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Two-Dimensional Electrophoresis/Phage Panning (2D-PP): A Novel Technology for Direct Antibody Selection on 2-D Blots[1]

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We describe a novel method, two-dimensional electrophoresis/phage panning (2D-PP), for the generation of antibodies against proteins in crude biochemical samples, such as cellular membrane fractions. These sources have traditionally presented problems as to the development of antibodies by conventional techniques. 2D-PP involves two-dimensional resolution of proteins, blotting of the proteins onto a nitrocellulose membrane, and screening of a phage antibody library and isolation of corresponding antibodies. By 2D-PP with detergent-insoluble "lipid rafts" as a target protein complex, we obtained specific phage pools against eight antigen spots (from a total of 39 spots). These antibodies were functional in Western blotting, enzyme-linked immunosorbent assaying (ELISA), and immunoscreening of a cDNA expression library. Propagation of anti-nitrocellulose phages was the major problem in 2D-PP, but was overcome by the use of the soluble anti-nitrocellulose antibody fragment. 2D-PP constitutes a key tool for functional analysis of proteins in complex fractions.

Key words: nitrocellulose membrane, phage display, proteomic analysis, single-chain antibody, two-dimensional electrophoresis.

Currently, major advancements are being made in the field of proteomics with mass spectrometric identification of proteins separated by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (*1*). Determination of the physiological roles of such proteins may necessarily require development of specific antibodies against the proteins to allow functional analysis. Advances in the development of antibodies by means of phage display technology are important in such studies since this can be used to produce many functional antibody fragments such as Fab (*2*) or single-chain Fv (scFv) (*3*), the classical hybridoma technology or animal immunization being bypassed (reviewed in Refs. *4* and *5*). Also, antibodies can be selected from phage antibody libraries against molecules that have previously been considered as difficult targets for a conventional immunization method; these molecules include self-antigens (*6, 7*) or cell surface proteins (*8–10*). Here, we describe a novel technology using a phage antibody system to generate antibodies against proteins in a crude biochemical fraction resolved by 2-D PAGE. We have designated the method 2-D electrophoresis/phage panning (2D-PP).

One of the potential problems of phage display technology is a strong bias for developing antibodies against immunodominant antigens when the phage library is screened against highly complexed targets (*11, 12*). To diminish this anticipated bias, we tried to select the binders against each individual component. Proteins in complex biological samples were first separated from each other by 2-D PAGE immobilized on a nitrocellulose membrane, and then each protein was excised and screened by a phage antibody library.

As a target of 2D-PP, we have focused on detergent-insoluble cell membrane microdomains, or lipid rafts, which have been described in prion diseases and Alzheimer's disease (*13*). There is currently no effective method for the isolation of antibodies against proteins separated by 2-D PAGE. Previously, two model studies were reported, in which pure proteins were subjected to 2-D PAGE, eluted from the gel and then used for phage panning (*14, 15*). In our study, we demonstrate the isolation of phage antibodies against unknown 2-D protein spots resolved from a complex lipid raft fraction, and the functional activity of these antibodies in immunobiochemical methods including immunoscreening of a cDNA expression library to identify the target antigens.

MATERIALS AND METHODS

Preparation of 2-D Protein Spots for Phage Panning—Lipid rafts were isolated from human neuroblastoma cell line SK-N-MC (ATCC No. HTB10) as described previously (*16*). Proteins in the fraction were solubilized with 9 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-HCl, and 0.5% IPG buffer (Amersham Pharmacia Biotech, Uppsala, Sweden) with vigorous shaking for 1 h, and then subjected to

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Abbreviations: ATCC, American type culture collection; Fv, variable region fragment; PBS, phosphate-buffered saline.

isoelectric focusing (the first dimension) with a linear immobilized pH gradient (range, 4–7) and an Immobiline dry strip (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The proteins were then separated by SDS-PAGE (10%) in the second dimension (*17*), and electrotransferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The 2-D blot was stained with colloidal gold total protein stain (Bio-Rad, Hercules, CA), and each of the visualized protein spots was excised with a Proteome works spot cutter apparatus (Bio-Rad) the diameter being 1 mm.

Phage Panning with 2-D Protein Spots—Phage antibodies were selected through 5 rounds of panning from the human synthetic single-chain Fv (scFv) library (Griffin.1 library, provided by Dr. G. Winter, MRC, Cambridge, UK, http://www.mrc-cpe.cam.ac.uk/~phage/), where basic manipulations of phages were performed according to the protocols for the library ("the library protocol," http://www.mrc-cpe.cam.ac.uk/~phage/g1p.html). For the experiments on each target antigen, four pieces of the excised protein spots were incubated with 1×10^{12} cfu of phage pool in each round. The experimental conditions for panning were as follows: method [i] is an original protocol and several modifications have been made to method [ii] to suppress the propagation of anti-nitrocellulose phages. A soluble anti-nitrocellulose scFv fragment was prepared from the anti-nitrocellulose phage obtained in this study according to the library protocol.

Method [i]: Protein spots were blocked with 10% skim milk in PBS (MPBS) containing 25% glycerol, and then incubated with phages (1×10^{12} cfu) in 1 ml of 10% MPBS containing 0.1% Tween 20 (MPBST) for 1 h. Then the spots were washed three times with PBS containing 0.1% Tween 20 (PBST) for 5 min, five times with 10% MPBS containing 25% glycerol for 20 min, and finally three times with PBS for 5 min. The bound phages were eluted with 100 mM triethylamine and propagated according to the library protocol.

Method [ii]: The antigen spots were first soaked in PBS containing 1% Tween 20 for 1 h for renaturation of the blotted proteins (*18*), and then blocked with 10% MPBST supplemented with a soluble anti-nitrocellulose scFv fraction. Next, phage antibodies (1×10^{12} cfu) in 200 μl of 10% MPBST supplemented with anti-nitrocellulose scFv were reacted with the antigen spots overnight (round 1) or for 3 h (rounds 2–5). Protein spots were washed once with PBS (round 1), or washed three times with PBST for 5 min and once with PBS for 5 min (rounds 2–5). In rounds 4 and/or 5, the anti-nitrocellulose scFv was omitted from the blocking and phage reactions. Bound phages were eluted as above.

Purification of ScFv—The scFv used in this study is tagged with a histidine hexamer and secreted into culture supernatant when expressed in *Escherichia coli* (see the library protocol). The secreted scFv was purified with a metal affinity resin (TALON™; Clontech, Palo Alto, CA) according to the manufacturer's protocol.

DNA Finger Printing of Phage Clones—The scFv insert of each phage clone was amplified by PCR (*19*) using primers 5′-CGGATAACAATTTCACACAGGAAAC (sense) and 5′-CTATGCGGCCCCATTCAGATC (antisense). The products were digested with *Hae*III and the restriction patterns were analyzed by agarose gel electrophoresis.

Phage Western Blotting—The lipid raft fraction was subject to SDS-PAGE (1-D) or 2-D PAGE, and then electroblotted onto nitrocellulose membranes. The membranes were stained with colloidal gold (Bio-Rad), renatured as described above (when needed), and then blocked with 10% MPBST for 1 h. Then the membranes were incubated with phages (5×10^{10} cfu/ml) in 10% MPBST for 1 h and washed with PBST three times for 5 min each. The membranes were incubated with anti-M13 antibodies conjugated with peroxidase (Amersham Pharmacia Biotech) in 1:7,000 diluted 5% MPBST for 1 h and washed as above. The bound phages were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

Immunoscreening of a cDNA Expression Library with Phage Antibodies—A human brain 5′-stretch plus λTriplEx cDNA library (Clontech) was screened with the phage antibodies isolated in this study. The λ phage plaques were grown and transferred to a Hybond-ECL nitrocellulose membrane according to the manufacturer's protocol. The screening with phage antibodies was carried out in the same manner by phage Western blotting, as described above.

Phage ELISA—A 96-well plate (Corning, Corning, NY) was coated with each of the following antigens (50 μg/ml, dissolved in PBS): human vimentin (Progen, Heidelberg, Germany), bovine actin (Sigma), human thyroglobulin (Biogenesis, England, UK), bovine serum albumin (BSA, Life Technologies), and hen egg-white lysozyme (ICN Biomedicals, Aurora, OH). After blocking with 10% MPBS for 1 h, monoclonal phage antibodies mo17–2 and mo23–7 (1×10^{10} cfu in 100 μl 10% MPBS) were incubated in each well for 1 h. After washing, 1:7,000 diluted anti-M13 peroxidase-conjugated antibodies (Amersham Pharmacia Biotech) were reacted in the wells for 1 h. After washing of the wells, bound phages were detected with an ABTS substrate kit (Nacalai Tesque, Kyoto).

Mass Spectrometry—The 2-D protein spots visualized with a copper staining kit (Bio-Rad) were cut out and subjected to in-gel digestion with trypsin (*20*). The masses of tryptic peptides were determined with a MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) (*21*) mass spectrometer (Voyager RP, Applied Biosystems), and peptide mass fingerprinting analysis was performed with MS-FIT software.

RESULTS

The lipid raft fraction is enriched with cholesterol, sphingolipids, and associated proteins such as glycosylphosphatidylinositol (GPI)-anchored, doubly acylated or transmembrane proteins, and is characterized by insolubility in non-ionic detergents such as Triton X-100 (*22–24*). This feature makes the protein components in lipid rafts hard to analyze in an aqueous solution. However, lipid rafts could be analyzed successfully using 2D-PP, in which the target antigens were immobilized on a nitrocellulose membrane during the antibody screening.

We used human synthetic scFv library Griffin.1 for phage antibody screening. For optimization of the conditions of 2D-PP, phage antibodies against BSA and thyroglobulin were isolated from the library by panning with polystyrene tubes (Maxisorp™; Nalgenunc, Rochester, NY) according to the library protocol. To find the membrane most suitable for the phage reaction, four different membranes

(nitrocellulose, PVDF, negatively-charged PVDF and nylon) were subjected to dot blot analysis using anti-BSA phages (Fig. 1A). Among them, nitrocellulose membranes gave the best contrast between the positive and negative signals of chemiluminescence, the others showing higher levels of background phage binding. Next, the blocking effect of the skim milk concentration on Western blotting was examined. The phage antibody specific to a non-raft protein, thyroglobulin, was reacted with a 1-D blot of lipid raft proteins in the presence of 0–10% skim milk. As shown in Fig. 1B, the nonspecific binding of anti-thyroglobulin phage to raft proteins was completely blocked in the presence of 10% skim milk. Furthermore, we determined the concentrations of phages suitable for panning with the membrane. Various concentrations of anti-BSA and irrelevant (anti-thyroglobulin) phages were reacted with BSA blots in 10% skim milk, and then the titers (colony forming units, cfu) of the bound phages were compared between the specific and irrelevant phages (Fig. 1C). The amounts of specific phages bound to BSA were higher than those of irrelevant phages at any concentrations of phages. The ratio between the specific and nonspecific phage binding (specific/nonspecific) became larger with higher concentrations of the phages: 13 at 10^{10} cfu/ml and 37 at 10^{12} cfu/ml. Thus, we employed higher concentrations of phages (10^{12} to 10^{13} cfu/ml) for the reaction with antigen spots. We also checked the effect of a high concentration (25%) of glycerol (*25*) in the blocking and washing steps of panning on the selection of positive phages. As a model experiment, the excised 2-D blot of BSA on nitrocellulose was screened for the Griffin.1 library. As shown in Table I, clones specific to BSA were more effectively enriched in the presence of glycerol in the blocking and washing steps than in its absence of glycerol: 24 out of 24 clones were specific after the 5th round of panning with glycerol.

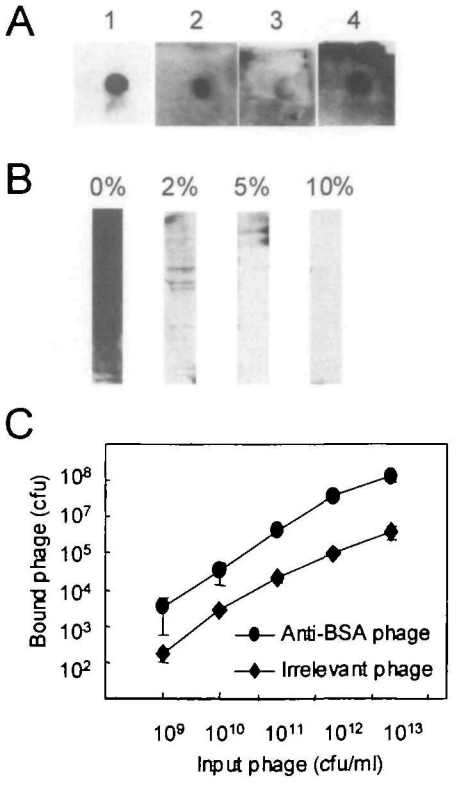

Fig. 1. **Optimization of conditions for phage panning.** (A) Difference in phage detection on chemically distinct membranes. BSA (50 ng) was dotted onto the following membranes, (1) nitrocellulose (Hybond-ECL), (2) PVDF (Fluorotrans, Pall, East Hills, NY), (3) negatively charged PVDF (Fluorotrans G, Pall), and (4) nylon (Biodyne A, Pall), and then blocked with 5% skim milk and incubated with anti-BSA phage (10^{11} cfu/ml) for 1 h. After washing, phage binding was evaluated by chemiluminescent detection as described under "MATERIALS AND METHODS." (B) Blocking effect of skim milk on Western blotting with phage antibodies. After blocking with various concentrations of skim milk, a 1-D blot of the lipid raft fraction on a nitrocellulose membrane was reacted with phages specific to a non-raft protein, thyroglobulin. (C) Evaluation of the optimal phage concentration for phage binding to an antigen blot. An excised nitrocellulose membrane (1 mm in diameter) was coated with BSA (100 μg/ml), and then reacted with various concentrations of phages after blocking with 10% skim milk. Bound phages were eluted with 100 mM triethylamine and titrated according to the library protocol. Values are the means ± SD of 3 independent experiments.

TABLE I. **Effect of glycerol in the blocking and washing steps on 2D-PP.**

Conditions for blocking and washing	Frequency of positive phage clones				
	Rounds of selection				
	1	2	3	4	5
With glycerol[a]	–	–	1/24	1/24	24/24
Without glycerol[b]	–	–	1/24	3/24	5/24

Phages (1×10^{12} cfu/ml in 10% MPBST) were panned against an excised 2-D blot of BSA using the Griffin.1 library. After each round, 24 phage clones were analyzed by *Hae*III fingerprinting and the specificity to BSA was checked for the unique clones by dot blot analysis. [a]Blocked for 1 h with 10% MPBST containing 25% glycerol. Washed 3 times for 5 min with PBST, 5 times with 10% MPBST containing 25% glycerol, and 3 times for 5 min with PBS. [b]Blocked for 1 h with 10% MPBST. Washed 3 times for 5 min with PBST.

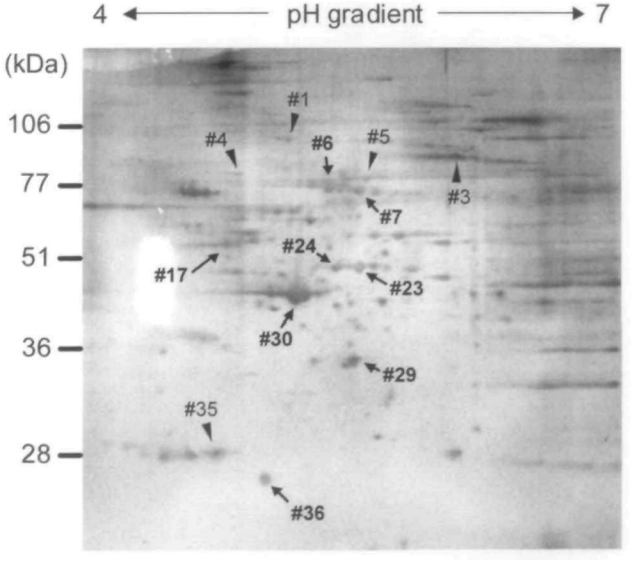

Fig. 2. **2-D PAGE of lipid rafts (160 μg as protein).** The protein spots on a nitrocellulose membrane were visualized by colloidal gold staining. The antigen spots against which phage antibodies were generated in this work are indicated by arrows. The spots indicated by arrowheads are the proteins identified by mass spectrometry.

Based on these results (Fig. 1 and Table I), we decided experimental conditions such as the incubation conditions and the number of panning rounds for method [i] described in "MATERIALS AND METHODS."

As the antigen source for panning, the lipid raft fraction of human neuroblastoma SK-N-MC cells was subjected to 2-D PAGE and then transferred to a nitrocellulose membrane. The protein spots were visualized by colloidal gold staining, since it exhibits high sensitivity comparable to that of silver staining of polyacrylamide gel (nanogram amounts of proteins are detectable) and is not known to have an inhibitory effect on antibody binding (*26–28*). We detected about 200 individual protein spots on the 2-D membrane (pH 4–7) with 160 μg protein of lipid rafts (Fig. 2). Since the nitrocellulose itself had the potential for antigenicity in our preliminary experiments, each antigen spot was excised (1 mm in diameter) and used for the phage reaction. The 32 antigen spots (#1–#32) that were detectable in every run were initially subjected to phage panning using the Griffin.1 library with original protocol method [i] (see "MATERIALS AND METHODS"). The enrichment of the specific binders in phage pools was evaluated by 1-D Western blotting of the lipid raft fraction (Fig. 3A). After 5 rounds of selection, specific antibodies were generated to three antigen spots, #17, #23, and #24 (3/32, 9.4%). We failed to obtain specific binders to other antigens, while the propagation of anti-nitrocellulose phages was observed in 25 antigen spots (25/32, 78.1%), such as #6, #7, #19, and #30 (Fig. 3A). No binders were enriched on the remaining four antigens (for example, spot #20).

We tried modification of the panning procedure in order to reduce the anti-nitrocellulose binders and to recover specific binders more efficiently (method [ii], see "MATERIALS AND METHODS"). To achieve optimum binding of specific phages to the antigens, renaturation treatment of antigen spots with Tween 20 (*18*) was introduced to each round of panning. To compete with anti-nitrocellulose phages, a soluble anti-nitrocellulose scFv fraction was added for the blocking and phage reactions.

Using the total 36 protein spots (#1–#39 except for #17, #23, and #24), the 5 rounds of panning with Tween 20 treatment and anti-nitrocellulose scFv were again per-

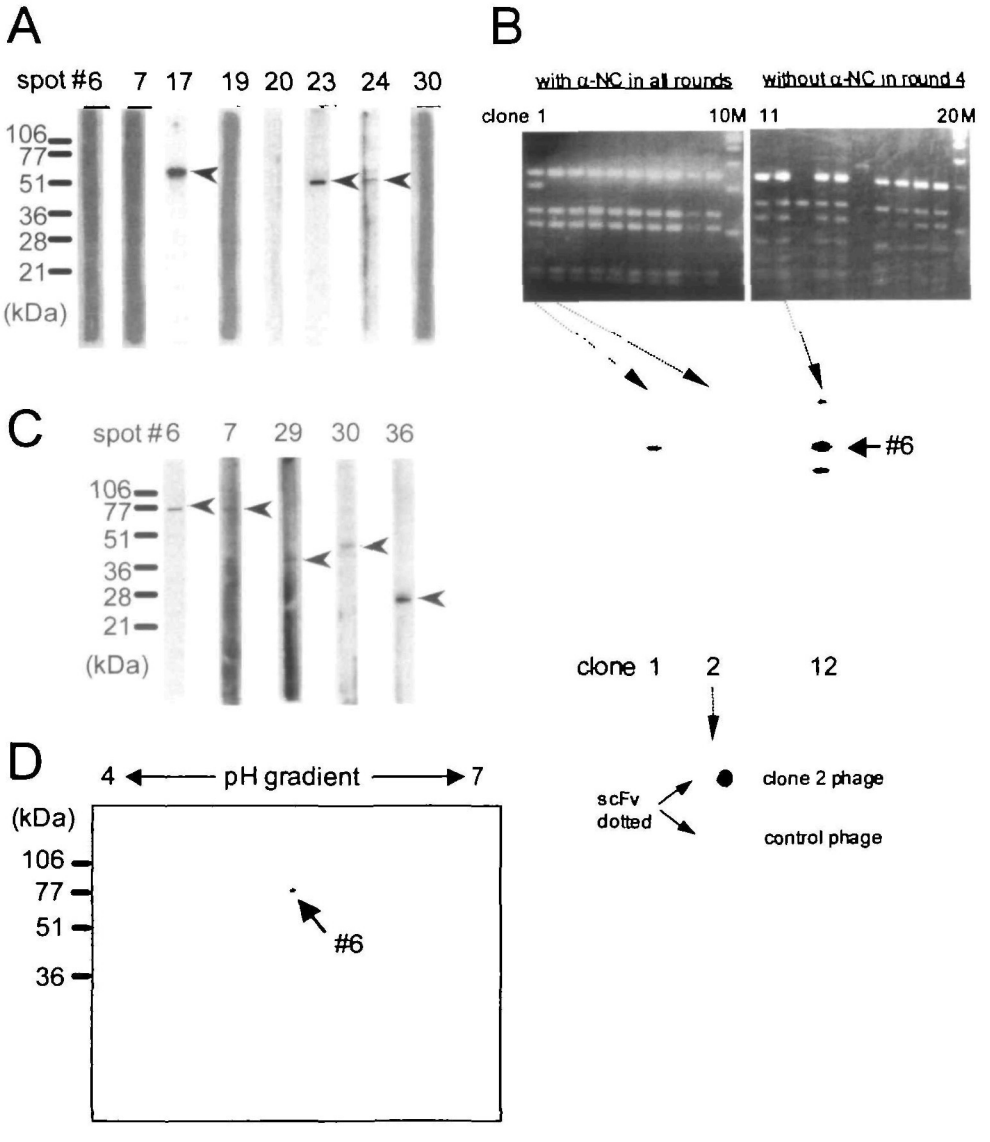

Fig. 3. **Characterization of phage antibodies isolated by 2D-PP against lipid raft proteins.** (A) 1-D Western blotting using polyclonal phage pools selected by means of method [i]. The signals corresponding to the target antigens are indicated by arrowheads. (B) Effects of the anti-nitrocellulose scFv fraction (α-NC) on phage panning. Top; the phage library was panned by means of method [ii] for 5 rounds against spot #6 with α-NC in all rounds (left) or with α-NC in all rounds except round 4 (right). *Hae*III fingerprinting of 10 randomly chosen phage clones is illustrated for each experiment (clones 1–10 and 11–20). M, DNA size markers. Middle; 1-D Western blotting with representative monoclonal phages. Bottom; dot blot analysis of purified scFv (anti-thyroglobulin) with clone 2 and control phage antibodies. (C) 1-D Western blotting using polyclonal phages selected by means of method [ii]. The protein bands corresponding to the target antigens are indicated by arrowheads. (D) 2-D Western blotting using the polyclonal phage pool against spot #6.

formed. When the soluble anti-nitrocellulose scFv was added through all 5 rounds, anti-nitrocellulose backgrounds were not detected on Western analysis. However, phages against the scFv dominated over the specific phage antibody clone, which were presumably selected and expanded against the anti-nitrocellulose scFv fraction. A representative experiment is illustrated in Fig. 3B: the dominant clones on spot #6 (clones 2–10 on fingerprinting, the top panel) are not reactive with the antigen on 1-D Western blotting (middle panel) but with scFv (dot blot presented at the bottom), indicating that the dominant clone was an anti-scFv phage. Only clone 1 reacted with the #6 antigen (middle panel).

We then performed the panning in the absence of anti-nitrocellulose scFv in round 4 or 5 (or both). This procedure sufficiently suppressed the predomination of anti-scFv phages and the most dominantly enriched clone on spot #6 (clone 12) showed binding to the #6 antigen (Fig. 3B). We employed these experimental conditions for the final panning procedure, method [ii]. We applied method [ii] to the panning on 36 antigen spots (#1–#39 except for #17, #23, and #24), and reactive phage antibodies were newly obtained against 5 antigen spots, #6, #7, #29, #30, and #36 (5/36, 13.9%) (Fig. 3C). Anti-nitrocellulose phages, however, were enriched on 28/36 spots (77.8%), suggesting that the anti-nitrocellulose phages potentially propagated even in the presence of anti-nitrocellulose scFv. The rest (3/36) showed no signal. Each of the polyclonal phage pools obtained by 2D-PP was functional in 2-D Western blotting, as expected. A representative blot experiment is illustrated in Fig. 3D. The specific phage pool reacted with multiple spots of the same molecular weight (#6), suggesting that the #6 antigen was modified, such as by phosphorylation.

The phage antibodies obtained in this work were applied to the immunoscreening of a cDNA expression library to identify its target antigens. Since the phages were selected against antigens immobilized on nitrocellulose membranes and fully functional on Western blotting, these antibodies were expected to recognize polypeptides immobilized on nitrocellulose on immunoscreening. Using monoclonal phage antibodies mo7–4.16 and mo17–2 (specific to spot #7 and #17, respectively), 1×10^6 λ clones of the human brain cDNA expression library were screened. For each phage antibody, one or two immunopositive λ phage plaques were isolated and their insert sequences were determined. On the BLAST (basic local alignment search tool) (*29*) search of nucleotide sequence databases, antigen #7 was identified as annexin VI and #17 as vimentin. In order to verify these results, we performed 2-D Western blotting and ELISA using their commercial antibodies and antigens. The antibodies against annexin VI or vimentin specifically reacted with spot #7 (Fig. 4A) or #17 (not shown) respectively, on the 2-D blot of lipid rafts. Phage antibody mo17–2 specifically reacted with human vimentin on ELISA (Fig. 4B). We

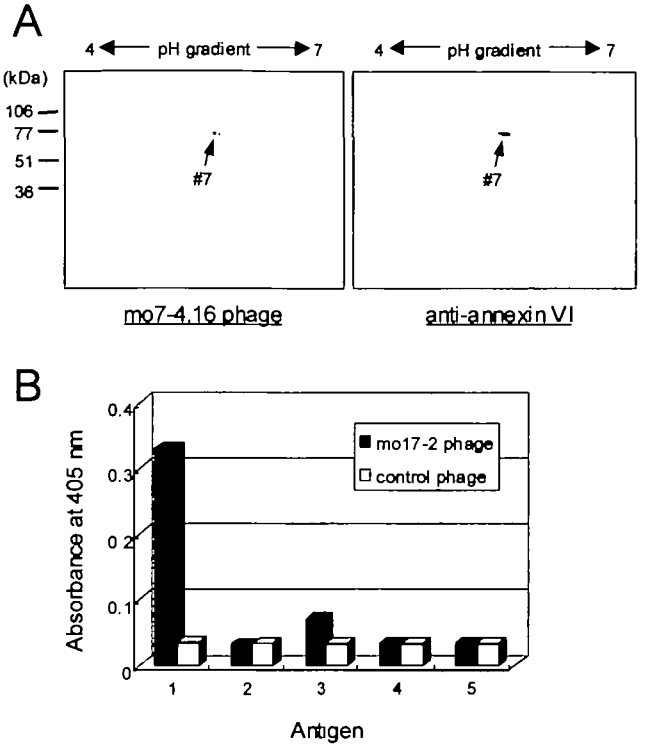

Fig. 4. **2-D Western blotting and ELISA using anti-#7 and #17 phage antibodies.** (A) 2-D Western blotting using anti-#7 monoclonal phage mo7–4.16 (left) and commercial anti-annexin VI (right) antibodies. (B) Specific binding of monoclonal phage antibody mo17-2 to vimentin on ELISA. The binding of phage clones mo17–2 and mo23-7 (control) was determined, by ELISA, to vimentin (1) and unrelated proteins (2–5); actin (2), thyroglobulin (3), bovine serum albumin (4), and hen egg-white lysozyme (5).

TABLE II. **Profiles of lipid raft proteins on 2D-PP.**

Antigen spot	Selection method[c]	Identification[d]	Molecular weight (kDa)	Isoelectric point	Average hydrophobicity[f]
#17[a]	[i]	Vimentin	53.7	5.06	–0.823176
#23[a]	[i]	Ubiquinol-cytochrome *c* reductase core I protein	52.6	5.94	–0.135417
#24[a]	[i]	Ubiquinol-cytochrome *c* reductase core I protein	52.6	5.94	–0.135417
#6[a]	[ii]	Vacuolar ATPase (isoform VA68)	68.3	5.35	–0.189951
#7[a]	[ii]	Annexin VI	75.9	5.42	–0.451560
#29[a]	[ii]	G protein β2 subunit	37.3	5.60	–0.177059
#30[a]	[ii]	Actin	41.7	5.29	–0.199733
#36[a]	[ii]	n.i.	27[e]	5.0[e]	–
#1[b]	–	Neuronal kinesin heavy chain	117.4	5.65	–0.744575
#3[b]	–	α-1 Catenin	100.1	5.95	–0.367550
#4[b]	–	Tumor rejection antigen (gp96) 1	92.5	4.76	–0.712703
#5[b]	–	DnaK-type molecular chaperone	73.7	5.87	–0.523197
#35[b]	–	SNAP-23	23.4	4.89	–0.810426

[a]To which the specific phages were generated in this study. [b]Randomly chosen. [c]See "MATERIALS AND METHODS." [d]Antigens #7 and #17 were identified by immunoscreening and the others by mass spectrometry. [e]Apparent values on the 2-D gel. [f]Calculated with the SOSUI system. n.i., not identified.

concluded that antigens #7 and #17 were annexin VI and vimentin, respectively. Immunoscreening with phages against other spots (#6, #23, #29, and #36) did not give a positive signal. Then, the protein spots successfully identified on 2D-PP and 5 other randomly chosen ones with negative results were subjected to mass spectrometric protein identification. The antigens except for spot #36 were identified by peptide mass fingerprinting analysis. The molecular weights and isoelectric points of the identified proteins are given in Table II. The SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) was used to predict the hydropathic profiles and the presence of transmembrane helices of the proteins. All of the identified proteins exhibited negative average hydrophobicity values (Table II), and no transmembrane region in these proteins was predicted.

DISCUSSION

We have developed a novel method that facilitates the selection of phage antibodies directly on two-dimensionally separated protein blots. 2D-PP enables us to obtain antibodies against molecules which have been considered to be difficult targets for a conventional method, obviating the purification of antigens in crude biochemical samples or animal immunization. Actually, antibodies specific to the components of the lipid raft fraction were successfully isolated and these phages could be functionally used for immunobiochemical methods such as Western blotting and ELISA. Furthermore, we demonstrated the applicability of these phage antibodies to the immunoscreening of a cDNA expression library, where annexin VI and vimentin were identified as the target antigens in the lipid rafts. It is known that annexin VI acts as a linker between membrane lipids and the cytoskeleton (*30*), and that vimentin is associated with SNAP-23, for instance, it is Triton X-100 insoluble (*31*). Therefore, these molecules can be observed in detergent-insoluble domains or the lipid raft fraction, and the results of our immunoscreening are reasonable.

The phage panning procedures involving nitrocellulose membranes, however, produced a large quantity of background antibodies, such as anti-nitrocellulose phages, and the efficiency, whether specific antibodies were obtained or not, was limited. As listed in Table II, eight positives were obtained on panning against a total 39 spots (20.5%), and there seemed to be some factors affecting the efficiency of 2D-PP.

The efficacy appeared to be unrelated with the spot sizes, molecular weights, isoelectric points or hydropathicity of the antigen proteins (Fig. 2 and Table II). All of the proteins listed in Table II were categorized as cell membrane-attached proteins or the subunits of membrane protein complexes. At present we do not know which specific characteristics of the proteins affect the results of 2D-PP.

On panning by methods [i] and [ii], we encountered the unexpected propagation of anti-nitrocellulose phages. This problem needs to be resolved, since it would affect the efficiency of 2D-PP more seriously than the characters of the antigen molecules. Each phage clone may have a proper rate of propagation through the panning procedures when the nitrocellulose membrane is used as an immobilization matrix of proteins. When antigen-binding clones proliferate with lower rates of propagation than that of background ones, such as anti-nitrocellulose or anti-blocking reagents phages, the number of positives in the polyclonal phage pool becomes lower, as the panning rounds proceed. In this situation, it will be necessary for a substantial number of individual phage clones to be analyzed for successful selection of positive clones. A robot-based technology, such as an antibody array system, has been developed for the screening of a large number of individual clones (*32*), however, it needed a large amount of antigens for selection. Another solution of the anti-nitrocellulose phage problem is to avoid the use of nitrocellulose membranes, as expected. However, our trial of alternate use of nitrocellulose and PVDF was not successful, which was inconsistent with the results of Liu and Marks (*33*). Theoretically, an immobilization matrix that has a high protein binding capacity but low affinity with background phages, and no antigenicity by itself is most suitable for 2D-PP, in particular for targeting the small quantities of antigens. Possibly, the microstructures of the membrane surface also affect the effi-ciency of 2D-PP. We observed that phage antibodies often failed to react with an antigen blot when reprobed after stripping of the antibody complex on the blots, whereas the usual antibody molecules such as IgG showed reproducible reactivity in such reprobing experiments. This was presumably because the antigens near the surface were stripped off during the reprobing procedure, and the phage antibody particles, about 900 × 10 nm in size (*34*), would not have access to the antigen molecules immobilized deep in the pores of the nitrocellulose membrane, although IgG (about 10 nm) could reach them. In this context, an immobilization matrix without pores will be advantageous for protein blotting in 2D-PP, which may be obtained using a new material such as conductive polymers.

In post-genomic bioscience, our 2D-PP will be a strong tool in combination with proteomic analysis performed by 2-D PAGE and mass spectrometry. 2-D PAGE/mass spectrometry is an increasingly used experimental system for the identification of proteins with high sensitivity. However, when specific antibodies are needed for further functional analysis of such identified proteins, each of the target proteins must be individually prepared in a large amount and subjected to laborious animal immunization to generate antibodies. In this context, 2D-PP is highly advantageous, since it can rapidly and directly give us antibodies against each protein separated by 2-D PAGE, even from insoluble crude fractions. Also, "the antibody catalog" can be made when antibodies are selected for each component in some characteristic protein fractions. The antibody set is applicable to the development of therapeutics or antibody chips useful for diagnosis or protein expression profiling in many diseases.

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