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Antibody fragments from a 'single pot' phage display library as immunochemical reagents

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The display of repertoires of antibody fragments on the surface of filamentous bacteriophage offers a new way of making antibodies with predefined binding specificities. Here we explored the use of this technology to make immunochemical reagents to a range of antigens by selection from a repertoire of $>10^8$ clones made *in vitro* from human V gene segments. From the same 'single pot' repertoire, phage were isolated with binding activities to each of 18 antigens, including the intracellular proteins p53, elongation factor EF-1α, immunoglobulin binding protein, rhombotin-2 oncogene protein and sex determining region Y protein. Both phage and scFv fragments secreted from infected bacteria were used as monoclonal and polyclonal reagents in Western blots. Furthermore the monoclonal reagents were used for epitope mapping (a new epitope of p53 was identified) and for staining of cells. This shows that antibody reagents for research can be readily derived from 'single pot' phage display libraries.

Key words: immunochemical reagents/immunoglobulins/phage/scFv

Introduction

Animal immunization has provided a wealth of valuable antisera and monoclonal antibodies as research reagents. However, the display of small peptides (Smith, 1985) and folded proteins (Bass *et al.*, 1990; McCafferty *et al.*, 1990) on filamentous phage, offers another source of such reagents. Thus antibody fragments have been made in bacteria by selection from repertoires of variable domains displayed on the surface of filamentous bacteriophage (for review see Hoogenboom *et al.*, 1992; Marks *et al.*, 1992a). Heavy and light chain variable domains are displayed by fusion to the viral coat protein, allowing phage with antigen binding activities (and encoding the antibody fragments) to be selected by panning on antigen. The selected phage can be grown after each round of panning and selected again, and rare phage ($<1/10^7$) isolated over several rounds of panning (McCafferty *et al.*, 1990). By interposing an amber mutation between the antibody fragment and coat protein, the fragments can also be secreted from infected bacteria as soluble fragments (Hoogenboom *et al.*, 1991). As in the immune system, selected antibodies from the primary library can be mutated, and those with improved affinities isolated (Hawkins *et al.*, 1992; Marks *et al.*, 1992b).

The repertoires of fragments, displayed on phage as single chain Fv fragments (McCafferty *et al.*, 1990) or Fab fragments (Hoogenboom *et al.*, 1991), are encoded by rearranged V genes. Diverse repertoires of heavy (V_H) and light (V_L) chain V genes can be derived from populations of lymphocytes (Sastry *et al.*, 1989; Ward *et al.*, 1989) using the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Orlandi *et al.*, 1989), and paired at random to encode diverse repertoires of fragments (Huse *et al.*, 1989). Immunization enriches the mRNA for V_H and V_L genes encoding antigen binding activities (Burton, 1991; Winter and Milstein, 1991), but requires a different library for each antigen.

By contrast, the use of larger and more diverse repertoires, either from V genes rearranged *in vivo* (Marks *et al.*, 1991a) or *in vitro* (Hoogenboom and Winter, 1992), can allow the isolation of antibodies of different binding specificities, without immunization, from the same library, as proposed by Milstein (1990). Such libraries have been used to produce antibodies against haptens, and foreign and self antigens (Marks *et al.*, 1991a; Hoogenboom and Winter, 1992; Griffiths *et al.*, 1993). Here we have explored the use of the same 'single pot' library to isolate antibodies with specificities that have proved difficult by hybridoma technology, for example against highly conserved intracellular proteins, and to use the phage and the encoded scFv fragments as immunochemical reagents.

Results

Selection of clones of different specificities

We built diverse repertoires of rearranged V_H genes *in vitro* from a bank of 50 cloned human V_H gene segments (Tomlinson *et al.*, 1992) and random nucleotide sequence encoding CDR3 lengths of 4-12 residues. The library was subjected to four or five rounds of selection against 18 antigens, including haptens, foreign and self-antigens, as well as intracellular antigens (Figure 1). Phage or secreted scFv fragments were screened for binding to the selecting antigen by ELISA, as a 'polyclonal' population and also as isolated clones. In all cases, phage with binding activities were detected to the selecting antigen. For some clones (see legend to Table I), binding activities were detected as phage but not as scFv fragments using supernatants from infected bacteria.

To obtain a diverse set of fragments for each antigen, clones with binding activities were screened for V_H family by hybridization, and for CDR3 length by PCR (see Materials and methods). Different clones were selected for sequencing. A range of CDR3 loop sizes were detected for several antigens, for example, loops of four, five, nine and

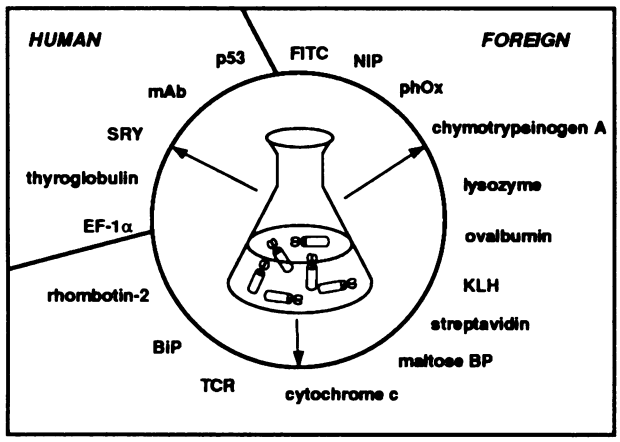

Fig. 1. Specificities from a 'single pot' human synthetic library. FITC, fluorescein isothiocyanate; NIP, 4-hydroxy-5-iodo-3-nitrophenylacetyl; phOx, 2-phenyl-5-oxazolone; KLH, haemocyanin from keyhole limpet; maltose BP, maltose binding protein; TCR, soluble chimeric murine T cell receptor; BiP, recombinant rat immunoglobulin binding protein; EF-1α, human elongation factor-1α; SRY, human sex-determining region Y protein; mAb, anti-erythrocyte rhesus D antibody Oak-3; p53, human tumour supressor protein p53.

ten residues for the hapten NIP. Over all the antigens, V_H segments were found from each of the V_H families except the small V_H2 family, as were all the CDR3 loop lengths represented in the library (Table I). Eight residue CDR3 loops were frequent. As *in vivo* (Zouali and Theze, 1991), segments of the V_H3 family (35/54) were well represented.

Western blots

Phage and scFv fragments were used as 'polyclonal' and monoclonal reagents for Western blots. 'Polyclonal' phage, prepared by PEG precipitation, were used in Western blotting (Towbin *et al.*, 1979) to detect a panel of purified antigens on nitrocellulose after PAGE (Figure 2A); anti-EF-1α phage were also used to detect EF-1α in COS cell lysates (not shown). Similarly 'polyclonal' scFv fragments, concentrated 10-fold by dialysis against PEG from the bacterial supernatants, were used to detect the panel of antigens (Figure 2B); and to detect p53 in lysates of the breast cancer (T47D) cells with high levels of mutant human p53 protein (Bartek *et al.*, 1990). p53 was not detected in lysates of a human osteosarcoma cell line (SAOS-2) with a genetic deletion of both p53 genes (Masuda *et al.*, 1987) (Figure 3).

Monoclonal scFv fragments derived from segments of the

Table I. Deduced V_H-CDR3 sequences and germline origin of selected antigen-specific synthetic antibodies isolated from a single pot human synthetic library

Self	CDR3 sequences	V_H segment
anti-RhD mAb (Oak-3)	RRPTGHWH	DP-53
	RHNGNWD	DP-53
thyroglobulin	VARYNMYIPP	DP-49
	SKGAWFVRPP	DP-49
	MKSSARPV	DP-66
EF-1α	VYPFRBTN	DP-51
	INGKKFDY	DP-73
p53	NQNV	DP-45
	NQHV	DP-45
	FTRKRD (#30)	DP-53
SRY	QNLHNNQLV	DP-2
	RTPAVLSQB RNV	DP-42
	MAIQT	DP-47
	SWRGLMM	DP-70

Haptens	CDR3 sequences	V_H segment
NIP	VMSSADGHR	DP-38
	RGMN	DP-42
	KGGFD	DP-42
	GGTR	DP-42
	SGRD	DP-42
	RRGPIGAYYP	DP-47
phOx	TRAYRFDY	DP-47
	LRNFSFDY	DP-47
	RNAWGLIAARRT	DP-53
FITC	SSKWSMRN	DP-7
	ALQGPAYST	DP-35
	EHRTSIPT	DP-42
	LRARPV	DP-67

Foreign	CDR3 sequences	V_H segment
rhombotin-2	QVYQA	DP-46
	YTRKRFRRPER	DP-47
recombinant	TRHARFDY	DP-14
rat BiP	TWPTK (#17)	DP-32
	GYPTPFNY (#2)	DP-33
	GKRYFTK	DP-42
	TRRSRFDY	DP-67
	GRHSRFDY	DP-67
mouse TCR	GKGPSYTAILSF	DP-10
	SYYHLFDY	DP-46
	GFAYIFDY	DP-74
maltose BP	NWRNSFPQ	DP-14
	SSNPWK	DP-14
	ELMYFV	DP-48
	TPIHRRRQFNTG	DP-53
	YRTAHPL	DP-67
turkey egg	SYNEIVPI	DP-3
lysozyme	NYQLBVPQ	DP-3
cytochrome *c*	RIGTPFDY	DP-3
KLH	LRHGF	DP-45
	RRHRAFDY	DP-47
ovalbumin	GWNASD	DP-7
chymotrypsinogen A	KKBI	DP-58
	SKBI	DP-58
streptavidin	DWRMIEG	DP-49
	QWEGIRS	DP-69

The encoded sequences of V_H-CDR3, the third complementarity determining region of the antibody heavy chain are given. Nomenclature of the heavy chain germline segments is as in Tomlinson *et al.* (1992). The specificities were detected as phage and also in bacterial supernatants as soluble scFv fragments, except for anti-thyroglobulin, EF-1α, SRY, turkey egg lysozyme, ovalbumin, chymotrypsinogen A and streptavidin, which were initially detected only as phage antibodies. B represents translation of amber codon to glutamine in *E.coli* TG-1 *supE* strain. Abbreviations as in Figure 1.

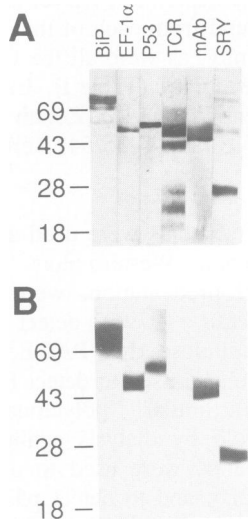

Fig. 2. Western blotting with polyclonal phage and fragments antibodies. 2 μg of purified antigens were electrophoresed under reducing conditions on 10% acrylamide gels, electroblotted to nitrocellulose and hybridized with polyclonal phage antibodies (**A**) or polyclonal scFvs (**B**). The expected molecular weights of BiP, EF-1α, p53, TCR, mAb and SRY are respectively 78, 53, 53, 43, 50 (γ3 heavy chain) and 26 kDa.

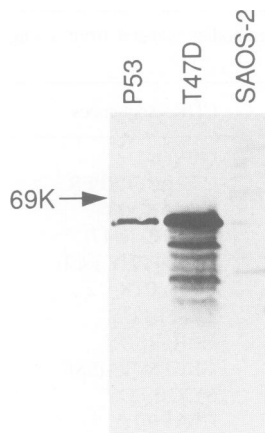

Fig 3. Detection of p53 in cell lysates by 'polyclonal' scFv fragments. Human p53 (2 μg) purified from SF9 insect cells infected with a recombinant baculovirus encoding wild type human p53 (Luckow and Summers, 1989; C.A.Midgley, unpublished), cell lysates (10^5 cells) of T47D human breast cancer cell line (overexpresses p53) and SAOS-2 human osteosarcoma cell line (in which both p53 genes are deleted).

human V_H3 family (and therefore binding to protein A) (Sasso *et al.*, 1991) were chosen for use as reagents, as they could be purified by binding to protein A–Sepharose (Hoogenboom and Winter, 1992), and as reagents readily detected with horseradish peroxidase (HRP)–protein A. (The fragments of other families, or indeed the V_H3 family, could be detected by their C-terminal peptide tag using the mouse monoclonal antibody 9E10 followed by HRP–goat anti-mouse IgG.) Analysis of the scFv fragments by gel filtration, for example those described here against p53 and BiP, indicated that the fragments were partly aggregated, as shown previously with scFv fragments derived from phage display repertoires (Griffiths *et al.*, 1993) or from monoclonal antibodies (Holliger *et al.*, 1993).

In lysates of COS cells, a purified monoclonal scFv

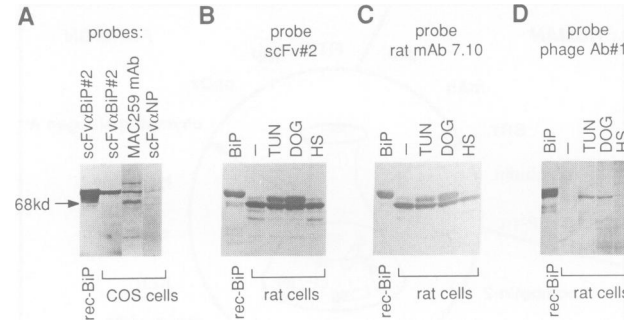

Fig. 4. Detection of hsp70 proteins by monoclonal anti-BiP scFv fragments and phage. (**A**) Detection of recombinant rat BiP and BiP in COS cell lysates with anti-BiP scFv#2, with controls of MAC256 monoclonal antibody (recognizing a KDEL motif shared by luminal ER proteins) and a scFv (directed against the hapten NP). (**B–D**) Specificity of anti-BiP scF fragments (B and C) or phage (D). Rat cells (RAT-1) were incubated for 19 h without drugs (–), or with 0.5 μg/ml tunicamycin (TUN), or with 10 mM 2-dioxy-D-glucose (DOG), or were heat shocked for 1 h at 42°C (HS). scFv#2 and rat mAb 7.10 recognize the hsp70 family. However, phage #17 reacts only with BiP, induced by TUN or DOG treatment but not by HS.

fragment (scFvαBIP#2) against recombinant rat BiP (immunoglobulin binding protein, and identical to glucose regulated protein grp78) (Munro and Pelham, 1986) detected a band of ~70 kDa, corresponding to 'cognate' cytosolic stress-70 proteins (hsc70) (Figure 4A). Likewise in lysates of rat fibroblast (RAT-1) cells (Figure 4B) the reagent mainly detected a single band of ~70 kDa, as does the control 7.10 mAb (Palter *et al.*, 1986) which is known to bind to several members of the hsp70 protein family including 'cognate' hsc70, heat inducible hsp70 and BiP (Figure 4C). The scFv fragments appear to be as sensitive as monoclonal antibodies as reagents. A solution of 10 μg/ml of scFv#2 reagent readily detected 15 ng recombinant BiP spotted onto nitrocellulose, resulting in strong staining by ECL.

BiP is present at low levels in fibroblasts; it is not heat inducible but the rate of its synthesis is increased when cells are starved for glucose or when induced by glycosylation inhibitors such as tunicamycin or 2-dioxy-D-glucose (Pouyssegur *et al.*, 1977; Olden *et al.*, 1979). When RAT-1 cells were treated, both the scFv reagent and the 7.10 mAb detected the appearance of a second band of ~80 kDa, presumably BiP (Figure 4B and C). Thus the scFv fragments appear to have a similar specificity to mAb 7.10. However, we also isolated more specific fragments that recognized only BiP, and not hsc70 (or the heat inducible hsp70) (Figure 4D), as illustrated here with a monoclonal phage reagent #17.

Epitope mapping

The epitope on p53 was mapped using monoclonal phage or scFv as a reagent (#30, CDR3: FTRKRD) Both phage and scFv fragment detected recombinant human p53 expressed in bacteria (Figure 5A), and also a series of p53 proteins deleted at the C-terminus (Figure 5B). This localized the epitope to the N terminus of p53. Mapping of two p53 proteins deleted at the N-terminus (Figure 5C) indicates that the epitope lies between residues 27 and 44 since a p53 fragment encoding amino acids 44–393 was not detected. The results were confirmed with the soluble scFv fragment reagent (not shown). The localization of epitope is consistent with the observation that the binding of the scFv fragment is not blocked by the DO-1 antibody (not shown), which

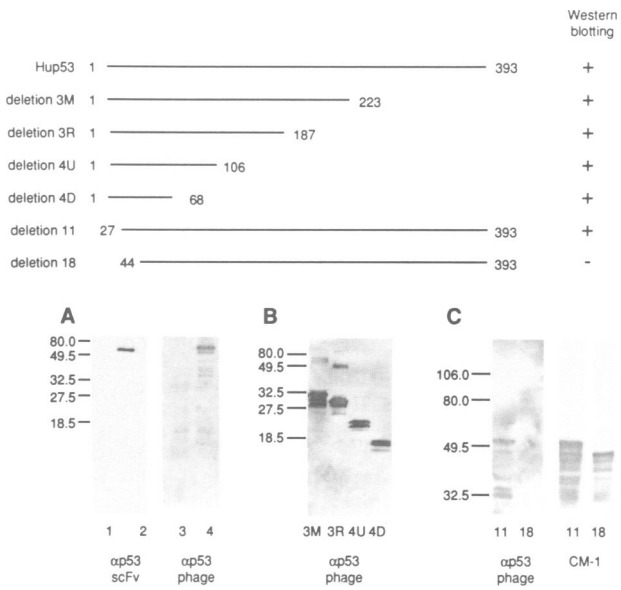

Fig. 5. Mapping the epitope in p53 recognized by an scFv fragment. The figure shows the portions of the p53 fragments expressed in bacteria and their reactivity with an anti-human p53 scFv fragment or corresponding phage on Western blots. (**A**) Western blot of lysate from induced *E.coli* containing pT7-7 vector only (lanes 1 and 3) or pT7-7Hup53 which direct the expression of full length human p53 (lanes 2 and 4). (**B** and **C**) Western blots of lysate from induced *E.coli* containing 3′ (B) or 5′ (C) truncated pT7-7Hup53. Anti-p53 phage antibodies detected all of the deleted proteins except deletion 18. CM-1 rabbit polyclonal anti-human p53 antibodies were used as positive control in (C).

recognizes an immunodominant site within the first 27 amino acids of the protein (D.Lane, unpublished data).

Immunocytochemistry

Monoclonal anti-p53 scFv fragments (#30) were used to stain the breast cancer cell line T47D. Clear nuclear staining of the T47D cells was seen when the scFv was used at a concentration of 10 μg/ml. At this concentration only a very low level of non-specific staining was seen when the same reagents were applied to the human SAOS-2 cells (Figure 6) or mouse C6 cells (and indicating that the scFv fragment does not bind mouse p53). We failed to find conditions for specific staining of the nucleus with the anti-p53 phage; presumably the phage particle is too large to penetrate.

Discussion

We have shown that phage with a range of binding specificities, can be isolated from a 'single pot' phage antibody library (Figure 1) within a few days and without animal immunization. We could isolate binding specificities to haptens, foreign and self-antigens, including secreted proteins (lysozyme and ovalbumin), cell surface proteins (T cell receptor), intracellular proteins from the cytoplasm (thyroglobulin, EF-1α), proteins from the lumen of the endoplasmic reticulum (BiP) and proteins from the nucleus (p53, SRY and rhombotin-2).

Phage and scFv fragments from the single pot library could be used without further affinity maturation as immunological reagents for Western blotting, epitope mapping and cell staining. We also used phage and scFv fragments in monoclonal and polyclonal formats. Polyclonal reagents are

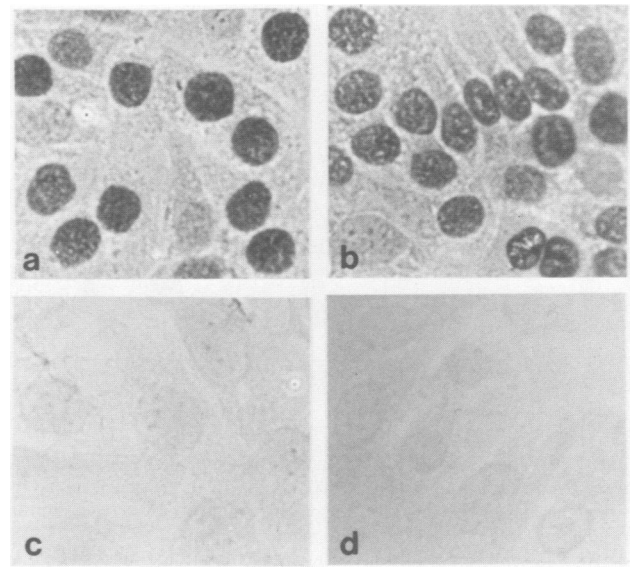

Fig. 6. Immunocytochemistry using monoclonal scFv. Staining of T47D (**a** and **b**) and SAOS-2 cells (**c** and **d**). (a) and (c) show staining with monoclonal anti-p53 scFv fragment, while (b) and (d) show staining by mouse monoclonal anti-p53 antibody (DO-1).

simpler to prepare, as the presence of binding phage can be readily detected by ELISA, and the phage population (or soluble scFv fragments) can then be used directly, or after affinity purification. In particular, scFv fragments derived from segments of the human V_H3 family (and therefore binding to protein A) could be readily purified with protein A−Sepharose, and as reagents readily detected with protein A−HRP conjugates.

Although for each of the antigens we could isolate phage clones with binding activities, we were sometimes unable to detect binding of the encoded soluble scFv fragments using bacterial supernatants. This may reflect the amplified detection of the phage by virtue of the 3000 subunits of the phage 'tail': if so, a more sensitive assay should be used for the soluble fragments. It might also reflect the greater binding avidities afforded by multivalent display on phage, and the moderate binding affinities of the monomeric scFv fragments from primary phage repertoires of 10^7-10^8 clones (Marks *et al.*, 1991a; Hoogenboom and Winter, 1992; Griffiths *et al.*, 1993).

We found that the scFv fragments in supernatants could be more readily detected by concentrating the supernatant, or by 'multimerization' of the fragments during purification. Although scFv fragments can associate to form dimers in bacterial supernatants (Griffiths *et al.*, 1993; Holliger *et al.*, 1993), and the dimers have greater binding avidities to solid phase antigen (Griffiths *et al.*, 1993), purification can also drive the formation of multimers (Figure 7). Presumably multimerization is promoted by dissociation of V_H and V_L domains of the same chain, followed by pairing with another chain, especially during acid elution and neutralization in concentrated solutions. Indeed the purified scFv fragments used to detect p53 and BiP in Western blotting, epitope mapping and cell staining were partly aggregated (Figure 7).

Aggregation of 'moderate affinity' scFv fragments is probably important for their use as immunological reagents as it enhances the avidity of binding and slows the rate of dissociation during washing. Clearly antigen should be provided in multimeric form, for example immobilized on

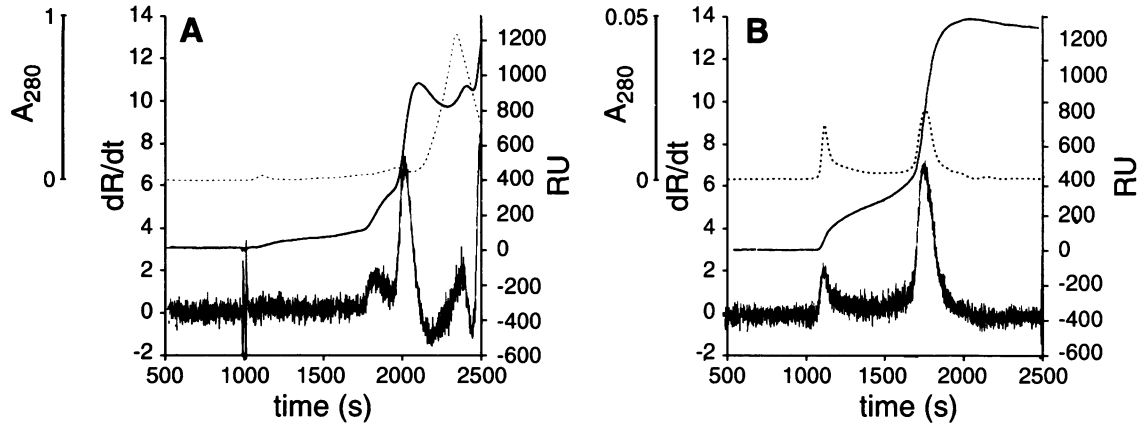

Fig. 7. Combined FPLC/BIAcore analysis of anti-BiP#2 scFv. Bacterial culture supernatant (**A**) and protein A affinity purified anti-BiP scFv#2 (**B**) were fractionated on a Superdex 200 column, with the eluent monitored by both UV 280 nm absorbance (upper, dotted line) and on-line BIAcore with immobilized 9E10 on the sensor chip (middle curve, light solid line). FPLC standards: of hen egg lysozyme (2455 s; M_r 14.3 kDa), hen ovalbumin (1900 s; 45 kDa), BSA monomer (1780 s; 68 kDa) and BSA dimer (1590 s; 136 kDa), were used to calibrate the FPLC column. The derivatized sensorgram (dR/dt, lower, heavy solid line) illustrates the rate of changes in mass of protein bound to the sensor chip as a function of time. Note that the various *y*-axes have been scaled with zero at different levels for clarity. In (A), the main peak of binding near 2020 s corresponds to monomeric scFv, with a small peak of more aggregated material at 1830 s. The disturbance near 1000 s is an artifact caused by pump filling; the exclusion volume corresponds to 1100 s on the traces shown. In (B) there is substantial material at or near the exclusion volume (1120 s); the main peak (1750 s) most closely corresponds to a mixture of trimer and tetramer, with only minor amounts of dimer and monomer.

nitrocellulose as in Western blots, or in fixed cells, and at high density to allow crosslinking with multimeric antibody. Furthermore the use of high density antigen should also facilitate the rebinding of multimeric and even monomeric fragments, slowing the net rate of dissociation from the solid phase. It would therefore be desirable to drive the self-aggregation of scFv fragments, for example by use of dimerization peptides (Pack and Pluckthun, 1992) or by shortening the flexible linker (Holliger *et al.*, 1993). However, scFv fragments are also aggregated by binding to protein A or to the bivalent mouse monoclonal antibody 9E10.

Not only have the single pot libraries provided a range of immunochemical reagents, but monoclonal specificities that are difficult to make by hybridoma technology, for example against the elongation factor 1α (EF-1α) (Merrick *et al.*, 1993) and immunoglobulin heavy chain binding protein (BiP). BiP is located in the lumen of the endoplasmic reticulum (see Gething and Sambrook, 1992), and is a member of a family of hsp70-related proteins that includes cytosolic 'cognate' hsc70 (p73) and heat inducible hsp70 (p72). BiP binds to partially folded or unassembled proteins, blocking their transport through the endoplasmic reticulum, and is highly conserved between species. Only one monoclonal antibody specific for BiP (Bole *et al.*, 1986), has been reported, and other less specific monoclonal antibodies have been used as reagents. For example, rat monoclonal antibody (7.10) raised against *Drosophila* hsp70 also binds to BiP (and to hsp70s and hsp-70-related proteins from a wide range of species) (Palter *et al.*, 1986), and antibodies against the C-terminal (KDEL) tetrapeptide of BiP, also bind to other KDEL proteins (including grp94 and protein disulfide isomerase) (Pelham, 1989). Presumably antibodies against BiP are retained in the lumen of the endoplasmic reticulum of B-cells and are difficult to display or secrete (Bole *et al.*, 1986). However, from the single pot library, we could readily isolate an anti-BiP reagent that reacts specifically with rat BiP in rat cell lysates, and also a more cross-reactive reagent that recognizes hsc70 (and with similar specificity to rat 7.10).

There is also a hint that the phage may recognize different epitopes, or at least those with a different bias, from monoclonal antibodies. For example, mouse antibodies to human p53 bind to two main immunodominant regions of the protein, at the N-terminal region and at the C-terminal region (Schlichtholz *et al.*, 1992). The immunodominant region lies between amino acids 1 and 25 at the N terminus or amino acids 370 and 381 at the C terminus (D.Lane, unpublished data); the epitope recognized by the anti-p53 scFv fragment appears to lie between residues 27 and 44. This portion of sequence differs significantly between mouse and human p53, and indeed as predicted the scFv reagent does not recognize mouse p53 in cell staining (or indeed by ELISA on purified mouse p53—not shown). However, it remains to be seen whether in general the phage libraries lead to a different spectrum of epitopes from natural immune systems. If so, such reagents could prove invaluable for mapping the functions of proteins.

In conclusion, single pot libraries are capable of providing immunochemical reagents of high specificity, and in future higher affinity fragments may emerge from larger libraries (Perelson and Oster, 1979; Waterhouse *et al.*, 1993), or by affinity maturation of selected antibodies by chain shuffling (Marks *et al.*, 1992b). The use of these libraries appears to open new possibilities for immunochemistry.

Materials and methods

Library construction

The human synthetic libraries (HSL) were constructed as in Hoogenboom and Winter (1992) using PCR primers designed to introduce V_H-CDR3 of random sequence varying in length from 4 to 12 residues. All 49 germline V_H segments amplified in Hoogenboom and Winter (1992) were used except for DP-44 (as this produces an identical product to DP-45; see Tomlinson *et al.*, 1992), and two additional V_H2 segments (DP-26 and DP-28) were incorporated. Note that several of these segments are not seen as rearranged genes, and lie outside the main V_H locus (unpublished data). DNA template encoding each of the 50 germline V_H segments was individually amplified using the V_H family based primers (Marks *et al.*, 1991a) VHBACKSfi and HSLP4-HSLP12 (Table II) in a volume of 50 μl with 250 μM dNTPs, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris—HCl (pH 8.8), 2 mM $MgCl_2$, 100 μg/ml BSA and 1 μl (1 unit) of Taq DNA

Table II. Oligonucleotide primers used to create the synthetic library

HSLP	5′-**GAC CAG GGT ACC TTG GCC CCA** [(A/C)NN]$_n$ *TCT TGC ACA GTA ATA CAC GGC CGT GTC*
JHSAL	5′-GCC TGA ACC GCC TCC ACC AGT CGA CAC GGT **GAC CAG GGT ACC TTG GCC CCA**
CDRFOR	5′-**CAG GGT ACC TTG GCC CCA**
CDRBACK	5′-*GTG TAT TAC TGT GCA AGA*

Each germline V_H segment (Tomlinson *et al.*, 1992) was amplified using V_H family based primers (Marks *et al.*, 1991a) VHBACKSfi and HSLP. The HSLP primers were designed to introduce a J segment and a random D segment of 4–12 residues (A/C, N, N)$_n$ where $n = 4-12$. The 3′ portion of the oligonucleotide was designed to anneal with FR3 of the germline V_H gene segments. The JHSAL primers were designed to introduce a *Sal*I restriction site (underlined) to the 3′ end of each amplified germline V_H segment for cloning, and the CDRFOR and CDRBACK primers to amplify CDR3. Regions of primers based in the J segment are marked in bold, and in italics for regions based in FR3

polymerase. Template was provided as 10^6 bacteria of a stock infected with recombinant M13 encoding each V_H segment. 25 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min) were used, with limiting VHBACKSfi and HSLP primers (0.25 pmol/μl) to exhaust the primers.

In a second PCR step, a *Sal*I restriction site was introduced at the 3′ end of the J_H segment. 30 pmol of the same VHBACKSfi oligonucleotide and 30 pmol of JHSAL (Table II) were added, and the PCR continued for additional 15 cycles. After checking that a band of the appropriate size was observed on agarose gel electrophoresis for each of the 450 samples (9 × 50), the PCR products of each of the amplifications encoding the nine different CDR3 loop lengths were pooled, cut with *Nco*I and *Sal*I, and cloned into *Nco*I–*Xho*I-cut pHEN1-Vλ3 (Hoogenboom and Winter, 1992) to provide nine phagemid libraries, each of at least 10^7 different clones.

Each library was rescued separately with helper phage VCS-M13 (Stratagene), then pooled with each other, and with two earlier libraries (Hoogenboom and Winter, 1992), each of at least 10^7 different clones and encoding loops of five or eight residues (the latter with five random and C-terminal Phe-Asp-Tyr), to create a single pot library of $>10^8$ clones.

Selection of phage library

Phage were panned for binding using immunotubes (Nunc; Maxisorp) coated with each antigen overnight at room temperature (Marks *et al.*, 1991; Griffiths *et al.*, 1993). The library was subjected to four or five rounds of selection on the various antigens.

The following antigens were coated at 100 μg/ml: human mAb (γ3, κ) anti-erythrocyte rhesus (D) monoclonal antibody (Oak-3, from Barbara D.Gorick; Bye *et al.*, 1992); human thyroglobulin (Hu-Thy; Scipac); 2-phenyl-5-oxazolone (phOx)-BSA; 4-hydroxy-5-iodo-3-nitrophenylacetyl (NIP)-BSA; turkey egg-white lysozyme (TEL; Sigma); horse heart cytochrome *c* (Sigma); keyhole limpet haemocyanin (KLH; Sigma); chicken egg ovalbumin (Ovalbumin; Sigma); α-chymotrypsinogen A (type II from bovine pancreas; Sigma); bovine serum albumin–L-fluorescein isothiocyanate (FITC-BSA; Sigma) and streptavidin from *Streptomyces avidinii* (Sigma). Coating was in 50 mM $NaHCO_3$ pH 9.6 (Oak-3, TEL, KLH, ovalbumin and chymotrypsinogen A), or PBS (human thyroglobulin, NIP, phOx, cytochrome *c*, FITC-BSA and streptavidin).

The following antigens were coated at 20 μg/ml: recombinant rat BiP (see below); human elongation factor 1α (EF-1α, from P.Kristensen; Merrick *et al.*, 1993); human p53 isolated from SF9 insect cells infected with a recombinant baculovirus encoding wild type human p53 (Luckow and Summers, 1989; C.A.Midgley, T.Hupp and D.Lane, unpublished); soluble chimeric murine T cell receptor (TCR, from K.Karjalainen; Weber *et al.*, 1992); protein encoded by the human testis determining gene, SRY (sex-determining region Y) from V.R.Harley (Harley *et al.*, 1992); fusion of rhombotin-2 to maltose binding protein (maltose BP) and recombinant mouse rhombotin-2 from T.Rabbitts (Foroni *et al.*, 1992). Coating was in PBS (BiP, TCR) or 50 mM $NaHCO_3$ pH 9.6 (p53, EF-1α, SRY, maltose BP and rhombotin-2).

The rat BIP was engineered with six N-terminal histidine residues and a factor X cleavage site preceding the mature BiP polypeptide (His-BiP). Oligonucleotide primers were used to amplify the coding region of BiP from the plasmid R76 (from H.Pelham). The amplified product was inserted into the *Bam*HI–*Hin*dIII restriction sites of pQE40 vector (Qiagen) encoding the His6 tail, then transformed into *Escherichia coli* strain XL-1 Blue (Stratagene). Expression and purification of His6-tagged protein over Ni-NTA resin were performed by protocols provided by the manufacturer (Qiagen). After eluting from the Ni-NTN resin with 250 mM imidazole, 200 mM NaCl, 50 mM HEPES, pH 7.0, the His-BiP was further purified by ATP-agarose affinity chromatography (Flynn *et al.*, 1989). His-BiP was >95% pure.

Screening and sequencing of clones

As described (Marks *et al.*, 1991; Hoogenboom and Winter, 1992; Griffiths *et al.*, 1993), phage were rescued from single ampicillin-resistant colonies of infected (suppressor) *E.coli* TG-1 using the helper phage VCS-M13 (Stratagene); soluble scFv fragments were induced from single colonies of infected (non-suppressor) *E.coli* HB2151 (Hoogenboom *et al.*, 1991) by IPTG. Bacterial supernatants containing phage or scFv fragments were screened for binding to antigen by ELISA. The binding specificity was checked by ELISA using plates coated with one or more other antigens: phage isolated by selection on 20 μg/ml antigens were screened against all the other antigens of this set. For the fusion proteins chimeric TCR–murine Cκ and rhombotin–2-maltose BP, the specificity of the selected phage antibodies was further examined by ELISA using plates coated with mouse IgM, κ antibody and maltose BP, respectively.

Phage with binding activities were screened for CDR3 length by PCR and for V_H family by hybridization. Individual phagemid colonies were amplified using γ-^{32}P-labelled CDR-BACK and CDR-FOR primers (Table II). The size of the amplified CDR3 was followed by gel electrophoresis using 7.6 M urea–8% polyacrylamide gels. Template from the entire phage library was used to produce a ladder of CDR3 lengths. Colonies were probed as in Tomlinson *et al.* (1992) for V_H family using the family-specific oligonucleotide probes (Marks *et al.*, 1991b). Different clones, as shown by different CDR lengths in combination with V_H segments of different families were sequenced by the dideoxy method (Sanger *et al.*, 1977) using DyeDeoxy chain termination (Applied Biosystems Inc.) and an Applied Biosystems 373A DNA sequencer. The sequences were analysed using SeqEd (Applied Biosystems Inc.) and MacVector 3.5 (IBI Kodak, New Haven, CT).

Preparation of monoclonal and polyclonal reagents

For monoclonal scFv fragments, supernatants from 1 l cultures of infected HB2151 bacteria induced overnight with IPTG at 30°C (Griffiths *et al.*, 1993) were filtered with Sterivex-HV 0.45 μm filter unit (Millipore) and then purified on protein A–Sepharose (Pharmacia), as described in Hoogenboom and Winter (1992). Monoclonal phage was prepared by PEG precipitation of the supernatant of infected bacteria (Marks *et al.*, 1991). Polyclonal reagents were prepared from an aliquot of infected bacteria after the last round of selection. However, for the polyclonal scFv fragments, the supernatant was concentrated ~10-fold by dialysis against dry granular PEG-6000.

Western blotting

Polyclonal reagents were used to detect purified antigen on Western blots as follows. 2 μg of each purified antigen were run on 10% polyacrylamide gel and then electroblotted. Filters were blocked for 1 h at room temperature in 5% Marvel/PBS/0.2% Tween 20 (for scFv fragments) or 10% Marvel/PBS/0.5% Tween 20 (for phage). The scFv fragments (1:2 dilution of 10 × concentrated supernatant) or phage (10^{11} TU/ml) were added and incubated overnight with gentle shaking at 4°C. After washing with PBS–0.2% Tween 20 (or PBS–0.5% Tween 20 for phage), binding of scFv fragments was detected with a mixed reagent, HRP–protein A (Sigma P8651) mixed with the mouse monoclonal antibody 9E10, and followed by HRP–goat anti-mouse Ig (Sigma). Phages were detected with sheep anti-M13 1:1000 (provided by Cambridge Antibody Technology), followed by HRP–anti-goat antibody (Sigma). Peroxidase activity was detected using an ECL kit from Amersham.

Anti-p53 polyclonal scFv fragments (~10 μg/ml) were likewise used to detect p53 in T47D breast cancer cell lysates, and polyclonal anti-EF-1α phage antibodies (10^{11} TU/ml) to detect EF-1α in COS cell lysates. Lysates were fractionated on 10% SDS–polyacrylamide gels (Laemmli, 1970), and electrophoretically transferred onto nitrocellulose (Towbin *et al.*, 1979).

For detection of BiP in cell lysates with monoclonal reagents, cell lysates were prepared from 5 × 10^6 COS cells grown at 37°C in DMEM/10% FCS, or from 5 × 10^6 RAT-1 fibroblast cells (Munro and Pelham, 1986; Napier *et al.*, 1992). The RAT-1 cells were treated with 0.5 μg/ml tunicamycin, 10 mM 2-dioxy-D-glucose or heat shock (Munro and Pelham, 1986). The filters were treated as above, and BiP detected either with 20

μg/ml monoclonal α-BiP scFv reagent followed by 10 μg/ml HRP–protein A; or with 10^{11} TU/ml of α-BiP phage (as above, except that the HRP was detected by 3,3′-diaminobenzidine in 0.02% cobalt sulfate). The rat 7.10 α-BiP mAb (ascites diluted 1:500; Cambridge Research Biochemicals), and the rat anti-KDEL mAb from J.Butcher (Napier *et al.*, 1992) were detected with 10 μg/ml anti-rat HRP.

Epitope mapping

Plasmid pT7-7 Hup53 directs the expression of human p53 and deletion mutants truncated at the N- or C-terminus (Vojtesek *et al.*, 1992) in *E.coli* BL21 (DE3) cells. Total lysates from log phase cultures induced with IPTG were blotted much as above, except using 15% SDS–polyacrylamide gels. CM-1 polyclonal rabbit serum (1:300) against human p53, monoclonal α-p53 scFv fragment (10 μg/ml), or monoclonal α-p53 phage (10^{11} TU/ml) were used to detect p53 using 10 μg/ml HRP–protein A (Sigma P8651), or (for the phage) 1:500 rabbit anti-F1 phage anti-serum followed by HRP–swine anti-rabbit Ig (Dako). Peroxidase activity was detected using an ECL kit from Amersham.

Immunocytochemistry

Cell staining was performed as in Vojtesek *et al.* (1992). Briefly, T47D cells containing high levels of mutant human p53 protein, and SAOS-2 cells which contain a genetic deletion of both p53 genes, were passaged on plastic dishes and prefixed in cold methanol/acetone (1:1 by volume). A 2-fold dilution series (in PBS/10% fetal calf serum) of scFv fragment was spotted onto the fixed cells as 5 μl drops and incubated at room temperature for 2 h. The cells were washed three times with PBS then incubated with HRP-conjugated protein A (Sigma, 1 μg/ml). Alternatively, 9E10 was used followed by HRP–goat anti-mouse IgG (Sigma). The peroxidase activity was detected with 3,3′-diaminobenzidine in 0.03% nickel sulfate.

Sizing of scFv fragments by combined FPLC/BIAcore analysis

Bacterial cultures of anti-BiP scFv #2 supernatants (0.5 ml of 0.2 μm-filtered) and protein A affinity-purified fragments (100 μl of 1.6 mg/ml) were analysed as described by Griffiths *et al.* (1993). The samples were fractionated on a Superdex S200 column (Pharmacia) and were monitored both by absorbance at 280 nm and on-line by BIAcore with immobilized 9E10 on the sensor chip (Johnsson *et al.*, 1991). The buffer was PBS containing 0.2 mM EDTA and 0.05% NaN_3. The BIAcore reading was corrected for the baseline, and also then calculated as dR/dt, the rate of change of the signal.

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