNRS 8113, 61 avenue du Président Wilson, 94235 Cachan Cedex, FRANCE

^b Departments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, Rm. 3C-38, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA, 94110, USA

^d Departments of Developmental & Cell Biology and Physiology & Biophysics. University of California, Irvine, Irvine, CA 92697-4560

^e Hermes Biosciences, 61 Airport Blvd., Suite D, South San Francisco, CA, 94080, USA

Abstract

To generate a panel of antibodies binding human breast cancers, a human single chain Fv phage display library was selected for rapid internalization into the SK-BR-3 breast cancer cell line. Thirteen unique antibodies were identified within the 55 cell binding antibodies studied, all of them showing specific staining of tumor cells compare to normal epithelial cells. Two of the antibodies bound the ErbB2 oncogene while 6 bound the tumor marker transferrin receptor (TfR). By developing a scFv immunoprecipitation method, we were able to use LC-MS/MS to identify the antigen bound by one of the antibodies (3GA5) as FPRP (prostaglandin F2alpha receptor-regulatory protein)/EWI-F/CD9P-1 (CD9 partner 1) an Ig superfamily member that has been described to interact directly with CD9 and CD81 tetraspanins and to be overexpressed in adherent cancer cell lines. Although the 3GA5 scFv had no direct anti-proliferative effect, intracellular expression of the scFv was able to knockdown CD9P-1 expression and could be used to further define the role of the tetraspanin system in proliferation and metastasis. Moreover, the 3GA5 scFv was rapidly internalized into breast tumor cells and could have potential for the targeted delivery of cytotoxic agents to breast cancers. This study is the proof of principle that the direct selection of phage antibody libraries on tumor cells can effectively lead to the identification and functional characterization of relevant tumor markers.

Keywords

phage display; scFv; intrabody; tumor marker; internalization; CD9P-1; transferrin receptor

*Corresponding author: Marie-Alix Poul is to be contacted, E-mail address: poul@lbpa.ens-cachan.fr, Fax 33 (0)1 47 40 76 84.

^cContributed equally to this work

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1. Introduction

The identification and characterization of tumor specific markers remains a major goal in both understanding the cellular transformation observed in cancer and in developing targets for the molecular therapy of cancer. Molecules that are tumor-specific or overexpressed in cancer are likely to have functional roles that participate in cellular transformation and migration. Targeting of such molecules can result in an anti-tumor effect and therefore utility in cancer therapy. Examples of molecules involved in carcinogenesis that have been targeted successfully are ErbB2 (Baselga et al., 1998; Slamon et al., 2001), EGFR (Grunwald and Hidalgo, 2003; Mendelsohn and Baselga, 2003), the transferrin receptor (TfR) (Moura et al., 2004; Shinohara et al., 2000), Bcr-Abl kinase (Druker et al., 2001) and c-Kit (Demetri et al., 2002). Inhibitors can be obtained from small chemical molecules derived from high throughput screening of large chemical libraries or alternatively from monoclonal antibodies (mAbs). Of particular interest within the spectrum of tumor-specific and overexpressed molecules are those located at the cell surface, since they are readily accessible and can be used to target cancer cells with highly specific ligands like mAbs.

Antibody phage display technology is a strategy that can be used to isolate tumor specific antibodies able to bind their cognate antigens in the cellular context for therapeutic uses (Hoogenboom, 2005; Nielsen and Marks, 2000). For antibody phage display, antibody fragments, corresponding to the binding site of an immunoglobulin (Ig) either in scFv or an antigen binding fragment (Fab) format are fused to the pIII minor capsid protein and displayed at the surface of filamentous phage M13 (Bradbury and Marks, 2004). Repertoires of antibody variable (V) domains can be generated (Marks et al., 1991) and used to construct large libraries of human scFv or Fab, which can then be used to generate panels of antibodies to virtually any antigen (Marks and Marks, 1996; Sheets et al., 1998). Direct selection of tumor specific antibodies from phage display human antibody libraries on tumor cells provides an approach for generating large panels of human antibodies that recognize tumor specific markers (Gao C, 2003; Geuijen et al., 2005; Heitner et al., 2001; Liu et al., 2004; Marks and Marks, 1996; Mazuet et al., 2006; Poul et al., 2000). Due to their human origin, antibodies isolated from phage display human antibody libraries can be directly used without the need to modify them to reduce immunogenicity, as required for murine antibodies derived from hybridoma technology. Depending on the application, antibody fragments can also be engineered to yield antibodies with multiple binding sites (McCall et al., 1999), to improve avidity (Adams et al., 2006) or to modify pharmacokinetic properties (Adams et al., 1998). Antibody fragments can also be used to deliver other therapeutic molecules such as doxorubicin-containing liposomes, enzymes, or DNA, into the cytosol of cancer cells to achieve a therapeutic effect (Noble et al., 2004; Wu and Senter, 2005).

For this work, we employed a previously described methodology (Becerril et al., 1999) to directly select phage antibodies binding a human breast (SK-BR-3) tumor cell line. The methodology generated a panel of phage-antibodies (Ph-Abs) that not only bind, but also are internalized into, the target SK-BR-3 cell line and other breast tumor cell lines. Characterization of the resulting antibodies indicated that several bound the internalizing transferrin receptor. By developing a scFv immunoprecipitation method, we were able to use LC-MS/MS to identify the antigen bound by one of the antibodies (3GA5) as FPRP (prostaglandin F2 alpha receptor-regulatory protein)/EWI-F/CD9P-1 (CD9 partner 1) an Ig superfamily member that has been described to interact directly with CD9 and CD81 tetraspanins (Charrin et al., 2001; Stipp et al., 2001a). While the 3GA5 scFv had no direct antiproliferative effect, intracellular expression of the scFv was able to knockdown CD9P-1 surface expression and could be used to further define the role of the tetraspanin system in proliferation and metastasis. Moreover, the 3GA5 scFv was rapidly internalized into breast tumor cells and could have potential for the targeted delivery of cytotoxic agents to breast cancers.

2. Material and Methods

2.1. Cells, antibodies and plasmids

CHO, SK-BR-3, Hs-578T, Hs-578Bst, MCF7, HCC1937, MDA-MB-231 and LS-574-T cell lines were purchased from the American Type Culture Collection and cultured in DMEM complemented with 10% (v/v) fetal calf serum (FCS) and antibiotics at 37°C in a humidified atmosphere and with 5% CO₂ (except MDA-MB-231 cell, that were cultured without CO₂). Hs-578Bst cell culture medium was additionally complemented with 30 ng/ml EGF (Sigma). CHO-CD9P-1 cell line, 1F11 mAb, biotinylated 1F11 mAb (Charrin et al., 2001), anti-CD9 mAb, anti-CD81 mAb and plasmid pCMV-CD9P-1 were kindly provided by Dr. Eric Rubinstein (CNRS, Villejuif, France). Plasmid pKDEL-scFv- α D11 was a gift from Dr. Andrew Bradbury (LANL, Los Alamos, USA).

2.2. Selection of breast tumor cells internalizing phage antibodies

Phage antibodies were selected from the Sheets antibody library (Sheets et al., 1998) for internalization into SK-BR-3 cells as described previously (Poul et al., 2000). Single chain Fv diversity was determined by PCR amplification followed by DNA fingerprinting with *Bst*NI restriction. Phage antibodies were prepared using PEG-NaCl precipitation and tittered as described elsewhere (Marks et al., 1991).

2.3 FACS Analysis

The binding specificity of Ph-Abs was tested using fluorescence activated cell sorting (FACS). Briefly, cells were grown to 80–90% confluence and harvested by trypsinization. Phage antibodies (10¹¹ cfu total) were incubated with 5×10⁴ cells overnight at 4°C in FACS buffer (phosphate-buffered saline (PBS) (pH 7.4), 1% FCS) in a total volume of 100µl. After two washes with 200µl of FACS buffer, bound phages were detected by the addition of 100µl (1µg/ml) of biotinylated anti-M13 mouse mAb (Sigma) and streptavidin-PE (Biosource). After a 30 min incubation at 4°C, the cells were washed twice and resuspended in PBS containing 1% paraformaldehyde (PFA). Fluorescence was measured by flow cytometry in a FACScalibur (BD Biosciences), and median fluorescence intensity (MFI) was calculated using Cellquest Pro Software (Becton Dickinson). Phage antibodies 3GH7 and 3TF5, which recognizes TfR and ErbB2 respectively (Poul et al., 2000), and Ph-Ab Bot (clone 3D12) (Amersdorfer et al., 1997) specific for botulinum toxin, were used as controls. Alternatively, cells were stained with anti-CD9P-1 1F11, anti-CD9 and anti-CD81 mAbs (1 µg/ml) or with soluble scFv (10 µg/ml) followed by anti-c-myc 9E10 mAb (1 µg/ml) and fluorescent secondary antibodies.

2.4. Soluble scFv expression and purification

The scFv cDNA were subcloned from phagemid pHEN into expression vector pUC119mycHis (Schier et al., 1995) and, if required, pSyn-Cys-His6 (Liu et al., 2004). pUC119mycHis allows *E. coli* periplasmic expression of a scFv fused to c-myc epitope tag and hexahistidine (His)₆ tag at the COOH terminus of the scFv. pSyn-Cys-His6 directs the expression of scFv bearing a free C-terminus cysteine followed by a (His)₆ tag. Soluble scFv was produced as described elsewhere (Nielsen et al., 2006; Schier et al., 1995). Typically 0.5 mg of purified scFv was obtained from 500 ml of culture of *E. coli* TG1.

2.5. Western Blot

Cell grown to 90% confluence were lysed in lysis buffer (1% NP40, 150 mM NaCl, 1mM EDTA, 10mM Tris pH 8) containing with protease inhibitors (Gibco) (1 ml of lysis buffer/100 mm diameter culture dish). Clear lysates were obtained by centrifugation at 10 000 rpm for 10 min. Protein content was quantified with BC Assay Protein Quantification Test (Interchim). Twenty µg of proteins were combined in non reducing loading buffer (4% SDS, 10% glycerol,

62 mM Tris, pH 6.8, 0.002% Bromophenol Blue), resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were treated with soluble scFv (10 µg/ml), followed by anti-c-myc 9E10 mAb (1 µg/ml) and HRP-conjugated anti-mouse Ig (Rockland) which was subsequently revealed by enhanced chemiluminescence (Amersham). Alternatively, membranes were treated with anti-CD9P-1, anti-CD9 or anti-CD81 mAbs (1 µg/ml) and HRP-conjugated anti-mouse Ig.

2.6. Liposome internalization assays

Liposomes were prepared by extrusion through 0.1 µm polycarbonate filters, following hydration by a series of six cycles of rapid freeze-thaw, accomplished by alternatively placing the lipid dispersion in a dry ice bath and a hot water bath maintained at 60 °C (Szoka and Papahadjopoulos, 1980; Mamot et al., 2003). One set of liposomes were composed of distearoylphosphatidylcholine (DSPC; Avanti Polar Lipids), cholesterol (Chol; Calbiochem), *N*-(polyethylene glycol)₂₀₀₀-distearoylphosphatidylethanolamine (PEG-DSPE), and the fluorescent DiIC lipid tracer (DiIC18(3)-DS from Invitrogen) in a 3:2:0.015:0.03 molar ratio. A second series of liposomes were prepared using the lipids dioleoylphosphatidylcholine (DOPC; Avanti Polar Lipids), cholesterol, 6-(cholesteryl-succinyl)amino-2-(*N,N*-bis-carboxymethylamino)hexanoic acid nickel salt (Chol-NTA-Ni; kind gift of Hermes Biosciences), and PEG-DSPE (30:15:5:0.5 molar ratio). The second series was prepared in the presence of 20 mM 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) and 120 mM NaCl.

To construct immunoliposomes, reduced scFvs with a C-terminal cysteine were conjugated to Mal-PEG-DSPE as described previously (Nellis et al., 2005a; Nellis et al., 2005b). Briefly, purified scFv (0.5 mg/ml) was reduced with 2-mercaptoethylamine (Pierce) (20 mM final) for 45 min at 37°C. Reduced scFvs were subsequently recovered by purification on a PD10 column (Amersham) eluted with Hepes buffered saline (Hepes 10 mM, NaCl 150 mM, EDTA 3.4 mM, pH 7.0). For incorporation into preformed liposomes, micellar solutions of Mal-PEG-DSPE were inserted into liposomes by coincubation at 60°C for 30 min at the ratio of 0.5 mol % of the liposomal phospholipids (PL). The scFv were then conjugated at a ratio of 30 µg scFv/µmol PL to the fluorescently-labeled (DiIC) unilamellar liposomes (Szoka and Papahadjopoulos, 1980) (Hermes, South San Francisco) overnight at room temperature (RT). Unreacted maleimides were subsequently quenched with β-mercaptoethanol (2 mM final) for 1 h at RT. The conjugated liposomes were then purified on a CL-4B sepharose column using Hepes buffered saline pH 6.5, and the liposomal PL concentrations were determined using a standard phosphate assay (Bartlett, 1959). SK-BR-3 cells were seeded one day before the experiment on coverslips. After two washes with PBS, Ca²⁺, Mg²⁺, 1% FCS, the cells were incubated with the immunoliposomes (50 µM of phospholipids) either at 4°C or at 37°C for 3 h. Cells were washed three times with PBS, stained with DAPI, fixed with 4% PFA for 30 min at RT and mounted on slides with Vectashield (Vector Laboratories) before microscopy analysis using a confocal microscope (Zeiss, LSM 510). DiI was excited by the HeNe (543nm) laser and the emission was collected through a 560 nm long-pass filter.

Alternatively, a chelated liposome assay (CLIA) was adapted from (Nielsen et al., 2006). Briefly, purified (His)₆-scFvs (10 µg/ml) were premixed with HPTS-loaded Ni-NTA modified liposomes (0.1 mM PL; Hermes, South San Francisco) and NiSO₄ (0.2 mM) and incubated at RT for 20 min. Adherent SK-BR-3 cells were incubated either at 4°C or at 37°C for 4 h with the immunoliposomes. After three washes with PBS or imidazole (125 mM), the cells were trypsinized and the MFI of the cells was quantified using a FACS LSRII (BD Biosciences).

2.7. Immunoprecipitation with scFv antibodies

For initial antigen characterization, antigen overexpressing cells were grown to 90% confluence and labeled in 0.1 mg/ml Sulfo-NHS-LC-biotin (Pierce). After labeling, cells were washed twice with PBS containing 50 mM of glycine. Total protein was extracted using lysis buffer completed with protease inhibitor cocktail. After centrifugation at 13,000g for 10 min, clear lysates were depleted by incubation with either Protein A-sepharose (Sigma) or Ni-NTA-agarose (Quiagen) for 1 h at 4°C. Depleted cell lysates were then incubated with scFv at 26 µg/ml for 2 h at 4°C before the immune complexes were captured on Protein A-sepharose or Ni-NTA-agarose. The sepharose immobilizing immune complexes were then washed 5 times in lysis buffer and heated to 94°C for 4 min in non-reducing protein loading buffer. Immunoprecipitates were resolved by SDS-PAGE in duplicate. One gel was transferred to PVDF membrane and revealed with HRP-conjugated streptavidin (Gibco). The other gel was stained with Coomassie R250 and the specific protein band was excised with reference to the Western Blot. Alternatively, membranes were revealed with mouse mAb anti-TfR (clone H68.4) (Zymed).

2.8. Mass spectrometry analysis

The excised protein band was subjected to standard sample preparation for mass spectrometry. Briefly, gel slices were stripped of stain in 25 mM NH₄HCO₃/50% ACN, reduced in 10 mM DTT, alkylated in 55 mM iodoacetamide, digested in 12.5 ng/µl trypsin (Mass Spectrometry grade, Promega) at 37°C for 4 h, and then extracted in 50% ACN/5% TFA prior to mass analysis. The tryptic peptide digest was analyzed by LC MS/MS using a QqTOF mass spectrometer (QSTAR XL, Applied Biosystems/PE Sciex) as described (Liu et al., 2002). Protein identification was achieved through database searching using Protein Prospector search engine (<http://prospector.ucsf.edu>).

2.9. Epitope comparison of Ph-Ab 3GA5 and mAb 1F11

1F11-mAb is an anti-huCD9P-1 mouse mAb previously described (Charrin et al., 2001). The epitopes recognized by anti-CD9P-1 scFv-3GA5 and 1F11 mAb were checked for overlapping sequences using competitive ELISA on CHO cells stably transfected with pCMV-CD9P-1 (CHO-CD9P-1). Cells were grown in 96 well culture plates, incubated with soluble scFv 3GA5 or 1F11 mAb at increasing concentrations for 1 h at 4°C, and then Ph-Ab 3GA5 (final titer 10⁸ cfu/ml in PBS, 1% FCS) or biotinylated 1F11 mAb (final concentration 0.6 µg/ml) was added. These titres and concentrations were determined as the ones giving 50% of the maximal signal as measured by ELISA. Bound Ph-Ab and biotinylated 1F11 mAb were detected by incubation with HRP-conjugated anti-M13 mAb (Amersham) or HRP-conjugated streptavidin (Gibco), respectively. After washing, bound conjugates were detected using ABTS substrate (Sigma) and measured at 405 nm with an automatic plate reader (Spectra MAX 190, Molecular Devices).

2.10. Intracellular expression of scFv 3GA5

CHO or CHO-CD9P-1 cells were transfected with plamids pKDEL-scFv-3GA5, pKDEL-scFv-αD11 or pCMV-CD9P-1 using Lipofectamine 2000 (Invitrogen) as described by the manufacturer and analyzed 48 hours after transfection. Protein extracts (0.5 ml/60 mm diameter dishes) were incubated with protein G sepharose (Santa Cruz) overnight at 4°C. Depleted extracts were then incubated sequentially at 4°C with 1 µg of 1F11 mAb for 4 h and protein G sepharose (Santa Cruz) for 2 h. Agarose was washed 3 times with lysis buffer and the pellet was resuspended in non-reducing loading buffer. After SDS-PAGE, gels were transferred on PVDF membrane and revealed with anti Myc epitope 9E10 mAb (Sigma) or biotinylated-1F11 mAb, followed by HRP-conjugated anti-mouse Ig or HRP-conjugated streptavidin, respectively.

The intracellular localization of scFv was detected by confocal microscopy in which cells were cultured on cover slips before transfection using the standard staining procedure. Briefly, cells grown on cover slips were fixed for 10 min at RT with PBS, 4% PFA, permeabilized with ice-cold acetone for 30 s, rinsed twice with PBS and saturated with PBS, 1% BSA for 20 min at RT. For double staining, the scFv was first detected using 9E10 mAb (10 µg/ml) followed by FITC-conjugated goat anti-mouse Ig (Sigma). After elimination of the secondary antibody by careful washing, CD9P-1 expression was detected using biotinylated 1F11 mAb (1 µg/ml) followed by PE-conjugated streptavidin (0.1 µg/ml, Gibco). Cover slips were analyzed by confocal microscopy where FITC was excited at 488 nm (emission 495 to 535 nm) and PE was excited at 488 nm (emission collected between 560 and 600 nm).

2.11 Cell proliferation and adhesion assays

Cell proliferation was tested as described previously (Poul et al., 2000). For the cell adhesion assay, SK-BR-3 or HS-578T cells were trypsinized, resuspended at 2.10^5 cell/ml in complete culture or in medium without serum, supplemented with scFv (10 µg/ml) and 0.5 ml of the cell suspension was plated on 24 well plates. After 8 h, cells were washed gently to eliminate non adherent cells and adherent cells were counted under a microscope.

3. Results

3.1. Selection of SK-BR-3 specific scFvs

In order to obtain breast cancer cell specific antibodies, Ph-Abs were isolated from a human naïve scFv library (Sheets et al., 1998) on the basis of their ability to be endocytosed by SK-BR-3 breast cancer cells through receptor mediated endocytosis after a 15 min incubation at 37°C. To avoid the selection of antibodies specific for molecules also present on normal cells, the library was depleted by incubation with an excess of normal human fibroblasts (Poul et al., 2000). After 3 rounds of selection, 40% (55/135) of the selected Ph-Abs bound selectively to SK-BR-3 cells compared to normal human fibroblasts, as determined by cell ELISA. Clones that bound to ErbB2 oncogene product have been characterized previously using ErbB2 extracellular domain recombinant protein, revealing that around 50% of SK-BR-3 Ph-Ab binders were specific of ErbB2 (Poul et al., 2000). Twenty-eight non-ErbB2 SK-BR-3 Ph-Ab binders were further characterized with respect to their DNA sequence, cell binding specificity and cognate antigen identity.

3.1.1. Sequences of SKBR-3 binding phage antibodies—*Bst*NI fingerprinting of the V-region gene sequence of the selected Ph-Abs showed 11 different electrophoretic patterns (data not shown), among which two patterns were over-represented (the 3GH7 pattern being represented nine times and the 3TF12 pattern being represented ten times) while each of the nine remaining patterns were unique. V_H cDNA of all clones was sequenced. All of the V_H have a rearranged V segment from the VH3 family and a J segment from the JH4 family (data not shown).

3.1.2. Binding specificity of the selected phage antibodies to a panel of cell lines

—To discriminate between epithelial specific and breast carcinoma specific antibodies, the selected Ph-Abs were tested for binding to a panel of cell lines including Hs-578T and Hs-578Bst cell lines, which are derived from a unique breast cancer patient (Hackett AJ, 1977). The Hs-578T cell line, isolated from a breast carcinosarcoma of epithelial origin, has been described as tumorigenic in semi-solid medium while Hs-578Bst is a normal epithelial cell line isolated from normal tissue adjacent to the tumor. Although immortalized, the Hs-578Bst cell line is not tumorigenic in semi-solid medium. Ph-Abs were used for these studies rather than native soluble scFv because they generate stronger signals due to signal amplification that results from the multiple copies of the phage major coat protein pVIII bound

by the detecting antibody. Anti-transferrin receptor (TfR) Ph-Ab 3GH7 (Poul et al., 2000) and botulinum toxin Ph-Ab Bot (Amersdorfer et al., 1997) were used as positive and negative controls, respectively. Eight of the 11 unique Ph-Abs tested in this assay detected a marker expressed much more strongly on Hs-578T cell line than on the normal counterpart Hs-578Bst cell line (Table 1). Other breast cancer cell lines (MCF7, HCC-1937, MDA-MB-231) and one colon cancer cell line (LS-574-T) were also tested to identify Ph-Abs targeting different antigens. Seven out of 8 Ph-Abs that bound Hs-578T cells bound all the cancer cell lines tested, with six of them giving a higher signal on SK-BR-3 cells than on Hs-578T cells and only one, Ph-Ab 3GA5, showing a higher binding signal on Hs-578T cells. The remaining Ph-Ab 3GA9 bound all breast cancer cell lines but not the colon cancer LS-574-T cell line. Three of the 11 unique SK-BR-3 positive Ph-Abs were Hs-578T negative, with two (2TB4 and 3TH8) showing significant staining only on SK-BR-3 and one (2TF5) having a distinct staining profile. Overall, this screening identified at least 5 different staining profiles (*i.e.* potential different antigens recognized) within the 11 distinct non anti-ErbB2 Ph-Abs tested (Table 1).

3.1.3. Antigen specificity of the selected antibodies—To further characterize the antigen specificity of the selected SK-BR-3 binders, the scFv cDNA of the binders were subcloned into the pUC119mycHis expression vector (Schier et al., 1995). Soluble scFvs bearing myc and (His)6 tags at the C-terminus were produced in *E. coli* and purified from periplasmic extracts using immobilized metal affinity chromatography. Direct Western Blotting of SK-BR-3 protein extracts, in reducing or not reducing conditions, using soluble scFvs (data not shown) gave no signal indicating that binding of all the scFvs tested required native antigen or that the affinity of the scFvs was too low to efficiently detect the antigen in Western Blot conditions. Surface molecules of SK-BR-3 cells were then biotinylated prior to cell lysis and scFvs coupled to Ni-NTA agarose were used to immunoprecipitate SK-BR-3 biotinylated lysates. Immune complexes were eluted sequentially using imidazole and elution fractions tested by Western Blot, in non reducing conditions, using HRP conjugated-streptavidin (Figure 1A). A 130 kD and a 80 kD protein were co-eluted with scFv-3GA5 and scFv-3GA9, respectively. The six scFvs that shared a similar cell line staining profile by FACS (3GH7, 3TF12, 3TF2, 3GH9, C3.2 and 3TG9) immunoprecipitated a 90 kD antigen. Since antibodies to TfR (MW 90 kD) had already been isolated following a similar strategy of selection (Gao et al., 2003; Poul et al., 2000), immunoprecipitates were analyzed with an anti-TfR mAb. A positive staining was obtained with C3.2 and 3GH7 scFvs (Figure 1B) as well as with 3TF12, 3TF2, 3GH9 and 3TG9 scFvs immunoprecipitates (data not shown). For the three remaining scFvs (2TB4, 3TH8 and 2TF5), no obvious immunoprecipitated band was detected.

3.2. Identification of 3GA5 cognate antigen

Although Ni-NTA agarose has a universal ability to isolate cellular proteins co-immunoprecipitated with (His)6-tagged scFv, this method also leads to non-specific background from proteins that bind to Ni-NTA agarose, even when depletion is conducted extensively (Figure 2A). To explore other methods that could be used to isolate cognate antigens of sufficient quantity for mass spectrometry analysis, Protein A-agarose was tested for the ability to capture immunoprecipitates with scFvs. In our study, around 25% of the scFvs selected from this phage antibody library bound to Protein A-agarose (not shown). This result is consistent with the observation that Protein A binds around 50% of scFvs containing a human V_H3 segment (Akerstrom et al., 1994), and that scFvs containing a V_H3 family V-segment gene are over represented in the Sheets library (Sheets et al., 1998).

3.2.1. Isolation of 3GA5 cognate antigen by immunoprecipitation—ScFv 3GA5 bound to Protein A-agarose and immunoprecipitated a single protein with apparent MW of 130 kD using Protein A-agarose. Compared with Ni-NTA agarose, Protein A-agarose gave a much

cleaner background (Figure 2A), which allowed easy isolation of the protein band from a Coomassie-stained separation gel

3.2.2. Identification of 3GA5 cognate antigen by LC-MS/MS—Purified protein bands from 3GA5-immunoprecipitation using Protein A-agarose were subjected to in-gel digestion and the resulting peptide digests were analyzed by LC MS/MS. Peptide sequencing unambiguously identified 11 unique peptide sequences (Figure 2B), which matched the KIAA1436 gene (NCBI accession number 7243270), corresponding to FPRP/EWI-F/CD9P-1 (Figure 2C). The specificity of 3GA5 was further confirmed by differential staining between CHO cells transfected with human CD9P-1 cDNA (CHO-CD9P-1 cells) and wild type CHO cells using 3GA5 Ph-Ab (Figure 3A). Binding of Ph-Ab 3GA5 was specific to CHO-CD9P-1 cells compared to CHO cells. Moreover, immunoprecipitation of CHO-CD9P-1, SK-BR-3 and MCF-7 but not CHO cell extracts using soluble scFv 3GA5 showed a specific band, in non reducing conditions, detected by mAb 1F11 that targets human CD9P-1 (Charrin et al., 2001). This confirmed that 3GA5 recognizes CD9P-1 in breast cancer cells (Figure 3B). CD9P-1 belongs to a newly defined family of proteins with high structural similarity. Other members of this subfamily besides FPRP include CD101, IGSF3 and EWI-2 proteins (Stipp et al., 2001a). CD9P-1 has been described as the major direct partner of CD9 and CD81 tetraspanins within the so-called tetraspanin Web, but its function remains unclear (Charrin et al., 2001; Stipp et al., 2001a). Interestingly, CD9P-1 expression level has been positively correlated with increased metastatic properties in adherent cancer cells (Charrin et al., 2001). On the contrary, CD9 expression level was shown to decrease with the metastatic potential in various cancers (Boucheix et al., 2001). The identification of CD9P-1 as the cognate antigen of scFv 3GA5 indicates that the direct selection of phage antibody against tumor surface targets may provide an efficient approach to the discovery of tumor specific antigens at the protein level.

3.3. Functional evaluation of scFv-3GA5

3.3.1. Internalization properties of scFv-3GA5—Since Ph-Ab 3GA5 had been isolated for its ability to be endocytosed by SK-BR-3 cells, we further tested the internalization properties of derived soluble scFv-3GA5 on those cells using scFv-conjugated immunoliposomes. Soluble scFv-3GA5 with a C-terminal cystein was covalently conjugated to DiIC fluorescently labelled liposomes. After 3 h of incubation at 37°C, SK-BR-3 cells showed a specific fluorescence uptake when using scFv-3GA5-immunoliposomes rather than liposomes bearing no scFv (Figure 4A). The fluorescent staining observed under the microscope after incubation with scFv-3GA5-immunoliposomes at 37°C showed perinuclear granulations while incubation at 4°C resulted in a ring corresponding to the cell membrane. Nontargeted liposome controls showed neither cell surface binding nor intracellular labelling.

Alternatively, a CLIA assay, where scFvs were directly conjugated through their (His)6 tag to pre-charged fluorescent liposomes bearing Ni-NTA at their surface (Nielsen et al., 2006), was also performed. After 4 h of incubation and mild PBS washes, cell-associated fluorescence intensity, as quantified by FACS, was identical whether the incubation had been performed at 4°C or at 37°C. On the opposite, imidazole washes, that reverse scFv-3GA5 binding to fluorescent liposomes, removed most of the cell associated fluorescence when incubation was performed at 4°C while it was only slightly reduced when incubation was performed at 37°C (Figure 4B), indicating that liposomes have delivered their content to the cell. Together, these data suggest that scFv-3GA5 provides for both binding and internalization of liposomes into CD9P-1 positive cells.

3.3.2. scFv-3GA5 does not down-regulate CD9P-1 nor modulate cell proliferation and adhesion—To evaluate the effect of soluble scFv-3GA5 on CD9P-1 positive cells, a

reagent was required to detect CD9P-1 independently of the presence of bound scFv-3GA5. Competition binding experiments showed that scFv-3GA5 and 1F11 mAb (Charrin et al., 2001) bind non-overlapping epitopes on CD9P-1 since they could both bind CD9P-1 positive cells at the same time, as tested by cell ELISA (data not shown). In addition to SK-BR-3 cells, Hs-578T cells were used to test scFv-3GA5 effect since they expressed the highest level of CD9P-1 considering both total (Figure 5A) or surface expressed CD9P-1 (Figure 5B). Notably, despite a lower level of CD9P-1, SK-BR-3 cells displayed significantly more CD9 and CD81 than HS-578-T cells (Figure 5A and B). The effect of scFv-3GA5 (0.1 to 10 µg/ml, incubation 24 to 48 h) on CD9P-1 cell surface expression was tested on both SK-BR-3 and Hs-578T cells using mAb 1F11 for FACS staining. No significant modulation of surface level of CD9P-1 was induced by scFv-3GA5 at any concentration. Similarly, CD9 and CD81 surface levels were not affected by scFv-3GA5 treatment (data not shown). In addition, scFv-3GA5 (10 µg/ml) was evaluated for its ability to inhibit the cell proliferation or the adhesion of SK-BR-3 or Hs-578T cells. No effect was seen on both parameters (data not shown).

These data led to the conclusion, that despite the fact that CD9P-1 can efficiently promote internalization of CD9P-1 targeted immunoliposomes into CD9P-1 positive cells, 3GA5-dependent CD9P-1 internalization neither affect the surface level of CD9P-1 nor CD9P-1-associated CD9 and CD81 tetraspanins in SK-BR-3 en HS-578T cells.

3.4. Phenotypic knock out of CD9P-1 in CHO cells

Since soluble scFv-3GA5 was not able to efficiently down-regulate CD9P-1 surface level, an eukaryotic expression vector was constructed with anti-CD9P-1 scFv-3GA5 cDNA fused to a signal peptide and a reticulum endoplasmic (ER) retention signal for intracellular expression of scFv-3GA5 (Persic et al., 1997) in order to (1) direct scFv-3GA5 expression to the secretory pathway, (2) allow its binding to neo synthesized CD9P-1 in transit into the ER and (3) inhibit neo synthesized CD9P-1 cell surface expression.

3.4.1. Intracellularly expressed scFv 3GA5 binds CD9P-1—To test the antigen binding activity of intracellularly expressed scFv-3GA5, CHO cells were transiently transfected with pCMV-CD9P-1 and pKDELscFv plasmids. Cell lysates prepared from transfected cells were analyzed for the expression of CD9P-1 and scFvs by Western Blot (Figure 6A). CHO cells transfected with pCMV-CD9P-1 only showed a unique 130kD band as detected with anti-CD9P-1 mAb 1F11 as well as CHO co-transfected with pCMV-CD9P-1 and pKDELscFv-αD11, a vector encoding an irrelevant anti-NGF scFv (Persic et al., 1997). CHO cells transfected with pCMV-CD9P-1 and pKDELscFv-3GA5 showed an additional lower band (around 105kD) probably corresponding to a non-glycosylated form of CD9P-1 resulting from ER sequestration of neo-synthesized CD9P-1. The ability of intracellular scFv-3GA5 to interact with CD9P-1 was also directly demonstrated by immunoprecipitation of lysates from co-transfected cells with mAb 1F11. Single chain Fv-3GA5 co-immunoprecipitated with CD9P-1 (Figure 6A) while irrelevant scFv-αD11 did not. In summary, intracellularly expressed scFv-3GA5 can bind and sequester CD9P-1 in the ER, resulting in a lack of CD9P-1 post-translational modifications normally acquired in the secretory pathway.

3.4.2 Intracellular expression of scFv 3GA5 inhibits surface expression of CD9P-1—CHO cells co-transfected transiently with pCMV-CD9P-1 and pKDEL-scFv plasmids were also analyzed by confocal microscopy (Figure 6B). Both anti-CD9P-1 scFv-3GA5 and irrelevant anti-NGF scFv-αD11 mainly localized in cytoplasmic vesicles corresponding to the ER, as observed using anti-myc tag mAb. When CD9P-1 was expressed alone or in combination with scFv-αD11, it localized both in the cytoplasm and at the cell membrane as observed with 1F11 mAb while co-expression with scFv-3GA5 clearly

diminished the display of CD9P-1 on the cell surface. Single chain Fv-3GA5 intracellular expression was also able to decrease CD9P-1 membrane level of CHO cells stably expressing CD9P-1. Indeed, CD9P-1 cell surface level of those cells transfected with pKDEL-scFv- α D11 was 193 (MFI as measured by FACS), similar to mock transfected cells. Interestingly, CHO-CD9P-1 cells transiently transfected with pKDEL-scFv-3GA5 in the same conditions showed a dramatic decrease of CD9P-1 surface level (MFI = 75) (Figure 6C). This data suggest that expression of scFv-3GA5 as an intrabody may significantly reduce the expression of endogenous CD9P-1 in breast cancer cells and could be used as a tool to further define the role of CD9P-1 in breast cancer biology.

4. Discussion

Phage antibodies able to internalize rapidly into the SKBR-3 carcinoma breast cancer cell line were obtained using a protocol based on a 15 min incubation at 37°C of the phage antibody library with the cells. Within the 55 SKBR-3 binding antibodies analyzed, approximately 50% were specific of ErbB2 (with two unique antibodies) (Poul et al., 2000), and approximately 40% bound TfR (with six unique antibodies) a receptor known to be rapidly internalized after binding of its natural ligand (transferrin) (Dautry-Varsat, 1986). The six fully human anti-TfR scFvs are currently being characterized with respect to their affinity for cells, yield of production in *E. coli*, and their efficiency in inhibition of SKBR-3 cell proliferation, to identify the best candidate for transferrin receptor-mediated drug delivery strategies (Daniels et al., 2006; Qian et al., 2002). Five additional binders (non ErbB2 and non TfR binding) specific for cancer cells and showing differential binding pattern on a panel of breast cancer and colon cancer cell lines were further characterized to identify the antigen recognized by using immunoprecipitation.

Only two antibodies (3GA5 and 3GA9) immunoprecipitated a cell surface protein detectable by Western Blot. Immunoprecipitation using Ni-NTA agarose, which captures scFvs via their (His)6 tag, led to immunoprecipitation of many intracellular proteins, complicating antigen identification by in gel digestion and LC-MS-MS. In contrast, immunoprecipitation with Protein A agarose gave much cleaner immunoprecipitates. Since approximately 25% of the scFvs in the parental phage antibody library have human VH3 VH domains and bind protein-A, the results suggest that when possible Protein A-sepharose be used for immunoprecipitation.

Mass spectrometry analysis of the 130 kD antigen immunoprecipitated by scFv 3GA5 led to the identification of CD9P-1, which was initially described as a major partner for the CD9 and CD81 tetraspanins (Charrin et al., 2001; Stipp et al., 2001b). Tetraspanins are a large super family of evolutionarily conserved cell-surface proteins characterized by four transmembrane domains and two extracellular loops. At the molecular level, tetraspanins have been shown to interact with each other and also with other transmembrane proteins like integrins (Stipp et al., 2003) forming a molecular network known as the "Tetraspanin Web". The Tetraspanin Web function is related to spatial and temporal organisation of membrane complexes/domains (Hemler, 2003). At the cellular level, tetraspanins have been implicated in various physiological processes including cell motility, metastasis, cell proliferation and differentiation. CD9P-1 has been shown to interact directly with the large extracellular loop and/or the fourth transmembrane domain of CD9 tetraspanin through a stoichiometric association (Charrin et al., 2001). Considering the putative implication of CD9P-1 in the acquisition of a transformed phenotype (Charrin et al., 2001) and the lack of information on its role within the tetraspanin web, we explored using scFv-3GA5 to obtain more information on CD9P-1 function.

Single chain Fv-3GA5 targeting led to rapid internalization of immunoliposomes into SK-BR-3 cells as demonstrated by both fluorescence microscopy and quantitative flow cytometry

analysis. We therefore evaluated the ability of 3GA5 to downregulate cell surface CD9P-1. Despite the fact that 3GA5 was rapidly endocytosed, no variation of cell surface levels of CD9P-1 or of partner CD9 and CD81 were observed following 2 days incubation with saturation conditions of soluble scFv-3GA5. This might be due to rapid recycling of CD9P-1 to the cell surface. Sustained incubation of soluble scFv-3GA5 with SKBR-3 cells, or with HS-578T cells which express higher levels of CD9P-1, also had no effect on cell proliferation nor on cell adhesion. These indicate that targeting CD9P-1 with the 3GA5 scFv does not down regulate the receptor nor cause associated anti-tumor effects. However, 3GA5 can be used to delivery nanoparticles specifically to CD9P-1 expressing tumor cells. Such an approach merits further study with respect to the ability to achieve a tumor specific cytotoxic effect *in vitro* and *in vivo* (Nielsen et al., 2002; Park et al., 2002; Noble et al., 2004; Mamot et al., 2005).

Single chain Fv-3GA5 mass was evaluated at around 28 kD by size exclusion chromatography (not shown) indicating that it does not spontaneously form dimers (also called “diabodies”) which have been observed with other scFvs, which would enhance its affinity for cells and perhaps favor more efficient binding and/or internalization. Internalisation *via* CD9P-1 might be more efficient in the immunoliposome format due to increased avidity due to multivalent binding, since each liposome bears 30 molecules of scFv/liposome. In conclusion, the ability of 3GA5 to internalize into target cells *via* the tumor marker CD9P-1 makes 3GA5 a highly suitable candidate for targeted immunotherapy *via* liposomal drug format.

Since soluble scFv 3GA5 did not downregulate CD9P-1 when added to culture media, intracellular expression of single chain Fv antibodies, so called ‘intrabodies’, was explored as a means to knock down CD9P-1. Intracellular expression of antibody fragments like scFvs can result in correct folding and conserved antigen-binding properties in certain examples (Cardinale et al., 2004). The intracellular scFv trafficking can also be directed by fusion to a specific intracellular localization sequence (Persic et al., 1997). As a result of these properties, the interaction of intrabodies with Ag can achieve a “phenotypic knockout” that may give insights into Ag function (Visintin et al., 2004) and, in the case of tumor associated antigens, revert a transformed phenotype (Tanaka and Rabbitts, 2003). Intracellular expression of scFv 3GA5 was tested for the ability to knock down the surface display of CD9P-1. The ER retention signal KDEL sequestered scFv 3GA5 in the ER and this sequestration resulted in a significant decrease of the surface display of CD9P-1, possibly because 3GA5 bound to the newly synthesized CD9P-1 polypeptide. Other evidence for this phenotypic knock out of surface CD9P-1 is that nearly 50% of CD9P-1 from the total cell extracts showed smaller apparent molecular weights of 105 kD, which indicated the glycosylation of CD9P-1 during post-translational modifications along the secretory pathway was hampered. The remaining approximately 50% of CD9P-1 with molecular weight of 130 kD, could be due to the incomplete knock-down effect resulting from transient expression of scFv 3GA5 and/or to low CD9P-1 cell surface recycling rate.

In summary, we have demonstrated that direct selection of phage antibody libraries on tumor cell lines can be used to generate panels of antibodies with differential tumor cell binding. Antibody specificity can be identified using immunoprecipitation and Western blotting using mAbs to known antigens, or by immunoprecipitation with the novel scFv and antigen identification by LC-MS-MS. Resulting scFv can be used to study whether the antibody-antigen pair have associated anti-tumor biology amenable to blockade with a naked antibody approach. In the case of the 3GA5 scFv and CD9P-1 antigen studied here, this was not the case. Antibodies generated using this approach, however, are also rapidly internalized by cells expressing antigen, opening up targeted therapeutic approaches using immunotoxins, immunoliposomes, or antibody-drug conjugates.

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Abbreviations

Ph-Ab	phage antibody
Ig	immunoglobulin
Ag	antigen
scFv	single chain variable fragment
TfR	transferrin receptor
RT	room temperature
LC-MS-MS	liquid chromatography-tandem mass spectrometry

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Figure 1. Antigen immunoprecipitation using soluble scFv-3GA5, scFv-3GA9 and scFv-C3.2
 (A) Single chain Fvs coupled to Ni-NTA-agarose were used to precipitate SK-BR-3 biotinylated cell lysates. Immune complexes were eluted using imidazole and eluted fractions were tested (upper band) by Western Blot in reducing conditions using HRP conjugated-streptavidin or stained with Ponceau Red (lower band); lane 1 (biotinylated crude extract), lanes 2 to 6 (eluted immune complexes from Ni-NTA agarose column) (B) Immunoblotting of scFv Bot, 3GH7, 3GA5, 3GA9 and C3.2 immunoprecipitates of SK-BR-3 cell extracts with anti-TfR mAb.

Figure 2. LC-MS/MS identification of 3GA5 cognate antigen

(A) Western Blot of scFv 3GA5 immunoprecipitated biotinylated SK-BR-3 cell lysates. The immune complexes were precipitated either with Protein A-agarose (lane 1), or with Ni-NTA-agarose (lane 2), and were tested by Western Blot, using HRP conjugated-streptavidin. (B) The purified band from scFv-3GA5 immunoprecipitation using Protein A-agarose was subjected to an in-gel digestion and subsequently analyzed by LC-MS/MS. Each peptide was sequenced by tandem mass spectrometry. (C) Alignment of the 11 peptides identified on the sequence of CD9P-1.

Figure 3. Confirmation of specificity of 3GA5 to its cognate antigen CD9P-1

(A) Wild-type CHO cells and CHO cells transfected with human CD9P-1 cDNA were stained with Ph-Ab-3GA5. The binding of Ph-Ab-3GA5 is specific to CHO-CD9P-1. (B) Immune complexes from the immunoprecipitation of CHO, CHO-CD9P-1, SK-BR-3 and MCF7 cell lysates with 3GA5 were tested by Western Blot using mAb 1F11, specific to human CD9P-1.

Figure 4. Internalization of scFv 3GA5

(A) SK-BR-3 cells were incubated with 3GA5-DiI liposomes for 3 h either at 4°C or 37°C, washed with PBS and analyzed by confocal microscopy. (B) SK-BR-3 cells were incubated with 3GA5-NiNTA liposomes for 3 h either at 4°C or at 37°C, after trypsinization the cells were washed either with PBS or with imidazole and analyzed by flow cytometry.

Figure 5. Analysis of CD9P-1 and associated CD9 and CD81 tetraspanins

(A) CHO-CD9P-1, CHO, SK-BR-3, Hs-578Bst and Hs-578T cell lysates were subject to Western Blot analysis using anti CD9P-1, anti-CD9 and anti-CD81 mAbs. (B) The cell surface expression of CD9P-1, CD9 and CD81 on CHO-CD9P-1, CHO, SK-BR-3, Hs-578Bst and Hs-578T cells was analyzed by flow cytometry, by staining the cells with mAb 1F11, anti-CD9 and anti-CD81 Ab, MFI (Mean Fluorescence Intensity).

Figure 6. Phenotypic knock out of CD9P-1 in CHO cells

(A) CHO cells were transfected with pCMV-CD9P-1 and/or pKDEL-scFv plasmids as indicated. Two days later, cell lysates were prepared, immunoprecipitated with anti-CD9P-1 mAb 1F11 and analyzed by Western Blot in non-reducing conditions using biotinylated 1F11 or anti-myc tag mAbs. (B) The transfected cells were also stained with biotinylated 1F11 and anti-myc-Tag mAbs and analyzed by confocal microscopy (C) CHO-CD9P-1 cells were transfected with pKDEL-scFv plasmids and analyzed for CD9P-1 membrane expression two days later by flow cytometry using anti-CD9P-1 mAb 1F11. Labeling is revealed using PE conjugated anti-mouse Abs. The negative control is performed using the secondary antibody alone. The percentage of the CD9P-1 positive cell population is indicated as well as the mean fluorescence intensity (MFI) of this positive cell population.

FACS analysis of a panel of cell lines stained with unique Ph-Ab selected on SKBR3 cells
Bound phages were detected with anti-M13 biotinylated mouse mAb and streptavidin-PE. Results are expressed in MFI (Mean Fluorescence Intensity).

Cell line	Origin	Bot *	3TF5 **	3GA5
SK-BR-3	Breast, adenocarcinoma, established from metastatic cells in pleural effusion	10	2871	1165
Hs-578T	Breast, carcinosarcoma of epithelial origin	7	12	2185
Hs-578Bst	Breast, myoepithelial origin	7	10	215
MCF7	Breast, adenocarcinoma, established from from metastatic cells in pleural effusion	11	66	663
HCC1937	Breast, primary ductal carcinoma	8	15	1344
MDA-MB-231	Breast, adenocarcinoma, established from metastatic cells in pleural effusion	7	7	12
LS-174-T	Colon, colorectal adenocarcinoma	12	103	1633

	3GH7 ***	3TF12	3TF2	3GH9	C3.2	3TC9	3TH8	2TB4	3GA9	2TF5
SK-BR-3	533	253	542	380	565	577	448	277	920	1407
Hs-578T	404	76	395	109	314	244	21	12	120	22
Hs-578Bst	6	6	6	6	12	6	5	8	7	9
MCF7	125	100	210	90	130	162	13	19	340	839
HCC1937	1227	310	953	450	790	548	23	ND	479	212
MDA-MB-231	140	ND	ND	ND	ND	ND	ND	ND	170	30
LS-174-T	651	640	527	887	981	1019	41	23	13	584

* irrelevant Ph-Ab
** anti-ErbB2 Ph-Ab
*** anti-TTR Ph-Ab, ND, not determined.