CONSTRUCTION, BACTERIAL EXPRESSION AND CHARACTERIZATION OF A BIFUNCTIONAL SINGLE-CHAIN ANTIBODY-PHOSPHATASE FUSION PROTEIN TARGETED TO THE HUMAN ERBB-2 RECEPTOR

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We have constructed genes expressing single-chain antigen binding proteins (scFv) which recognize the human erbB-2 receptor. These genes encode the heavy and light chain variable domains of an erbB-2 receptor specific monoclonal antibody, MAb FRP5, connected by a peptide linker. In order to express a bifunctional molecule, a bacterial alkaline phosphatase gene was fused 3′ to the scFv gene. The scFv(FRP5) and scFv(FRP5)-alkaline phosphatase fusion protein (scFv(FRP5)-PhoA) expressed in *E. coli* specifically recognize the human erbB-2 protein and compete with MAb FRP5 for binding to the receptor. The bound scFv(FRP5)-PhoA protein can be detected directly on tumor cells using a substrate for alkaline phosphatase, showing that the chimeric protein retains both binding and enzymatic activity.

The erbB-2 protein, a member of the receptor tyrosine kinase family[1,2], is over-expressed in approximately 30% of primary human breast and ovarian tumors[3-6]. Patients whose tumors display elevated erbB-2 levels appear to have a worse prognosis[7-9]. Therefore, the routine immunohistochemical detection of erbB-2 in breast and ovarian tumors may become desirable in the clinic. We have isolated mono-

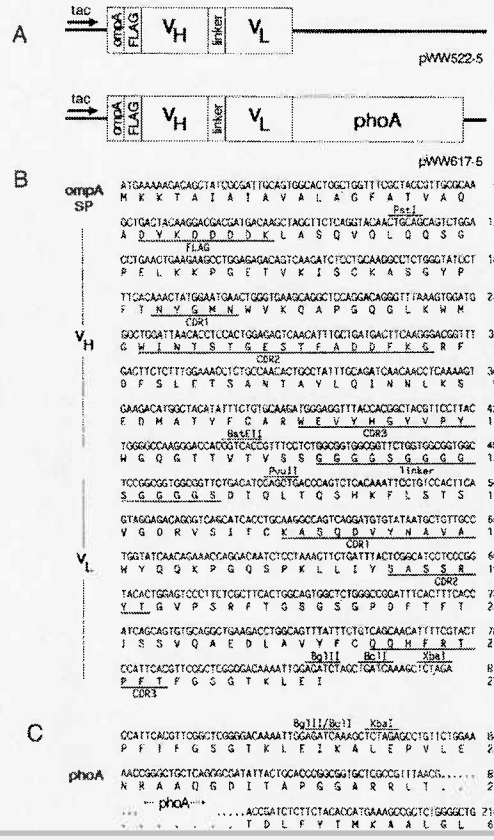

FIGURE 1 (A) Scheme of the scFv(FRP5) and scFv(FRP5)-phoA expression plasmids. Plasmid pWW522-5 contains the gene for the expression of scFv(FRP5) inserted into the plasmid pFLAG-1. The fusion gene consists of the IPTG inducible *tac* promoter, the OmpA signal sequence (OmpA SP), 24 nucleotides encoding the FLAG epitope (FLAG), the PCR amplified cDNA fragment of the MAb FRP5 V_H, a sequence encoding a 15 amino acid connecting linker (linker), and the PCR amplified cDNA fragment of the MAb FRP5 V_L. Plasmid pWW617-5 contains the fusion gene for the expression of scFv(FRP5)-PhoA. The 5′ end of the gene is identical with that described for the scFv(FRP5). At the 3′ end of the V_L domain an open reading frame was created and fused to a gene encoding a modified PhoA protein (phoA). (B) Nucleotide sequence and deduced amino acid sequence of the scFv(FRP5) gene. The sequence shows: the OmpA signal peptide (OmpA SP) from plasmid pFLAG-1 (bp 1–63); the FLAG epitope (bp 64–87); the PstI/BstEII fragment encoding the V_H domain (bp 106–444); the synthetic 15 amino acid linker (bp 454–498); and the PvuII/BglII fragment encoding the V_L domain (bp 505–817). The complementarity determining regions (CDR) in the deduced amino acid sequence of the FRP5 heavy and light chain variable domains are underlined. The sequence positions bp 87–105, bp 445–504, and bp 818–836 are from a synthetic linker sequence designed for the subcloning of variable domain cDNAs. (C) Partial nucleotide sequence and deduced amino acid sequence of the scFv(FRP5)-PhoA gene. The 5′ sequence of the gene from bp 1–780 is identical with that shown in (B). An open reading frame was created by BglII/BclI cleavage and religation (bp 812–817). The modified *E. coli phoA* gene was cloned 3′ of the V_L domain at the XbaI site. The Pro at amino acid position 256 corresponds to position 6 of the processed wild type PhoA. The nucleotides at position 895 to 2121, not shown in this sequence, correspond to bp 427 to 1653 of the published *phoA* sequence[13]. The XbaI site at bp 822–827 and the SacI site at bp 2236–2241 were introduced for sub-cloning by site directed mutagenesis.

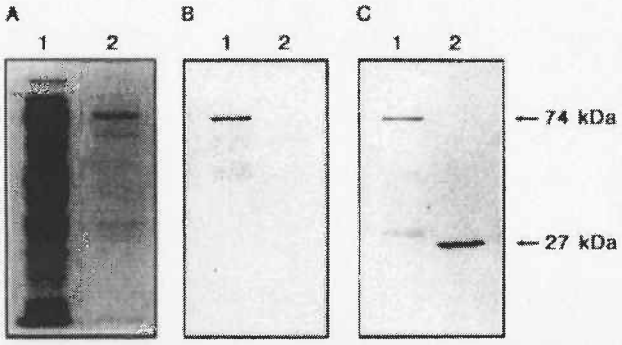

FIGURE 2 SDS-PAGE analysis of scFv(FRP5) and scFv(FRP5)-PhoA proteins purified from *E. coli* lysates. The scFv proteins were purified by affinity chromotography using the MAb M1 which binds to the FLAG epitope. The purified proteins were analysed on 9% SDS-PAGE. (A) A Coomassie stained gel of the (lane 1) soluble proteins of a lysate of *E. coli* cells expressing plasmid pWW617-5; (lane 2) the 74 kD scFv(FRP5)-PhoA protein purified by affinity chromatography. The identity of the purified scFv proteins was confirmed by a blotting analysis using anti-PhoA serum (Panel B) or M1 anti-FLAG MAb (Panel C). In both panels the 74 kD scFv(FRP5)-PhoA protein is shown in lane 1 and the 27 kD scFv(FRP5) protein is shown in lane 2. The filters were incubated with AP-coupled, goat anti-rabbit IgG (B) or AP-coupled, sheep anti-mouse IgG (C) and bound antibody was detected with a substrate for alkaline phosphatase.

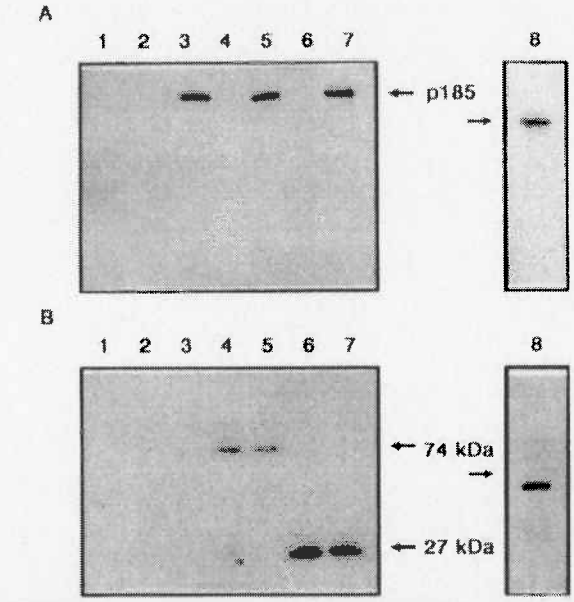

FIGURE 3 The scFv(FRP5) and scFv(FRP5)-PhoA proteins bind the erbB-2 protein. Aliquots of SKBR3 cell extract were incubated with scFv(FRP5) (A and B, lanes 1, 6, 7) or scFv(FRP5)-PhoA (A and B, lanes 1, 4, 5, 8) followed by additional incubation of the mixtures with either pre-immune serum (A and B, lane 1), scFv(FRP5) specific antiserum (A and B, lanes 4 and 6), PhoA specific antiserum (A and B, lane 8), or 21N erbB-2 specific antiserum (A and B, lanes 5 and 7). As controls two aliquots of the extract were incubated with 21N antiserum (A and B, lane 3) or anti-scFv(FRP5) serum (A and B, lane 2). The complexes formed were collected with Protein A sepharose and the released proteins were separated by 7.5% SDS-PAGE, blotted onto PVDF membranes, and an immunoblotting analysis was done using the 21N antiserum (A, lanes 1 to 8), anti-scFv(FRP5) serum (B, lanes 1 to 7) or the anti-PhoA serum (B, lane 8). Bound antibodies were detected with ^{125}I-Protein A. The arrows indicate the positions of the 185 kD erbB-2 protein, the 27 kD scFv(FRP5) and the 74 kD scFv(FRP5)-PhoA.

clonal antibodies (MAbs) which specifically bind the extracellular domain of the erbB-2 receptor[10]. cDNA encoding one of these MAbs, FRP5, has been used to construct single chain antigen binding protein (scFv)[11] genes consisting of the variable domains of the heavy chain (V_H) and light chain (V_L) joined by a short peptide linker. Two genes, one encoding an erbB-2 specific scFv protein, and the other an scFv-alkaline phosphatase (PhoA) fusion protein, were constructed. Both scFv proteins, which are produced in bacteria, retain specific erbB-2 binding activity, and the scFv(FRP5)-PhoA protein also displays alkaline phosphatase (PhoA) enzymatic activity. A simple immunohistochemical reaction can be used to detect erbB-2 molecules on the surface of tumor cells after binding of the chimeric scFv(FRP5)-PhoA protein.

RESULTS

Construction of scFv(FRP5) and scFv(FRP5)-PhoA expression plasmids. The FRP5 light chain (V_L) and heavy chain (V_H) variable region cDNAs were isolated by reverse transcription of poly(A) containing RNA and subsequent PCR amplification as described[11]. The amplified DNA sequences were joined into one open reading frame by an oligonucleotide encoding 15 amino acids $(Gly,Gly,Gly,Gly,Ser)_3$. To construct a gene encoding a protein with erbB-2 binding activity and with enzymatic activity, the alkaline phosphatase (*phoA*) gene of *E. coli* was fused to the 3′ end of the scFv(FRP5) gene. Figure 1A schematically shows the pWW522-5 plasmid containing the scFv(FRP5) gene, and the pWW617-5 plasmid containing the scFv(FRP5)-PhoA gene. For expression in *E. coli* both the scFv(FRP5) and the scFv(FRP5)-phoA genes were inserted into the plasmid pFLAG-1, which contains an IPTG inducible *tac* promoter[12]. The 5′ coding sequences specify the *ompA* signal peptide, which promotes secretion of recombinant proteins into the periplasmic space, followed by a sequence encoding the FLAG epitope, which allows the isolation of recombinant protein via antibody affinity chromatography. The sequence of the scFv(FRP5) coding region is shown in Figure 1B and, in Figure 1C, a portion of the scFv(FRP5)-phoA gene is shown. The amino acid at position 256 (Pro) corresponds to the sixth amino acid of the processed PhoA protein[13].

Expression and purification of scFv(FRP5) and scFv(FRP5)-PhoA from *E. coli*. The expression plasmids pWW522-5 and pWW617-5 were transformed into the phoA negative *E. coli* strain CC118[14]. Single colonies were grown, scFv expression induced with IPTG, and bacterial lysates were prepared. scFv proteins were purified from the soluble fraction of bacterial lysates by immunoaffinity chromatography using the M1 FLAG affinity column. Figure 2A shows the Coomassie blue stained gel of the cleared lysate of pWW617-5 expressing cells. Following passage over the M1 FLAG affinity column the predominant protein which bound to the column and was eluted by treatment with EDTA was the 74 kD scFv(FRP5)-PhoA protein. When the same purification protocol was performed using pWW522-5 expressing cells, the predominant protein visible on the Coomassie stained gel was the 27 kD scFv(FRP5) protein (data not shown). The identities of the 74 kD scFv(FRP5)-PhoA protein and the 27 kD scFv(FRP5) protein were further confirmed by immunoblot analysis with antiserum specific for PhoA (Fig. 2B) or the FLAG epitope (Fig. 2C).

The scFv(FRP5) and scFv(FRP5)-PhoA proteins bind the erbB-2 protein. Affinity purified scFv(FRP5) and scFv(FRP5)-PhoA were tested for their ability to bind the erbB-2 protein in solution. SKBR3 cells, a human breast

tumor cell line which expresses approximately 1 × 10^6 molecules of the erbB-2 protein per cell[15], was used as a source of antigen. Aliquots of SKBR3 extract were incubated with scFv(FRP5) or scFv(FRP5)-PhoA to allow the formation of erbB-2-scFv complexes. The mixtures were then incubated with either preimmune serum, anti-scFv(FRP5) serum, anti-PhoA serum, or 21N anti-erbB-2 serum[15]. As a control, two aliquots of the SKBR3 extract were incubated with 21N serum or anti-scFv(FRP5) serum. Immune complexes were collected by treatment with Protein A-Sepharose and the precipitated proteins were analyzed by SDS-PAGE and immunoblotting. The filters were treated with 21N serum, anti-scFv(FRP5) serum, or anti-PhoA serum. Bound antibodies were detected by incubation with ^{125}I-Protein A. The results are shown in Figure 3. Preimmune serum does not precipitate the erbB-2 protein or the scFv proteins (Fig. 3A and B, lanes 1). The anti-scFv(FRP5) serum does not precipitate the erbB-2 protein from the SKBR3 extract (Fig. 3A, lane 2).

The scFv proteins present in the mixtures were precipitated with either anti-scFv(FRP5) serum (Fig. 3B, lanes 4 and 6), or as part of a complex with 21N anti-erbB-2 serum (Fig. 3B, lanes 5 and 7). The precipitation of the p74 scFv(FRP5)-PhoA protein from the mixture with anti-PhoA serum is shown in Figure 3B lane 8. The p185 erbB-2 protein was precipitated from the SKBR3 cell lysate either with 21N antiserum (Fig. 3A, lanes 3, 5 and 7) or as part of a complex with scFv(FRP5)-PhoA with anti-PhoA serum (Fig. 3A, lane 8). The results show that both scFv(FRP5) and scFv(FRP5)-PhoA form complexes with the erbB-2 protein which can be immunoprecipitated with the 21N serum (Fig. 3B) or anti-PhoA serum (Fig. 3A).

Although the anti-scFv(FRP5) serum efficiently precipitates scFv(FRP5) and scFv(FRP5)-PhoA from the mixtures (Fig. 3B), it fails to bring down complexes formed between the scFv proteins and the erbB-2 receptor (Fig. 3A). This suggests that either the binding of scFv proteins to the erbB-2 protein is blocked by anti-scFv(FRP5) serum or scFv(FRP5) proteins bound to the erbB-2 protein are no longer recognized by the antiserum.

The scFv(FRP5)-PhoA protein is bifunctional. The bifunctional nature of the scFv(FRP5)-PhoA protein was demonstrated as follows. Varying amounts of affinity purified erbB-2 protein were blotted onto membrane strips and subsequently incubated with 0.1 μg/ml of scFv(FRP5)-PhoA (Fig. 4A, Panel 1), MAb FRP5 (Fig. 4A, Panel 2) or scFv(FRP5) (Fig. 4A, Panel 3). The membranes in Panels 2 and 3 were treated with a second antibody, AP-coupled, sheep anti-mouse IgG. The bound MAb and scFv proteins were detected using Fast Red as a phosphatase substrate[16]. Incubation of the scFv(FRP5) treated filters did not result in detectable reaction product, whereas the bound scFv(FRP5)-PhoA protein retained enzymatic activity. Since equal amounts of the monovalent scFv(FRP5)-PhoA and the bivalent MAb FRP5 were included in each reaction and the intensities of the bands in Panels 1 and 2 are equivalent, the scFv(FRP5)-PhoA and MAb FRP5 appear, in this assay, to be equally sensitive.

Binding properties of MAb FRP5 and scFv(FRP5)-PhoA. A competition experiment was performed to determine if MAb FRP5 and the two scFv proteins bind the same domain of the erbB-2 protein. An ELISA assay using fixed SKBR3 breast tumor cells was carried out by incubating cells with 50 nM scFv(FRP5)-PhoA in the presence of increasing amounts of either MAb FRP5 or scFv(FRP5). The relative activity of the bound scFv(FRP5)-PhoA was determined at 405 nM (Fig. 4B).

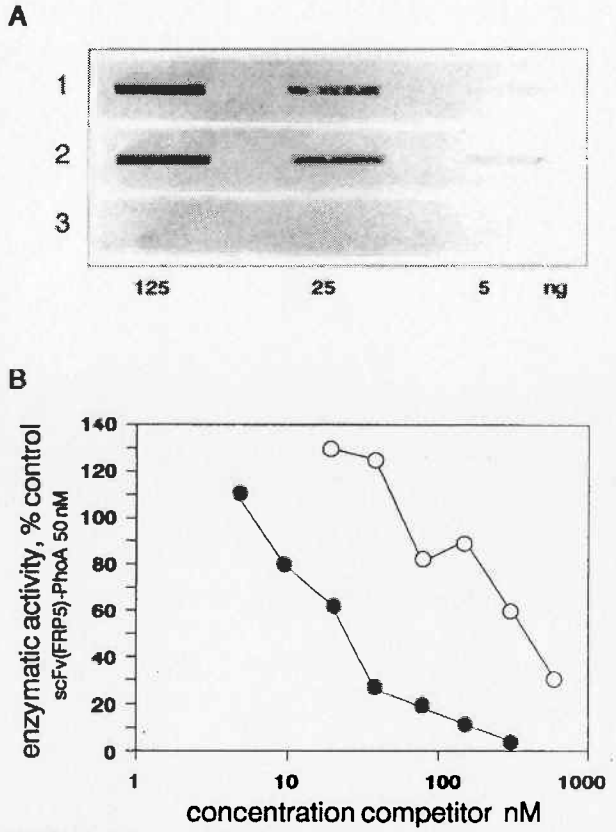

FIGURE 4 (A) The scFv(FRP5)-PhoA protein detects erbB-2 in an immuno-slot-blot analysis. Five, 25 and 125ng of affinity purified erbB-2 protein were blotted onto a PVDF membrane using a slot blot apparatus. Strips were incubated with 0.1 μg/ml: (1) scFv(FRP5)-PhoA; (2) MAb FRP5; (3) scFv(FRP5). The strips in lanes 2 and 3 were then treated with AP-coupled, sheep anti-mouse IgG. Bound phosphatase activity was detected with the substrate Fast Red. (B) Competition analysis. SKBR3 cells were fixed with 2% formaldehyde and incubated with 50nM scFv(FRP5)-PhoA and the following competitors: MAb FRP5 (solid circles) at concentrations ranging from 300 to 4.7nM in two-fold dilution steps, or scFv(FRP5) (open circles) at concentrations ranging from 600 to 18.8nM. The relative activity of bound scFv(FRP5)-PhoA was determined as the absorbance at 405nm and is shown as percentage of controls without competitor.

The concentration of MAb FRP5 and scFv(FRP5) needed to compete binding of the scFv(FRP5)-PhoA by 50% is, respectively, 20 nM and 200 nM. In another ELISA experiment the apparent affinities of both MAb FRP5 and scFv(FRP5)-PhoA were found to be, respectively, 0.82 nM and 7.2 nM (data not shown). These results suggest that the genetically engineered proteins bind to the same domain on the erbB-2 protein as does the MAb. The apparent binding affinity of the bacterially produced scFv(FRP5)-PhoA protein is 9-fold lower than that of the original MAb FRP5.

Immunocytochemical detection of erbB-2 with scFv(FRP5)-PhoA. SKBR3 cells were fixed in 3.7% formaldehyde and incubated with scFv(FRP5)-PhoA (Fig. 5A and B) or with scFv(FRP5) (Fig. 5C and D). Bound scFv(FRP5)-PhoA was detected using Fast Red as a substrate. SKBR3 cells incubated with scFv(FRP5)-PhoA showed surface staining of erbB-2 in normal light and by immunofluorescence. Cells treated with the control scFv(FRP5) protein showed no surface staining.

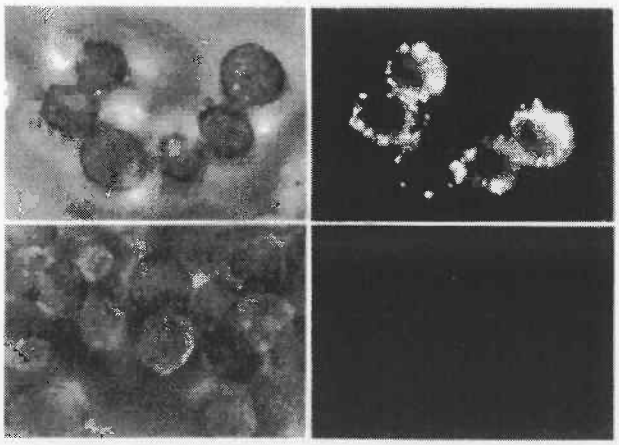

FIGURE 5 The scFv(FRP5)-PhoA protein detects the erbB-2 protein in an immunocytochemical analysis. SKBR3 cells were fixed with 3.7% formaldehyde and incubated with (A and B) 1 μg/ml scFv(FRP5)-PhoA; (C and D) 1 μg/ml scFv(FRP5). Bound PhoA was detected with the substrate Fast Red. In (A) and (C) the cells are shown in phase contrast, in (B) and (D) the immunoflourescent image of the respective fields is shown.

DISCUSSION

Overexpression of the erbB-2 receptor protein in breast and ovarian tumors appears to correlate with poor patient prognosis[5-9]. Thus, the erbB-2 receptor has potential importance, both as a diagnostic marker and as a target for cancer therapy. MAbs directed against tumor cell proteins such as erbB-2 have numerous applications[17,18] but in some instances the size of the antibody and its subunit structure make its manipulation for specific purposes difficult. The smallest domain of an antibody necessary for specific binding is the Fv domain. Recent technological advances have made the cloning and production of scFv molecules routine[11,19,20]. This approach also allows the direct fusion of the Fv domain of an antibody to genes encoding effector molecules and the high level expression of these bifunctional molecules in bacteria. Recombinant scFv molecules have already demonstrated some utility in tumor therapy as single chain immunotoxins directed towards cell surface expressed proteins[21,22].

Both of the scFv proteins we have produced retain their affinities for erbB-2, and the scFv(FRP5)-PhoA fusion retains enzymatic activity following binding. The scFv proteins were expressed in *E. coli* using a system which employs an amino terminal OmpA signal peptide to facilitate the secretion of soluble scFv protein into the periplasmic space. However, the yield of soluble scFv proteins was higher in total bacterial lysates containing CHAPS than in periplasmic extracts without the addition of a detergent (W. W., unpublished results). Approximately 30% of the scFv protein could be purified from total bacterial lysates in a single affinity purification step. The remaining recombinant protein, which was insoluble, appeared to be correctly processed at the signal peptide cleavage site, but could only be recovered by solubilization under denaturing conditions (W. W., unpublished results).

The recombinant scFv(FRP5)-PhoA had an approximately 9-fold reduced affinity for the erbB-2 receptor when compared with the parental MAb. A reduced affinity for antigen has been described for other scFv proteins[22-24]. This may be due to the peptide linker connecting the two variable domains or to the order of these domains in the protein. Nevertheless, the affinity of the recombinant scFv(FRP5)-PhoA is sufficiently high to allow rapid, specific binding to cells expressing the erbB-2 receptor. The bacterially produced scFv(FRP5)-PhoA is an example of a new type of immunohistochemical reagent that can detect an antigen on cell or tissue samples in a one step reaction.

EXPERIMENTAL PROTOCOL

Cloning of FRP5 V_H and V_L cDNAs. Poly(A) containing RNA isolated from the FRP5 hybridoma cell line was used for first strand cDNA synthesis following standard conditions[25] with MCK2 primer 5′ TCACTGGATGGTGGGAAGATGGA 3′, specific for the kappa constant region, or MCH2 primer 5′ AGATCCAGGGGCCAGTGGATAGA 3′, specific for the IgG1 constant region. V_H and V_L domain cDNAs were amplified using PCR as described[11]. For amplification of the V_H domain, oligos VH1FOR and VH1BACK were used[11]. Amplification of the V_L domain was accomplished using oligos MCK2 and VK1BACK[11]. The PCR fragments were cloned into Bluescript KS+ (Stratagene) and sequenced. This revealed that the V_L sequence had been amplified due to oligo MCK2 priming in both directions. PvuII and BglII restriction enzyme sites were introduced into the FRP5 V_L sequence in a separate PCR reaction using the oligonucleotides 5′ GACATTCAGCTGACCCAG 3′ and 5′ GCCCGTTAGATCTCCAATTTTGTCCCCGAG 3′.

Assembly of the FRP5 scFv genes. A linker sequence for joining the V_H and V_L domains was constructed using oligonucleotides and inserted into a modified Bluescript KS+, in which the two internal PvuII sites had been destroyed. The linker encodes a peptide of 15 amino acids $(GGGGS)_3$[20]. The FRP5 V_L fragment was inserted into the resulting plasmid pWW15 as a PvuII/BglII fragment. The V_H fragment was inserted 5′ of the V_L fragment as a PstI/BstEII fragment. To obtain a scFv(FRP5)-PhoA fusion gene, an open reading frame was created at the 3′ end of the V_L domain by BglII/BclI cleavage and re-ligation. A XbaI restriction enzyme site was introduced near the 5′ end and a SacI restriction enzyme site into the 3′ non-coding region of the phoA gene by PCR using a pBR322 derivative carrying transposon *Tn*-phoA[14] as a template and the oligonucleotides 5′ CCCTCTAGAGCCTGTTCTGGAAAAC 3′ and 5′ CCCGAGCTCTGCCATTAAG 3′. After digestion with XbaI and SacI, the 1419 bp phoA fragment was introduced 3′ of the V_L domain using XbaI and SacI sites present in the cloning linker. Finally, the scFv(FRP5)-PhoA gene was isolated as a HindIII/SacI fragment, and sub-cloned into pUC19 yielding plasmid pWW615-5. For the expression in *E. coli* the scFv(FRP5) and scFv(FRP5)-PhoA genes were introduced into the plasmid pFLAG-1 (IBI Biochemicals)[12]. Insertion of the scFv(FRP5) gene into pFLAG-1 yielded the expression plasmid pWW522-5. The plasmid pWW617-5 was obtained by insertion of the scFv(FRP5)-PhoA gene into pFLAG-1. In both constructs the scFv genes were fused in frame to the OmpA signal peptide sequence and a 24 nucleotide sequence encoding the FLAG epitope, a synthetic amino acid sequence that allows Ca^{2+} dependent affinity purification of recombinant proteins with the M1 anti-FLAG MAb affinity matrix (IBI Biochemicals)[12].

Expression and purification of scFv(FRP5) and scFv(FRP5)-PhoA in *E. coli*. Plasmids pWW522-5 and pWW617-5 were transformed into the phoA negative *E. coli* strain CC118[14]. Single colonies were grown overnight at 37°C in LB medium containing 0.4% glucose and ampicillin (100 μg/ml). The cultures were diluted 30-fold in 500ml of the same medium and grown at 28°C. At an OD_{550} of 0.5, the cultures were induced for 45min at 28°C with 1mM IPTG. Cells were harvested by centrifugation and lysed by sonication in 20ml PBS containing 5mM CHAPS (Boehringer Mannheim), 1mM $CaCl_2$, 10% glycerol. The lysates were cleared by ultracentrifugation at 45,000g for 45min at 4°C. The supernatants containing soluble scFv proteins were collected and applied to a M1 FLAG affinity column[12]. The column was washed three times with 10 bed volumes of PBS containing 1mM CHAPS, 1mM $CaCl_2$, 10% glycerol. Bound proteins were eluted in 1ml fractions of PBS containing 1mM CHAPS, 5mM EDTA, 10% glycerol. The fractions containing scFv(FRP5) and scFv(FRP5)-PhoA were pooled and concentrated by ultrafiltration through a YM10 membrane (Amicon). The yield of purified scFv proteins was approximately 400μg/l *E. coli* culture.

Binding activity of the scFv and scFv(FRP5)-PhoA proteins. The binding of scFv and scFv(FRP5)-PhoA to the erbB-2 protein was tested in extracts of SKBR3 breast tumor cells[15]. Four million SKBR3 cells were suspended in 1ml of 50mM Tris-HCl (pH 7.5), 5mM EGTA, 1% Triton X-100, 0.15M NaCl, and 3mM PMSF and particulate matter was removed by centrifugation. Fifty μl aliquots of the cell extract were brought to a volume of 350μl with PBS and incubated for 1h on ice with scFv(FRP5) or scFv(FRP5)-PhoA at a concentration of 3μg/ml followed by additional incubation of the mixtures with either preimmune serum, anti-scFv(FRP5) serum, anti-PhoA serum, or 21N anti-erbB-2 serum[15]. Complexes were collected with Protein A-sepharose, proteins were released by boiling in sample buffer, separated by SDS-PAGE and electroblotted onto PVDF membranes (Millipore)[26]. Membranes were incubated with 21N antiserum, anti-PhoA serum, or anti-scFv(FRP5) serum, followed by treatment of the filters with ^{125}I-Protein A (Amersham).

Enzymatic activity of the scFv(FRP5)-PhoA protein. The bifunctional nature of the scFv(FRP5)-PhoA protein was tested as follows. Five-125ng of immunoaffinity purified erbB-2 protein were blotted onto a PVDF membrane using a slot-blot apparatus (BioRad). The membrane was cut into three strips which were incubated 1h at room temperature with 0.1μg/ml of MAb FRP5, scFv(FRP5) or scFv(FRP5)-PhoA. The first two strips were incubated an additional hour with AP-coupled, sheep anti-mouse IgG. Bound antibody or scFv fusion protein was detected by measuring phosphatase activity for 30min at 37°C in 100mM Tris-HCl, pH 8.2, 1mM $ZnCl_2$, 1mg/ml Fast Red TR salt (Sigma), and 0.4mg/ml AS-MX phosphate (Sigma).

Binding affinities. The binding affinities of MAb FRP5 and the scFv(FRP5)-PhoA protein to erbB-2 on SKBR3 human breast tumor cells were measured by ELISA. SKBR3 cells, in 96 well microtiter plates, were fixed for 30min with 2% formaldehyde/PBS and non-specific binding was blocked by incubation with 3% BSA/PBS. One hundred μl of MAb FRP5 was added in three-fold dilutions at concentrations ranging from 50 to 0.008μg/ml in 3% BSA/PBS for 2h at 4°C. Unbound MAb FRP5 was removed and the wells incubated 1h at room temperature with 100μl of AP-coupled, sheep anti-mouse IgG. Specifically bound MAb was detected with an AP substrate by incubation in 200μl of 1M Tris-HCL (pH 8.0), 0.4mg/ml *p*-nitrophenylphosphate disodium (Sigma). The binding of MAb FRP5 to erbB-2 was determined by measuring the absorbance at 405nm. For the determination of the binding of scFv(FRP5)-PhoA to SKBR3 cells, 100μl of scFv(FRP5)-PhoA was added in twofold dilution steps at concentrations ranging from 12.8 to 0.2μg/ml in 3% BSA/PBS. After 2h incubation at 4°C unbound scFv(FRP5)-PhoA was removed. The activity of bound and free scFv(FRP5)-PhoA was determined as the absorbance at 405nm after incubation of supernatants, or washed cells, in a total volume of 200μl of 1M Tris-HCl (pH 8.0), 2mM $ZnCl_2$, 0.4mg/ml *p*-nitrophenylphosphate disodium for 30min at 37°C. The amount of bound and free scFv(FRP5)-PhoA was determined by comparison of the activities to that of scFv(FRP5)-PhoA standards. The binding affinity was determined using the LIGAND program[27].

Competition experiments. The enzymatic activity of scFv(FRP5)-PhoA was determined by ELISA as described above. SKBR3 cells were incubated with 50nM scFv(FRP5)-PhoA in the presence of MAb FRP5 or scFv(FRP5) as competitor. The concentrations of MAb FRP5 or scFv(FRP5) ranged from, respectively, 300 to 4.7nM, or 600 to 18.8nM. The relative activity of bound scFv(FRP5)-PhoA was determined as the absorbance at 405nm as a percentage of controls without competitor.

Immonocytochemistry. For detection of the erbB-2 protein by immunocytochemistry, SKBR3 cells were fixed with 3.7% formaldehyde in PBS, unspecific binding was blocked with 3% BSA/PBS, and cells were incubated 1h at 37°C with 1μg/ml of scFv(FRP5)-PhoA or scFv(FRP5). Bound scFv(FRP5)-PhoA was detected using the Fast Red substrate solution described above. In this case 0.8mg/ml levamisole was included in the buffer to inhibit endogenous cellular phosphatase activity. The cells were illuminated under fluorescence and bright field conditions[16].

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