Isolation of anti-glutathione antibodies from a phage display library

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We have isolated anti-glutathione antibodies from a human synthetic phage antibody scFv library (Nissim,A., Hoogenboom,H.R., Tomlinson,I.M., Flynn,G., Midgley,C., Lane,D. and Winter,G., 1994, EMBO J., 13, 692-698). Glutathione (GSH) conjugates with carrier proteins, such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) and human lysozyme (LZM), were used as antigens. After four cycles of panning and affinity chromatography, clones that recognized GSH-conjugated proteins, but not BSA, KLH or LZM, were isolated. The isolated phage antibody and the soluble scFv fragments were characterized by immunoblotting, and the nucleotide sequences of the VH segments of selected clones were determined. The binding of several isolates to GSH–BSA was competitively inhibited by GSH in an ELISA. These observations have demonstrated that antibodies against GSH, a tripeptide, can be isolated from the library. We constructed the tertiary models of several scFv fragments and discussed the mechanism of antigen binding sites.

Keywords: anti-glutathione antibody/disulfide bond/glutathione/phage display library/protein folding

Introduction

The formation of disulfide bonds in secretory proteins is known to be crucial in the process of protein folding in vivo; however, its mechanism still remains unclear. We previously reported that the oxidation step in disulfide bond formation in the mutant human lysozyme C77A, in which Cys77 is replaced by alanine, involves a mixed disulfide with glutathione (GSH) at Cys95 (Taniyama et al., 1990). Subsequently, glutathione disulfide was found to be the source of oxidizing equivalents in the endoplasmic reticulum (ER) (Hwang et al., 1992). These observations suggest that the glutathionylated protein mimics the in vivo intermediate just prior to the formation of the disulfide bond Cys77–Cys95 of human lysozyme. Therefore an anti-GSH antibody may be a useful means to study the detailed mechanism of the involvement of GSH in the folding of a disulfide bonded protein in vivo. However, since GSH is a small flexible tripeptide and exists intracellularly as a self-component, obtaining an anti-GSH antibody by immunization would be difficult. We decided to isolate anti-GSH antibodies from a phage display library, because scFv is smaller than antibody and structure prediction of GSH–scFv complex is rather easy.

The recent success in producing recombinant antibodies in prokaryotic systems has opened up new perspectives in the fields of immunological research and therapeutic applications (Winter et al., 1994). The display of antibody fragments on the surface of filamentous bacteriophage (McCafferty et al., 1990; Hoogenboom et al., 1991) by fusion to a phage minor coat protein, and the selection of the phage with antigen, provides a powerful means of creating antibodies with binding specificity from V gene repertoires. The display and selection of antibody fragments on the surface of phage mimics immune selection (Marks et al., 1992), and antibodies have also been isolated without immunization from repertoires of V gene rearrangements in vivo (Marks et al., 1991) and in vitro (Hoogenboom and Winter, 1992). The same phage repertory may be used to generate many different binding specificities, including those that are difficult to raise by immunization. Recently, it was reported that antibodies against self-antigens, such as p53, thyroglobulin (Nissim et al., 1994) and TNFα (tumor necrosis factor) (Griffiths et al., 1994) were isolated from a phage antibody library.

We now report the isolation of anti-GSH antibodies from a phage display library, and the characterization of several clones.

Materials and methods

A phage display antibody library was kindly supplied by Dr G.Winter. GSH was purchased from WAKO Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) were from Sigma Chemical (St Louis, USA) and Calbiochem-Novabiochem Corporation (California, USA), respectively. Human lysozyme was prepared by the method described previously (Yoshimura et al., 1987).

Bacterial strains

Escherichia coli strain TG-1 [K12, Δ(lac-pro), supE, thi, hsdSΔ5/F′traD36, proAΔ32 ΔlacZΔM15] was used for the phage rescue. The non-suppressor E.coli strain HB2151 [K12, ara, Δ(lac-pro), thiF′proA+ ΔlacZΔM15] was used for the preparation of single-chain Fv fragments.

Conjugation of GSH with carrier proteins

A 1.5 mg amount of sulfo-succinimidyl 4-(N-maleimido-methyl) cyclohexane-1-carboxylate (Sufo-SMCC) (PIERCE, Illinois, USA) was dissolved in 1 ml conjugation buffer (100 mM sodium phosphate buffer, 0.9 M NaCl, 0.1 M EDTA, pH 7.0), and either 4 mg BSA or 4 mg KLH was added. The mixture was stirred for 2 h at 30°C and was loaded on a PD-10 column (Pharmacia Biotech, Uppsala, Sweden), as recommended by the supplier. Conjugated proteins were fractionated as the peak fractions at 280 nm absorbance, and were added by 4 mg GSH dissolved in 0.5 ml conjugation buffer. After an incubation at 4°C overnight, the reaction mixture was fractionated on a PD-10 column equilibrated with purification buffer (100 mM sodium phosphate buffer, 0.9 M NaCl, pH 7.0), and the protein fractions were pooled. The reduction of human lysozyme was carried out with the Reduce-ImmTM...
Reducing Kit (PIERCE, Illinois, USA), and the product was modified with GSH by the method of Ellman (1958).

Rescue of phagemid particles from the library

The phage library used for the selection of antibodies was described previously (Hoogenboom and Winter, 1992). This library contains a diverse repertory of human VH genes from 50 germline VH gene segments with variable third complementarity determining regions (CDR3) of 4–12 residues (Nissim et al., 1994), which are combined to a human V\(\lambda\) light chain gene segment. To rescue the phagemid particles, the library cells (approximately 10\(^8\) cells) (TG-1) were inoculated into 50 ml of 2\(\times\)TY medium (1.6% tryptone, 1% yeast extract and 1% NaCl) containing 100 \(\mu\)g/ml ampicillin and 1% glucose and were grown at 37°C with shaking until OD\(_{600}\) reached 0.5. A 10 ml aliquot of this culture was infected with 2\(\times\)10\(^9\) plaque forming units (p.f.u.) of VCS-M13 helper phage (Stratagene, California, USA), and the mixture was incubated for 30 min at 37°C without shaking. The phage particles were purified from the culture and were concentrated by three PEG-precipitation steps, as described (Marks et al., 1991).

Selection of GSH binders by panning and by affinity column chromatography

The purified phage particles were panned for binding using Nunc Maxisorp immuno test tubes (Gibco-BRL, Maryland, USA) coated with GSH-conjugated BSA (Marks et al., 1991; Nissim et al., 1994) or were affinity purified on a GSH-bound Sepharose 4B column (Pharmacia Biotech, Uppsala, Sweden). To coat the test tubes, GSH-conjugated BSA and GSH-conjugated KLH in PBS (0.1 M NaCl, 33 mM Na\(_2\)HPO\(_4\), 17 mM NaH\(_2\)PO\(_4\)) were used at a concentration of 20 \(\mu\)g/ml with an overnight incubation. For each round of panning, the wells were washed 15 times with PBS containing 0.1% (v/v) Tween-20, and 15 times with PBS, and were eluted with 100 mM triethylamine. The eluate was immediately neutralized with 1.0 M Tris–HCl, pH 7.4, and was subjected to the next round of panning. Selection of phage by column chromatography was carried out as described by Griffiths et al. (1993). After the panning solution was loaded onto the GSH-bound Sepharose 4B column, which was equilibrated with PBS containing 2% skimmed milk (MPBS), the column was washed with 10 ml MPBS, 10 ml PBS (pH 7.2) and 10 ml of 50 mM Tris–HCl buffer (pH 9.0) containing 500 mM NaCl. Finally, the phage were eluted with 5 ml of 100 mM triethylamine and were neutralized with 1.0 M Tris–HCl buffer (pH 7.4).

Screening and sequencing of clones

The phage were rescued from single colonies of the infected E.coli suppressor strain TG-1, using the VCS-M13 helper phage (Clackson et al., 1991), and the soluble scFv fragments were prepared from the E.coli non-suppressor strain HB2151 (Marks et al., 1991). The phage and the soluble scFv fragments were screened for binding to GSH by ELISA. The 96-well ELISA plates were coated with GSH-conjugated KLH (1 \(\mu\)g/ml). The binding of phage to GSH was assayed by a detection module kit (Pharmacia Biotech, Uppsala, Sweden). The binding of the soluble scFv fragments was detected with the mouse monoclonal antibody 9E10 (4 \(\mu\)g/ml), which recognizes the c-myc tag peptide (Munro and Pelham, 1986), and peroxidase-conjugated anti-mouse Fc antibody (Sigma Chemical, St Louis, USA) was used as the second antibody.

The nucleotide sequences of the VH segments of the phage antibodies with binding activities to GSH were determined by

<table>
<thead>
<tr>
<th>Table I. Frequency of binding clones from scFv libraries before and after selection</th>
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<tbody>
<tr>
<td>Rounds of selection (panning)</td>
</tr>
<tr>
<td>anti GSH</td>
</tr>
<tr>
<td>anti BiP(^a)</td>
</tr>
<tr>
<td>anti NIP(^b)</td>
</tr>
</tbody>
</table>

\(^a\)BiP, immunoglobulin heavy chain binding protein.

\(^b\)NIP, 4-hydroxy-5-iodo-3-nitrophenylacetyl.

GSH and NIP were conjugated with BSA and used as antigens. After panning, phage were prepared from 192 colonies each and their binding against antibodies were examined by ELISA.

Western blotting and dot blotting

Five micrograms of each antigen were fractionated on a 13.5% SDS-polyacrylamide gel (Laemmli, 1970), and then were electroblotted by the semidy method. The filters were blocked and were incubated with the scFv fragments (0.5 ml of the supernatant) or the phage (10\(^{10}\) p.f.u./ml). The scFv fragments were detected with the mouse monoclonal antibody 9E10, followed by an HRP-conjugated anti-mouse antibody (Sigma Chemical, St Louis, USA), and the phage were detected with an HRP-conjugated sheep anti-M13 phage antibody (Pharmacia Biotech, Uppsala, Sweden). The peroxidase activity was detected with an ECL kit (Amersham, Buckinghamshire, UK). For dot blotting, 2 \(\mu\)g of each antigen were dotted onto nitrocellulose membranes, and the membranes were treated in the same manner as the western blots.

Inhibition ELISA

The detection of phage scFv fragments bound to GSH was performed by ELISA. The 96-well plates were coated with 10 \(\mu\)g/ml GSH–BSA in PBS overnight at room temperature, and were blocked with MPBS. After adding 10 \(\mu\)l aliquots of various concentrations of GSH to each well, phage particles or scFv fragments were added, and antibody binding of the antibodies was detected by ELISA, as previously described.

Results

Selection of anti-GSH phage antibodies

We performed a selection of anti-GSH antibodies from a phage display library obtained from Nissim et al. (1994). This phage display library contains at least 10\(^7\) different clones.

To strengthen the binding of GSH to the immunotube, we prepared BSA-conjugated GSH (GSH–BSA), as described in the Materials and methods. Before screening, as a positive control, we confirmed that anti-NIP and anti-BiP phage antibodies were successfully selected from the Nissim library (Table I), as described (Nissim et al., 1994).

After excluding the phage that bound to BSA, the clones were screened by panning using GSH–BSA as an antigen. With each round of panning, we detected the anti-GSH antibody by ELISA. After the first two rounds of panning, we did not obtain any clones with GSH binding activities. Three rounds of panning produced four GSH-binding clones per 192 clones, and after four rounds, 56 of the 192 clones were found to bind GSH–BSA (Table I). For some clones, the binding activities were detected using phage supernatants from infected bacterial
Table II. Deduced VH-CDR3 sequences and germline origins of selected anti-GSH antibody

<table>
<thead>
<tr>
<th>Clone</th>
<th>CDR3 sequences</th>
<th>VH segment</th>
<th>Family</th>
<th>Germline</th>
</tr>
</thead>
<tbody>
<tr>
<td>20C9</td>
<td>SNRGNDW</td>
<td>VH1</td>
<td>DP-8</td>
<td></td>
</tr>
<tr>
<td>20D2</td>
<td>SGVGVMSR</td>
<td>VH1</td>
<td>DP-8</td>
<td></td>
</tr>
<tr>
<td>20D7</td>
<td>SSQGVMASA</td>
<td>VH1</td>
<td>DP-8</td>
<td></td>
</tr>
<tr>
<td>12D4</td>
<td>TMTMARFRH</td>
<td>VH1</td>
<td>DP-23</td>
<td></td>
</tr>
<tr>
<td>6D5</td>
<td>CREDL</td>
<td>VH1</td>
<td>DP-25</td>
<td></td>
</tr>
<tr>
<td>11F12</td>
<td>LTRNEKFSR</td>
<td>VH1</td>
<td>DP-25</td>
<td></td>
</tr>
<tr>
<td>20G11</td>
<td>TGLQRP</td>
<td>VH3</td>
<td>DP-45</td>
<td></td>
</tr>
<tr>
<td>18C2</td>
<td>AWDVRT(E)R</td>
<td>VH3</td>
<td>DP-45</td>
<td></td>
</tr>
<tr>
<td>20B5</td>
<td>GLELNLSRY</td>
<td>VH3</td>
<td>DP-45</td>
<td></td>
</tr>
<tr>
<td>20E9</td>
<td>DVPW(E)VRERIH</td>
<td>VH3</td>
<td>DP-45</td>
<td></td>
</tr>
<tr>
<td>20A2</td>
<td>WKRHLHEA</td>
<td>VH3</td>
<td>DP-47</td>
<td></td>
</tr>
<tr>
<td>20A4</td>
<td>WTSGGSSIV</td>
<td>VH3</td>
<td>DP-47</td>
<td></td>
</tr>
<tr>
<td>18B10</td>
<td>TQYHVRYHVL</td>
<td>VH3</td>
<td>DP-47</td>
<td></td>
</tr>
<tr>
<td>20E6</td>
<td>RMRRVSV</td>
<td>VH3</td>
<td>DP-53</td>
<td></td>
</tr>
<tr>
<td>19F3</td>
<td>TWDVRT(E)R</td>
<td>VH4</td>
<td>DP-66</td>
<td></td>
</tr>
</tbody>
</table>

*Human germline VH gene segments were assigned as reported by Tomlinson et al. (1992), (E) represents translation of amber codon to glutamine in E.coli supE strain.*

cultures, instead of scFv fragments. These clones bound neither BSA nor KLH by ELISA.

**Sequencing**

We determined the nucleotide sequences of the VH segments of these clones by the dideoxy method, as described in the Materials and methods, and selected different clones. Finally, we selected 15 clones, as shown in Table II. Some clones that contained a translation of an amber codon to glutamine in the E.coli supE strain were detected only as phage antibodies. The deduced CDR3 sequences varied for the individual clones, except for 20D2 and 20D7. These two clones have highly similar CDR3 sequences. The number of CDR3 residues varied from five to 11. Eight and 11 residues in the CDR3 loops were frequently found. Clone 6D5 has a short CDR3 sequence, and a strong GSH-binding affinity (see Figure 3). However, it is not stable and we did not use this clone for further characterization except for structure prediction. The VH segments were derived from the VH1, VH3 and VH4 families. The germlines were not highly variable and were mainly from the DP-8, DP-45 and DP-47 lineages.

**Western blotting and dot blotting**

After screening for the anti-GSH antibodies, we performed western and dot blotting by using phage and scFv fragments. The phage, prepared by PEG precipitation as described, were used in immunoblotting with an ECL kit to detect GSH conjugated BSA or KLH blotted onto Hybond-ECL membranes (Amersham, Buckinghamshire, UK) after PAGE. The scFv fragments were also prepared from bacterial supernatants. As shown in Figure 1, GSH–BSA and GSH–KLH were detected by using the scFv fragments of 18B10, 20C9 and 20G11, and by using the phage 18C2. Dot blotting (Figure 2) indicated that the scFv fragment of 20C9 bound to GSH–KLH, GSH–BSA, GSH–LZM and GSH itself, but not to BSA or LZM. Binding of the phage clone 18C2 to GSH–LZM and GSH–KLH was also shown. The binding to GSH–LZM as an antigen was weaker than the binding to GSH–BSA and GSH–KLH. This may be because the number of GSH molecules bound to LZM is smaller than those bound to BSA and KLH, or because there is no linker between LZM and GSH. From these results, the phage antibodies that we obtained are able to recognize and bind to a small peptide, GSH, and GSH-conjugated proteins, such as GSH–BSA, GSH–KLH and GSH–LZM. These observations demonstrate that these clones are useful for detecting GSH and proteins modified with GSH.

**Competitive inhibition of selected phage antibodies**

To examine the binding of selected phage antibodies to GSH, we performed an inhibition ELISA. We used GSH–BSA and a reduced form of GSH as an antigen and a competitive inhibitor, respectively. Typical examples are shown in Figure 3. As the amount of added GSH increased, the binding affinities of the clones 6D5 and 20C9 to GSH–BSA decreased, indicating that these clones recognize GSH. By adding 1 mg/ml GSH, the binding of clones 6D5 and 20C9 to GSH–BSA was completely inhibited, but that of clone 18B10 was only partially inhibited (data not shown). These results indicate that the GSH binding of clones 6D5 and 20C9 is stronger and more specific than that of clone 18B10.
Fig. 3. Competitive inhibition with GSH. The methods are described in the Materials and methods.

Fig. 4. Backbone model structures of the antigen binding sites of (a) 6D5, (b) 20C9 and (c) 18B10. The VH and VL segments are colored light pink and light blue, respectively. CDR3 in the VH segment is indicated by H3 s in red, and the other CDRs in the VH segment are indicated by H1 and H2 s in pink. The three CDRs in the VL segment are indicated by L1, L2 and L3 s in blue. Several residues of the CDRs, possibly related to GSH-binding, are indicated by circles at their Cα positions, with the one-letter amino acid code followed by the segment name, H or L. The GSH position is indicated by green circles, corresponding to the three amino acids.
Discussion

We have shown that antibodies with a range of GSH binding activities can be isolated from a phage antibody library without immunization. Glutathione exists in the ER at millimolar concentrations and maintains the oxidative environment of the ER (Hwang et al., 1992). Glutathione is also indispensable for the correct folding of various disulfide-bonded proteins; however, an increase of its concentration in blood and urine causes glutathionemia. Glutathione is a small self-tripeptide, which could not be used as an antigen for animal immunization because of immunological tolerance. Recently phage with a range of binding specificities against either self or foreign antigens were isolated from a single phage library (Marks et al., 1992; Griffiths et al., 1993; Nissim et al., 1994); nevertheless, there have been no reports on the isolation of phage antibodies against a flexible small compound, such as GSH. This success highlights the possibility of selecting self and low molecular weight compounds from a phage library.

The phage antibodies and the scFv fragments derived from segments of the human VH3 family were readily purified with the glutathione-Sepharose 4B column, which is routinely used for purifying glutathione-S-transferase. The sequences of the CDR3 domains of the isolated clones were comparatively variable, and generally contained a high number of residues. Both the isolated phage antibodies and glutathione-S-transferase recognize GSH, suggesting that some of the phages have a GSH-binding pocket mimicking that of the enzyme. Recently, the three-dimensional structures of various glutathione-S-transferases have been determined (Lim et al., 1994; McTigue et al., 1995; Ji et al., 1995; Wilce et al., 1995; Reinemer et al., 1996). These GSH-binding sites are composed of a variety of residues, but a distinctive tendency is observed. The residues Arg, Gln, Ser and Thr frequently form hydrogen bonds with the carboxyl group of γ-glutamate. The carboxyl group of Asp or Glu tends to form a salt bridge with the amide group of γ-glutamate. The Gln residues, as well as the Arg, Lys and Trp residues, interact with the terminal carboxylate atoms of the glutathione glycine. The glutathione peptide backbone is maintained by hydrogen bonds with the polar groups in the protein. In the CDR3 region sequences of the isolated clones, many of the aforementioned amino acid residues are found, as indicated in Table II.

In order to determine the recognition mechanisms of the current scFv fragments, tertiary models of 6D5, 20C9 and 18B10 were made. The frame structures of VL and VH segments were constructed from those in the HIL Fab fragment (Saul, F.A. and Poljak, R.J., unpublished results; PDB code, 1FAB). The common backbone structure of the CDR3 loop in the VL segment was based upon that in KOL (Kratzin et al., 1989; 2FB4). The CDR3 loops of 6D5, 20C9 and 18B10 in the VH segments were classified according to the amino acid sequences of the hypothetical rules proposed by Shirai et al. (1996), and the tertiary models were constructed using the CHA225 backbone structures (Love et al., 1993; 1HND), D1.3 (Bhat et al., 1994; 1VF5A), and 36–71 (Strong et al., 1991; 6FAB), respectively. The other CDR loops were constructed from the backbone structure of HIL Fab fragment, based upon the conventional classification of the canonical structures for the CDR loops except CDR3 in the VH segment (Chothia et al., 1989, 1992). The side-chain conformations were determined consistently using the dead-end elimination method (Tanimura et al., 1994). The entire fragment structures were optimized and the poor contacts were minimized by the conjugate gradient method, using the molecular mechanics program PRESTO (Morikami et al., 1993) with an AMBER all-atom force-field (Weiner et al., 1986).

In Figures 4a, b and c, the antigen binding sites of 6D5, 20C9 and 18B10, respectively, are shown with the hypothetical disposition of GSH. In every scFv fragment, several putative residues of CDR3 in the VH segment are considered to determine the binding activity, in cooperation with Arg91 on CDR3 in the VL segment and other polar side-chains of CDR1 in the VH segment. This recognition model for GSH seems similar to that of glutathione-S-transferase. Further investigation is necessary to understand the precise mechanism of GSH-binding.

Understanding the principles of protein folding in the cell will require the isolation and analysis of a folding intermediate. To this end, we inhibited the formation of the native disulfide bond of human lysozyme by mutation and successfully isolated the mutant C77A-a, which has a mixed disulfide with GSH at Cys95 (Taniyama et al., 1990). The C77A-a protein was predicted to mimic the intermediate of disulfide bond formation just prior to the formation of the Cys77–Cys95 bond. The GSH molecule was efficiently dissociated from C77A-a by PDI, but not by thioredoxin (Hayano et al., 1993), and the structural changes of the C77A-a protein were proved to be associated with disulfide bond formation at the last step of the folding process in vivo (Inaka et al., 1995). It was reported that the main function of PDI may be to relax the structure around the mixed disulfide bond (Nakamura et al., 1996). In view of these findings, it seems likely that the free thiol groups of the nascent polypeptides are modified by GSH after translocation to the ER, and then PDI catalyzes native disulfide bond formation using these mixed disulfides as substrates (Nakamura et al., 1996). Thus searching for proteins modified with glutathione will be a way to detect folding intermediates and to elucidate the folding mechanism through disulfide bond formation. The anti-GSH phage antibodies obtained here detected GSH–BSA, GSH–KLH, GSH–LZM and GSH by western and dot blotting. These phage antibodies will be useful in detecting GSH and proteins modified with glutathione.

Acknowledgements

We thank Dr G. Winter for providing the human synthetic phage antibody library and for valuable advice.

References

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Received 23 October 1997; revised 3 November 1997; accepted 22 December 1997