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Covalent disulfide-linked anti-CEA diabody allows site-specific conjugation and radiolabeling for tumor targeting applications

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An engineered anti-carcinoembryonic antigen (CEA) diabody (scFv dimer, 55 kDa) was previously constructed from the murine anti-CEA T84.66 antibody. Tumor targeting, imaging and biodistribution studies in nude mice bearing LS174T xenografts with radiolabeled anti-CEA diabody demonstrated rapid tumor uptake and fast blood clearance, which are favorable properties for an imaging agent. Current radiolabeling approaches result in random modification of the protein surface, which may impair immunoreactivity especially for smaller antibody fragments. Site-specific conjugation approaches can direct modifications to reactive groups located away from the binding site. Here, cysteine residues were introduced into the anti-CEA diabody at three different locations, to provide specific thiol groups for chemical modification. One version (with a C-terminal Gly-Gly-Cys) existed exclusively as a disulfide-bonded dimer. This cysteine-modified diabody (Cys-diabody) retained high binding to CEA and demonstrated tumor targeting and biodistribution properties identical to the non-covalent diabody. Furthermore, following reduction of the disulfide bond, the Cys-diabody could be chemically modified using a thiol-specific bifunctional chelating agent, for radiometal labeling. Thus, the Cys-diabody provides a covalently linked alternative to conventional diabodies, which can be reduced and modified site-specifically. This format will provide a versatile platform for targeting a variety of agents to CEA-positive tumors.

Keywords: reactive thiol groups/scFv fragment/site-specific labeling

Introduction

Antibodies specific for tumor-associated antigens can provide effective vehicles for *in vivo* delivery of agents for detection or therapy of cancer, such as radionuclides. The potential utility of cancer-targeting antibodies can be improved by protein engineering approaches, which can be used to modify characteristics such as affinity, immunogenicity and pharmacokinetic properties. In particular, recombinant antibody fragments have been produced with favorable characteristics, including retention of high affinity for target antigen, rapid, high-level accumulation in xenografts in murine models and quick clearance from the circulation resulting in high tumor to normal activity ratios. Furthermore, since antibody fragments do not persist in the circulation, they are less likely to be immunogenic than intact murine or even chimeric antibodies when administered to patients. With the advent of humanized and human antibodies, the issue of immunogenicity of recombinant antibodies is rapidly diminishing.

Recombinant fragments such as diabodies [dimers of single-chain Fvs, 55 kDa (Holliger *et al*., 1993)] or minibodies [scFv-C_H3 fusion proteins, 80 kDa (Hu *et al*., 1996)] have shown promise as *in vivo* imaging agents in preclinical studies when radiolabeled with single-photon emitting radionuclides such as In-111 or I-123 or positron emitters such as Cu-64 or I-124 for positron emission tomography (PET) in preclinical studies (Wu *et al*., 2000; Sundaresan *et al*., 2003). Targeting and imaging of I-123 radiolabeled single-chain Fv (scFv, 27 kDa) fragments has been demonstrated clinically, although the size and monovalency of scFv may limit their utility (Begent *et al*., 1996). Recent clinical imaging studies using I-123 radiolabeled diabodies appear promising (Santimaria *et al*., 2003).

Most current antibody radiolabeling approaches involve conjugation to random sites on the surface of the protein. For example, standard radioiodination methods using Chloramine T or Iodogen result in modification of random surface tyrosine residues. As the size of the antibody decreases, the protein may become inactive following iodination due to modification of key tyrosines in or near the binding site (Nikula *et al*., 1995; Olafsen *et al*., 1996). Alternative iodination approaches or radiometal labeling through conjugation of bifunctional chelates direct modifications to ε-amino groups of lysine residues, again randomly located on the surface of antibodies. Chemical modification of lysines located in or near the antigen-binding site could also potentially interfere with binding through steric hindrance if a bulky group is added (Benhar *et al*., 1994; Olafsen *et al*., 1995). Hence the issue of inactivation following radiolabeling becomes more pressing with smaller antibody fragments, if equal reactivity is assumed, because the binding site(s) represent a larger proportion of the protein surface and fewer 'safe' sites for conjugation are available.

Site-specific radiolabeling approaches provide a means for both directing chemical modification to specific sites on a protein and for controlling the stoichiometry of the reaction. Several strategies capitalize on naturally occurring moieties or structures on antibodies that can be targeted chemically. For example, the carbohydrate found on constant domains of

immunoglobulins can be oxidized and conjugated with bifunctional chelates for radiometal labeling (Rodwell *et al*., 1986). In one instance, an unusual carbohydrate moiety occurring on a hypervariable loop of a kappa light chain was modified for site-specific chelation and radiometal labeling (Leung *et al*., 1995). Others have exploited selective reduction of interchain disulfide bridges to enable modification using thiol-specific reagents. C-terminal cysteine residues on antibody Fab or Fab′ fragments have been used for direct labeling using ^{99m}Tc (Behr *et al*., 1995; Verhaar *et al*., 1996). Novel approaches include the identification of a purine binding site in antibody Fv fragments, allowing specific photoaffinity labeling (Rajagopalan *et al*., 1996).

More recently, genetic engineering approaches have been used to introduce specific sites for modification or radiolabeling of proteins and antibodies. Building on the above-mentioned work, glycosylation sites have been engineered into proteins to provide novel carbohydrate targets for chemical modification. (Leung *et al*., 1995; Qu *et al*., 1998). The six-histidine tail commonly appended to recombinant proteins to provide a purification tag has been used in a novel ^{99m}Tc labeling method (Waibel *et al*., 1999). Alternatively, a popular strategy has been to use site-directed mutagenesis to place cysteine residues on the surface of proteins to provide reactive sulfhydryl groups. This approach has been implemented by numerous groups to allow site-specific labeling of antibodies (Lyons *et al*., 1990; Stimmel *et al*., 2000) and other proteins (Haran *et al*., 1992; Kreitman *et al*., 1994).

Introduction of cysteine residues into engineered antibody fragments has also been used for stabilization or multimerization purposes. For example, introduction of strategically placed cysteine residues in the interface between the V_H and V_L domains of antibody Fv fragments has allowed covalent linkage and stabilization of these fragments (disulfide-stabilized Fv or dsFv) (Glockshuber *et al*., 1990; Webber *et al*., 1995). FitzGerald *et al*. described a disulfide-bonded diabody in which cysteine residues were introduced into the V_L/V_H interface for stability and demonstrated its utility for fluorescent imaging of tumors (FitzGerald *et al*., 1997). Others have appended cysteine residues to the C-termini of single-chain Fv fragments (scFv, formed by fusing V_H and V_L domains with a synthetic peptide linker) to allow multimerization into $scFv'_2$ fragments (Adams *et al*., 1993; Kipriyanov *et al*., 1995).

We have previously produced an anti-carcinoembryonic antigen (anti-CEA) diabody, assembled V_L–eight amino acid linker–V_H. Following radiolabeling (at random sites on the protein) with radioiodine or radiometals, this fragment exhibits rapid tumor targeting in a nude mouse/LS174T human colon carcinoma xenograft model in biodistribution and imaging studies (Wu *et al*., 1999; Yazaki *et al*., 2001b). In order to allow site-specific radiolabeling using thiol-specific reagents, this paper describes four mutant anti-CEA diabodies engineered by substitution or addition of unique cysteine residues. Two variants, with the C-terminal sequence -LGGC or -SGGC, were found to exist as a stable disulfide-linked dimer. The -LGGC Cys-diabody demonstrated equivalent antigen binding *in vitro* and tumor targeting *in vivo* and had the added advantage of allowing site-specific chemical modification following reduction of the interchain disulfide bridge.

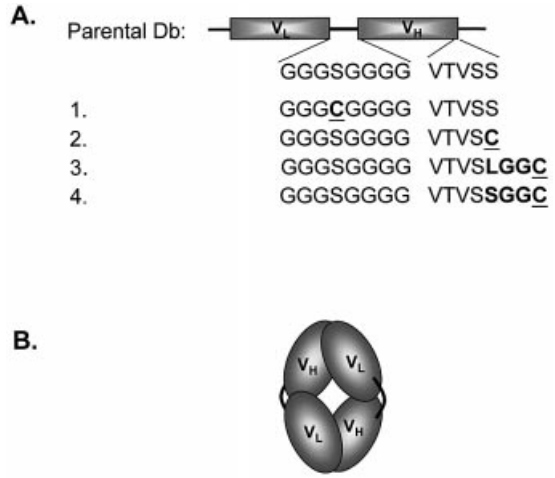

Fig. 1. Design of the anti-CEA Cys-diabodies. (**A**) Gene assembly of the parental diabody with the eight amino acid linker sequence between the V domains and the C-terminus five amino acid sequence present in the V_H domain shown; 1–4 show the Cys-diabody variants made with the added amino acids highlighted and the cysteine residue underlined in each construct. (**B**) Schematic drawing of a non-covalent bound diabody.

Materials and methods

Design of cysteine-modified diabodies (Cys-diabodies)

Four variants of anti-CEA diabodies (Figure 1) were constructed by PCR-based mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) of the pEE12 expression vector (Lonza Biologics, Slough, UK) containing the original anti-CEA diabody constructed with an eight amino acid glycine–serine linker (GS8) (Wu *et al*., 1999). The first variant contained a cysteine instead of serine in the GS8 linker between the variable domains (Cys-linker). The other three contained a cysteine at the C-terminus of the V_H. In one construct the terminal serine (Kabat residue 113) was replaced by a cysteine (VTVS-S changed to VTVS-C). The remaining two constructs contained two glycines inserted in front of the cysteine as a spacer. One retained the original C-terminal sequence with GGC appended (VTVS-SGGC) and in the other, serine 113 was exchanged to a leucine (VTVS-LGGC).

Mammalian expression, selection and purification

A total of 1×10^7 NS0 cells (provided by Lonza Biologics) (Galfre and Milstein, 1981) were transfected with 40 μg of linearized vector DNA by electroporation and selected in glutamine-deficient media as described (Yazaki *et al*., 2001a). Clones were screened for expression by ELISA, in which the desired protein was captured either by protein L or by a recombinant CEA fragment, N-A3 (You *et al*., 1998), and detected using alkaline phosphatase-conjugated goat anti-mouse Fab antibodies (Sigma, St Louis, MO). Supernatants were also examined by western blot for size analysis, using the alkaline phosphatase-conjugated goat anti-mouse Fab antibodies. The best producing clones were expanded. Cys-diabodies were purified from cell culture supernatant, using a BioCad 700E chromatography system (Applied Biosystems, Foster City, CA) as described (Yazaki *et al*., 2001a). Briefly, the supernatants were treated with 5% AG1-X8 (Bio-Rad Laboratories, Hercules, CA) overnight to remove phenol red and cell debris and then dialyzed versus 50 mM Tris–HCl, pH 7.4. Treated supernatant was loaded on to an anion-exchange

chromatographic column (Source15Q; Amersham Pharmacia Biotech, Uppsala, Sweden) and proteins were eluted with an NaCl gradient to 0.2 M in the presence of 50 mM HEPES, pH 7.4. Eluted fractions, containing the desired protein, were subsequently loaded on to a Ceramic Hydroxyapatite (Bio-Rad Laboratories) column and eluted with a KPi gradient to 0.15 M in the presence of 50 mM MES, pH 6.5. Fractions containing pure proteins were pooled and concentrated with a Centriprep 10 (Amicon, Beverly, MA). Elution was monitored by absorption at 280 nm. The concentration of purified protein per milliter was determined by measuring the OD_{280}, but also by applying a small sample on protein L using known amounts of parental diabody and later Cys-diabody standards quantitated by amino acid composition analysis (Wu *et al*., 1996).

Characterization of purified Cys-diabodies

Aliquots of purified proteins were analyzed by SDS–PAGE pre-cast 4–20% polyacrylamide Ready Gels (Bio-Rad Laboratories) under non-reducing and reducing (1 mM DTT) conditions and stained using MicrowaveBlue (Protiga, Frederick, MD). Samples were also subjected to size-exclusion HPLC on Superdex 75 (Amersham Biosciences). Retention time was compared with a standard of parental diabody. Binding to CEA was initially assessed by ELISA as described above. Competition/Scatchard was also carried out in ELISA microtiter plates wells coated with N-A3, using a fixed concentration (1 nM) of biotinylated chimeric T84.66 antibody and increasing concentration of non-biotinylated competitors (0.01–100 nM). Displacement was monitored with alkaline phosphatase-conjugated streptavidin (1:5000 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA) and color was developed with phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.8. All assays were carried out in triplicate.

Radioiodination

A 70 μg amount of purified Cys-diabody was radiolabeled with 140 μCi $Na^{131}I$ (Perkin-Elmer Life Sciences, Boston, MA) in 0.1 M phosphate buffer at pH 7.5, using 1.5 ml polypropylene tubes coated with 10 μg of Iodogen (Pierce, Rockford, IL). Following 5–7 min of incubation at room temperature, the sample was purified by HPLC on Superdex 75. Peak fractions were selected and diluted in normal saline/1% human serum albumin to prepare doses for injection. The labeling efficiency was 85%. Immunoreactivity and valency were determined by incubation of radiolabeled protein with a 20-fold excess of CEA at 37°C for 15 min, followed by HPLC size-exclusion chromatography on a calibrated Superose 6 column (Amersham Biosciences).

Biodistribution in tumor-bearing mice

Female athymic mice (7–8 weeks old) were injected subcutaneously in the flank with 10^6 LS174T human colon carcinoma cells (ATCC CL-188). At 7 days post-inoculation, mice bearing LS174T xenografts were injected with 1 μg of ^{131}I-labeled Cys-diabody (specific activity 1.7 μCi/μg) via the tail vein. Groups of five mice were killed and dissected at 0, 2, 4, 6, 18 and 24 h post-injection. Major organs were weighed and counted in a gamma scintillation counter. Radiouptakes in organs were corrected for decay and expressed as percentage of injected dose per gram of tissue (% ID/g) and as percentage of injected dose per organ (% ID/organ). Tumor masses ranged from an average of 0.580 g (0 h group) to 1.058 g (24 h group). Biodistribution data are summarized as means and corresponding standard errors (SEM). Animal blood curves were calculated using the ADAPT II software (D'Argenio and Schumitzky, 1979) to estimate two rate constants (k_i) and associated amplitudes (A_i).

Conjugation and radiometal labeling of Cys-diabodies

The VTVS-LGGC Cys-diabody was reduced and conjugated with a bifunctional chelating agent comprised of the macrocyclic chelate DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), a tetrapeptide linker and a hexanevinyl sulfone group for chemical attachment to thiol groups. This compound, DOTA-glycylleucylglycyl(ε-aminobis-1,6-hexanevinyl sulfone)lysine, abbreviated DOTA-GLGK-HVS, has been described in detail previously (Li *et al*., 2002). VTVS-LGGC Cys-diabody (2 mg in 0.5 ml of PBS) was reduced by treatment with 20 μl of 20 mM tris(carboxyethyl)phosphine (TCEP) (Pierce) in PBS for 2 h at 37°C under Ar and centrifuged through a Sephadex G25 spin column. DOTA-GLGK-HVS (58 μl of 20 mM in PBS) was added and the solution rotated at 10 r.p.m. for 4 h at 25°C. The conjugate was dialyzed against 0.25 M NH_4OAc, pH 7.0. The extent of modification was evaluated with isoelectric focusing gels (Li *et al*., 2002).

Radiolabeling of Cys-diabody conjugates with copper-64

Copper-64 (copper chloride in 0.1 M HCl; radionuclide purity >99%) was produced in a cyclotron from enriched ^{64}Ni targets at the Mallinckrodt Institute of Radiology, Washington University Medical Center (McCarthy *et al*., 1997). DOTA-GLGK-HVS-conjugated Cys-diabody (200 μg) was incubated with 7.3 mCi of ^{64}Cu in 0.1 M ammonium citrate, pH 5.5, for 1 h at 43°C. The reaction was terminated by addition of EDTA to 1 mM. Labeled protein was purified by size-exclusion HPLC on a TSK2000 column (30 cm×7.5 mm i.d.) (Toso-Haas, Montgomeryville, PA). The radiolabeling efficiency was 56% and the specific activity was 1.7 μCi/μg.

MicroPET imaging

CEA-positive (LS174T) and CEA-negative (C6 rat glioma) xenografts were established in nude mice by subcutaneous injection of $(1–2)\times10^6$ cells subcutaneously into the shoulder area 10–14 days prior to imaging. Mice were imaged using the dedicated small-animal microPET scanner developed at the Crump Institute for Molecular Imaging (UCLA) (Chatziioannou *et al*., 1999). Mice were injected in the tail vein with 57 μCi of ^{64}Cu-diabody. After the appropriate time had elapsed, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (7 mg/kg) injected intraperitoneally, placed in a prone position and imaged using the microPET scanner with the long axis of the mouse parallel to the long axis of the scanner. The acquisition time was 56 min (8 min per bed position; seven bed positions) and images were reconstructed using a MAP reconstruction algorithm (Qi *et al*., 1998). Images were displayed and regions of interest (ROIs) were drawn and quantitated using AMIDE (Loening and Gambhir, 2003).

Results

Expression, purification and characterization of Cys-diabodies

The Cys-diabodies were expressed in the mouse myeloma cell line NS0. The expression level was low (<1 μg/ml in T-flasks) for the Cys-linker and VTVS-C constructs, whereas the expression level for VTVS-SGGC and VTVS-LGGC was

between 5 and 20 μg/ml as determined by ELISA. Cultures were expanded in T-flasks and supernatants collected. The VTVS-SGGC and VTVS-LGGC Cys-diabodies were purified essentially as described (Yazaki *et al*., 2001a).

Analysis of the purified proteins by SDS–PAGE demonstrated that the two-step purification scheme yielded VTVS-SGGC and VTVS-LGGC diabodies that were >95% pure (Figure 2A), whereas the other two constructs could not be readily separated from other proteins present in the growth medium (not shown). Under non-reducing conditions both proteins migrated as single species in the range 55–60 kDa with the VTVS-LGGC version exhibiting slightly lower mobility (Figure 2A, lanes 1 and 2). Under reducing conditions both proteins demonstrated the presence of the expected 25 kDa monomer (Figure 2A, lanes 4 and 5). Thus, when purified under native conditions, both of these Cys-diabodies existed as essentially pure disulfide-bonded homodimers. Small amounts of the Cys-linker diabody were purified on protein L. It migrated as higher molecular weight proteins (aggregates) as well as monomers under non-reducing conditions. The aggregates were only partially reduced under reducing conditions. The VTVS-C version of Cys-diabody was never expressed in sufficient quantities to permit purification and biochemical characterization. The remainder of the work was focused on the VTVS-LGGC Cys-diabody as it expressed very well and existed as a homogenous covalent homodimer.

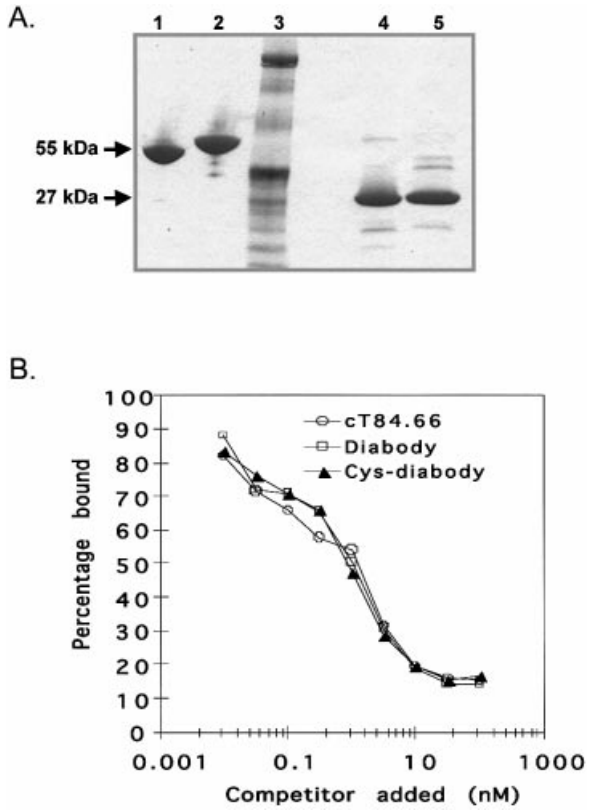

Fig. 2. Biochemical characterization of purified Cys-diabodies. (**A**) SDS–PAGE. Lanes: 1 = non-reduced -LGGC; 2 = non-reduced -SGGC; 4 = reduced -LGGC; 5 = reduced -SGGC. In addition to the primary bands, some impurities are visible. Lane 3 = molecular weight markers. (**B**) Competitive ELISA binding assay. Plates were coated with CEA and biotinylated intact, chimeric anti-CEA antibody was used as probe.

Size-exclusion chromatography demonstrated that the covalently linked Cys-diabody appeared slightly smaller than the regular diabody, as it eluted at 20.42 min (average of five experiments) as opposed to 20.13 min (average of four experiments) (not shown).

The binding activity of the Cys-diabody to CEA was initially demonstrated by ELISA. Affinity was measured by competition ELISA in the presence of competitors at different concentrations. As shown in Figure 2B, by competition assay the affinities of the Cys-diabody and parental diabody were essentially the same as that of the intact chimeric T84.66 antibody.

The immunoreactivity and valency of the Cys-diabody were analyzed following radioiodination by solution-phase incubation in the presence of excess CEA. Size-exclusion HPLC analysis demonstrated that 90% of the Cys-diabody shifted to high molecular weight complexes indicated by two peaks, suggesting that the Cys-diabody was bound to one and two CEA molecules (not shown).

In vivo biodistribution and targeting

The ^{131}I-labeled Cys-diabody was assessed for its ability to target tumor in athymic mice bearing xenografts of LS174T human colon carcinoma cells. As can be seen in Table I, the accumulation of the ^{131}I-labeled Cys-diabody reached 9.32% ID/g at 2 h and this level of localization was maintained at 4 and 6 h post-injection. Blood clearance was rapid and nearly

Table I. Biodistribution of ^{131}I-labeled T84.66 Cys-diabody in athymic mice bearing LS174T xenografts

Organ (%ID/g)	0 h	2 h	4 h	6 h	18 h	24 h
Tumor	2.49 (0.33)	9.32 (1.73)	10.02 (0.69)	9.15 (0.77)	6.43 (1.05)	4.79 (1.28)
Blood	36.34 (2.70)	5.87 (0.63)	4.04 (0.53)	3.28 (0.27)	0.55 (0.13)	0.36 (0.09)
Liver	8.40 (0.61)	3.16 (0.81)	2.66 (0.64)	1.67 (0.27)	0.64 (0.24)	0.32 (0.06)
Spleen	5.67 (0.80)	2.54 (0.39)	1.57 (0.20)	1.30 (0.14)	0.34 (0.11)	0.19 (0.04)
Kidney	18.58 (1.22)	4.03 (0.57)	2.99 (0.51)	2.09 (0.25)	0.47 (0.09)	0.37 (0.04)
Lung	9.90 (0.69)	3.40 (0.48)	2.50 (0.39)	1.94 (0.17)	0.46 (0.10)	0.27 (0.07)
Ratios						
Tumor:blood	0.07	1.59	2.48	2.79	11.69	13.31
Tumor:kidney	0.13	2.31	3.35	4.38	13.68	12.95
Tumor:liver	0.30	2.95	3.77	5.48	10.05	14.97
Tumor						
Tumor weight	0.58 (0.28)	0.70 (0.20)	0.69 (0.09)	0.63 (0.19)	1.02 (0.54)	1.06 (0.53)

Groups of five mice were analyzed at each time point. Tumor and normal organ uptake are expressed as percentage injected dose per gram (% ID/g). Table values are the means with corresponding standard errors of the means (SEM) shown in parentheses. The ratios presented are the averages of the tumor:blood, tumor:kidney and tumor:liver ratios for the individual mice.

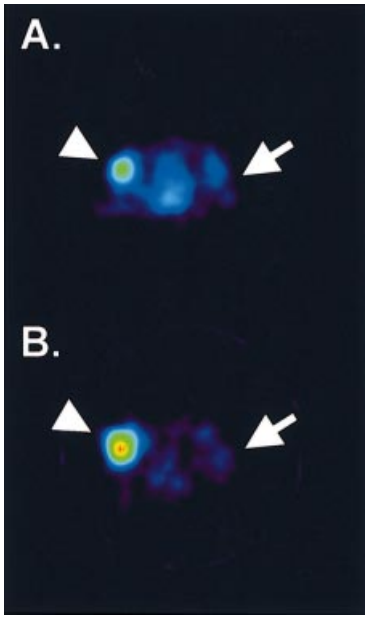

Fig. 3. Transverse slices of serial microPET scans of a mouse bearing bilateral C6 (arrow) and LS174T (arrowhead) xenografts, injected with ^{64}Cu Cys-diabody (57 μCi) and imaged at (**A**) 4 and (**B**) 18 h.

complete by 18 h (0.55% ID/g), with the half-life in the beta-phase being 2.68 h, essentially the same as that observed with the non-covalently bound iodinated diabody (2.89 h) (Yazaki *et al*., 2001b). Activities in other normal organs (liver, spleen, lung, kidneys) also fell rapidly and were below 1% ID/g by 18 h. These biodistribution results were essentially identical with those observed for the iodinated parental anti-CEA diabody (Wu *et al*., 1999).

In vivo imaging by microPET

Cys-diabody was conjugated with the macrocyclic chelate DOTA using a novel peptide–hexanevinyl sulfone derivative described in detail elsewhere (Li *et al*., 2002). This allowed efficient radiolabeling with ^{64}Cu, a positron-emitting radionuclide with a 12.7 h half-life, well matched to the targeting and clearance kinetics observed for diabodies in murine systems *in vivo*. MicroPET imaging studies were conducted on athymic mice bearing LS174T (CEA-positive human colorectal carcinoma) and C6 (CEA-negative rat glioma) xenografts. Specific targeting to the CEA-positive xenograft was observed at 4 and 18 h post-injection, with little evidence of activity in the CEA-negative tumor at 18 h (Figure 3). The positive tumor/control tumor uptake ratio deducted from ROI analysis was 4.6:1 at 18 h, demonstrating specificity. This ratio is comparable to that observed with the ^{64}Cu anti-CEA mininbody (3.4:1) (Wu *et al*., 2000). However, this particular protein–chelate–radionuclide combination (^{64}Cu-DOTA-GLGK-HVS Cys-diabody) resulted in elevated liver activity (19.4% ID/g at 4 h) in addition to kidney activity (55.1% ID/g at 4 h) (Li *et al*., 2002).

Discussion

This paper describes the design, production and evaluation of a novel antibody format, a covalently linked (disulfide-bonded) diabody which we refer to as the Cys-diabody. Four constructs were made, including a version with the cysteine incorporated into the intervariable domain linker or at the C-terminus with or without a short linker peptide. The initial intent was to introduce cysteine residues into the anti-CEA diabody in order to provide specific sites for chemical modification including conjugation and radiolabeling. We unexpectedly discovered that addition of the sequence GGC to the end of the protein resulted in a diabody in which the C-termini of the V_L–V_H subunits came together and formed a disulfide bond. Two slightly different versions of the Cys-diabody (with C-terminal sequences of LGGC or SGGC) resulted in essentially 100% formation of the disulfide linkage. This protein has two key improvements compared with a standard diabody: covalent linkage for greater potential stability and the feasibility of site-specific modification following reduction of the disulfide bond and generation of free reactive thiols. An added bonus is that the introduced cysteine residues are essentially 'protected' through the internal linkage and prevented from forming random disulfide bonds with small molecules (such as glutathione) or other sulfhydryl-containing proteins present in the cell. As a result, the Cys-diabody can be obtained in higher amounts and with greater purity than might be expected for proteins containing engineered cysteine residues that are unpaired and accessible to fortuitous disulfide formation. Unpaired cysteine residues and formation of aggregates are probably the reasons why the two other constructs did not express that well. A study comparing the effects of unpaired cysteines on yield, solubility and activity of recombinant antibody constructs in *Escherichia coli* shows that the removal of unpaired cysteines increases the yield, activity and purity significantly (Schmiedl *et al*., 2000). In eukaryotic cells, it has been shown that the penultimate cysteine (Cys575) in the secretory tail-piece in IgM (μtp) plays a crucial role in retention and degradation of the IgM subunits in the endoplasmic reticulum (ER) (Sitia *et al*., 1990). It was demonstrated that the IgM subunits were secreted when the Cys575 was removed by mutagenesis (Sitia *et al*., 1990) and when the intracellular redox potential was altered by β-mercaptoethanol (Alberini *et al*., 1990). Thus, the role of free thiol groups in preventing unhindered transport of proteins through the secretory pathway in eukaryotic cells has been proposed (Alberini *et al*., 1990; Sitia *et al*., 1990). Intracellular retention has been observed for acetylcholinesterase with a C-terminal cysteine (Kerem *et al*., 1993) and for proteins with the μtp appended to their C-terminus, such as cathepsin D (Fra *et al*., 1993) and engineered antibody fragments (Olafsen *et al*., 1998). Hence, ER retention and aggregation seem to be the fate of thiol-exposing proteins and probably explains the low recovery of our two other Cys-diabody constructs.

The crystal structure of the parental T84.66/GS8 diabody has recently been solved (Carmichael *et al*., 2003). In the crystal the Fv units of the diabody assumed a very compact, twisted structure, with the binding sites oriented in a skewed orientation at a tight 70° angle. The C-termini of the heavy chain variable regions (where we have appended the Cys residues) are ~60 Å apart. However, the structure that was solved is likely to represent one of many conformations that the parental diabody can adopt. The fact that the Cys-diabody forms with such high efficiency implies that the parental diabody is flexible and that the Fv domains can swivel such that the C-termini are juxtaposed. Figure 4 shows the crystal structure of the parental diabody (A) and a model where the Fvs have been rotated to bring the C-termini close enough for disulfide bridge formation [represented by the blue (Gly) and yellow

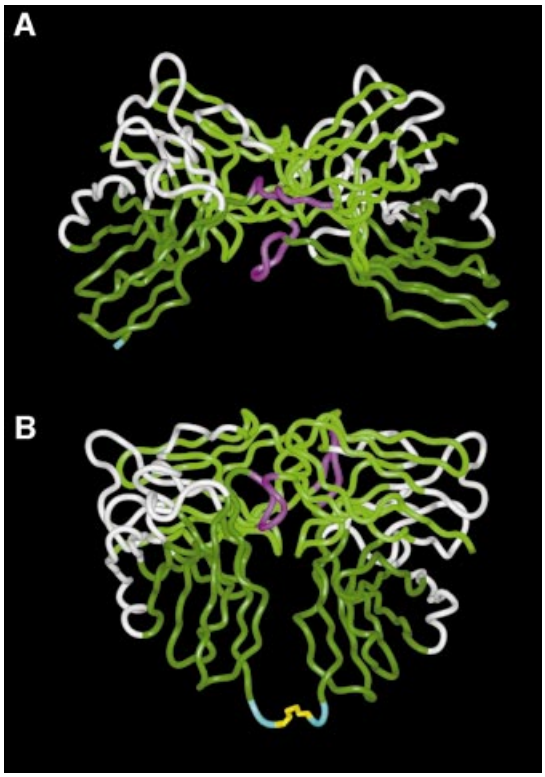

Fig. 4. (**A**) Published crystal structure of the parental anti-CEA diabody and (**B**) a model of the structure of -SGGC Cys-diabody where the Fvs have been rotated to bring the C-termini close enough for disulfide bridge formation [represented by the blue (Gly) and yellow (Cys) residues in the model].

(Cys) residues in the model] (B). Size-exclusion HPLC analysis confirms the notion that the native, non-covalent diabody is fairly flexible; it elutes at an earlier retention time, suggesting a larger Stokes radius. By contrast, the covalently linked Cys-diabody elutes at a later retention time, implying a more compact, more highly constrained molecule. Taken together, these results suggest that the native diabody has an open and flexible structure.

The VTVS-LGGC version expressed well, was purified in milligram quantities and was selected for further study. Initial biodistribution studies using radioiodinated Cys-diabody shows that this protein demonstrates equivalent targeting to parental anti-CEA diabody. Thus the tethering of the C-termini of the protein subunits in the diabody appears to have no detrimental effect on the *in vivo* tumor targeting and blood clearance properties of the diabody. However, the performance of the Cys-diabody immunoconjugate (^{64}Cu-DOTA-GLGK-HVS Cys-diabody) was not optimal *in vivo*, resulting in high kidney and liver activities. The microPET imaging studies shown here confirm the previous biodistribution experiments (Li *et al*., 2002). These results can be compared with the biodistribution determined for the parental T84.66 diabody conjugated of DOTA to random surface residues and radiolabeled with In-111 (Yazaki *et al*., 2001b). In that work, kidney activity was extremely elevated and liver activity was low. Use of the novel peptide–DOTA and conjugation to the free sulfhydryls following reduction of the disulfide provided a facile and site-specific means for labeling the protein with radiometal. In the present work following reduction, the Cys-diabody was readily modified with a bifunctional chelating agent, consisting of DOTA, a tetrapeptide linker and a hexanevinyl sulfone moiety as a thiol-specific reactive group. Furthermore, the DOTA conjugated diabody was efficiently labeled with Cu-64. The peptide linker (GLGK) was designed to favor lower kidney retention, owing to the incorporation of a lysine residue to provide a target for kidney brush border carboxypeptidase activity (Arano *et al*., 1999). The kidney activity was indeed lowered; however, the liver activity was increased. Experiments are in progress to optimize further the peptide linker chemistry in order to reduce the normal tissue accumulation of the Cys-diabody radioimmunoconjugate and its metabolites.

The novel covalently-linked Cys-diabody described here provides a platform for ready conjugation of a wide variety of effector moieties to this rapid targeting anti-tumor molecule. These would include alternative radiolabeling approaches; for example, we are developing an F-18 tag with thiol-specific chemistry for generating additional PET tracers. Other tags such as optical or fluorescent probes can be site-specifically attached. In addition, the C-terminal cysteine residues can be used for conjugation of other small molecules such as chemotherapeutic drugs. This engineered antibody fragment has a unique advantage: the presence of the cysteine thiol groups in an internal, protected format, which can be released when required for chemical modification.

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