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Paratope-based protein identification by antibody and peptide phage display

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Abstract

In this paper we report a novel application of single-chain antibody fragments (scFv) for protein identification utilizing the inherent information of the paratope for primary structure analysis. Combining the potential of antibody phage display and peptide phage display, selected scFvs are employed to select phage-displayed peptides mimicking an epitope of the protein of interest. Proof of principle is demonstrated by identification of the neuroblastoma protein NB-p260. This protein is recognized by apoptosis-inducing IgM antibodies present in the sera of healthy individuals. Identification of NB-p260 has been hindered by its high molecular weight in the range of 260–280 kDa and its instability in purified protein preparations. Employing our approach, we subjected a human synthetic scFv library to selection using sodium dodecyl sulfate-denatured NB-p260. Specific scFvs were further used for selection of a heptapeptide phage display library. From analyzed clones, peptide sequences were identified, two of which could not be related to known proteins by conservative amino acid replacement and one of which, obtained from several clones, could be related to the actin-binding protein ABP278 after two conservative amino acid replacements. The identity of NB-p260 with ABP278 was verified by specific antibodies directed against the N and C termini of ABP278.

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Monoclonal antibodies are molecules of outstanding interest in analytical, biochemical, and medical applications. The unique property of specificity makes them essential in a broad range of identification techniques. Utilizing phage display technology [1,2] a huge variety of antibody fragments has been successfully selected against purified proteins [3] and complex sources including cell surfaces and tissues [4–8]. Antibody phage display is fundamentally based on two types of libraries, synthetic ones and those built from lymphoid sources including peripheral blood, bone marrow, and tonsils. Whereas for generation of antibodies against defined target molecules semisynthetic library formats can provide a broad panel of specificities [9–12], the selection of antibodies against immunologically interesting structures such as tumor-associated antigens is most often

pursued by libraries of the latter type [13–15]. Given an unknown protein of interest it should be possible to select and employ recombinant antibody fragments for identification of the protein by utilizing their paratope for the selection of a randomized peptide library. This approach could provide a useful alternative if identification of the protein of interest causes serious problems, e.g., due to extremely low protein levels or instability under the conditions of purification.

Here we report the sequential combination of antibody phage display technology with peptide phage display to identify the neuroblastoma-derived tumor antigen NB-p260. This antigen has been shown to be involved in the induction of apoptotic cell death by natural IgM antibodies present in the sera of healthy individuals [16–19]. Identification of NB-p260, however, has been hindered by its low concentration, its high molecular weight in the range of 260–280 kDa, and its instability due to proteolytic activity in the purified protein preparations. Using the paratope-based identification technique, NB-p260 could be identified as

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ABP278, thereby demonstrating proof of principle of this novel combinatorial approach.

Materials and methods

Tissue culture

Human LA-N-1 NB cells (obtained from R.C. Seeger) were cultivated in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Tissue culture reagents were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

Purification of NB-p260

NB-p260 was purified from extracts of LA-N-1 cells following a published procedure [19] with slight modifications. All buffers utilized for the purification of NB-p260 were supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After preparation of extracts by treatment of 1×10^9 cells with 40 ml of 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100 (pH 8.3), insoluble components were removed by centrifugation and the supernatant was applied to anion-exchange chromatography (Econo Q; Bio-Rad, München, Germany). NB-p260-containing fractions were eluted in the breakthrough with 20 mM Tris-HCl, 5 mM EDTA, 0.1% (v/v) Triton X-100 (pH 8.3), pooled, and desalted by passing through a PD-10 column (Amersham Pharmacia Biotech, Freiburg, Germany) equilibrated with 50 mM sodium phosphate, 0.1% (v/v) Triton X-100 (pH 6.5). The desalted fractions were applied to cation exchange chromatography (Econo S; Bio-Rad) equilibrated in the same buffer. NB-p260-containing fractions eluted in the breakthrough were concentrated by ultrafiltration (Amicon Diaflo Ultrafilter XM300, Millipore, Schwalbach, Germany) and further purified to homogeneity by preparative SDS-PAGE (Prep Cell Model 491, Bio-Rad) using a 4% (w/v) polyacrylamide separating gel.

Panning procedure

Immunotubes (Nunc Maxisorp, Gibco Life Technologies, Karlsruhe, Germany) were coated with purified NB-p260 (25 µg/ml PBS)² overnight at 4 °C, rinsed

twice with PBS, and blocked with MPBS for 2 h at room temperature. The blocking buffer was discarded and 10^{12} to 10^{13} phages of the human synthetic scFv library Griffin-1 (preincubated for 30 min in 4 ml of MPBS) were added. After incubation for 30 min at room temperature under continuous rotation and another 90 min without rotation, the phage-containing supernatant was removed and the tube was washed 10 times with TPBS, followed by another 10 times with PBS. During the selection procedure each washing step was performed 25 times. Bound phages were eluted by incubation with 1 ml 0.1 M triethylamine for 9 min at room temperature (continuous rotation) and neutralized immediately after removal from the tube by adding 0.5 ml 1 M Tris-HCl, pH 7.4. Remaining phages in the immunotube were neutralized with 0.2 ml of 1 M Tris-HCl, pH 7.4. Eluted phages were amplified by reinfection of an exponentially growing culture of *Escherichia coli* TG1. After 30 min at 37 °C, the infected *E. coli* TG1 culture was centrifuged at 3300g for 10 min, plated on tryptone yeast extract plates supplemented with 100 µg/ml ampicillin and 1% w/v glucose, and grown at 30 °C overnight. After four rounds of selection single colonies were randomly picked for further characterization.

Polyclonal and monoclonal ELISA

For assessment of immunoreactivity, 75 µl of phages (diluted 1:2 with PBS-milk powder (4% w/v)) was applied to each well of a microtiter plate coated with NB-p260 at 4 °C overnight and blocked with PBS-milk powder (4% w/v) at room temperature for 2 h. After incubation for 90 min at room temperature on a rocker platform, wells were rinsed three times each with PBS and TPBS, followed by adding to each well 100 µl anti-M13-horseradish peroxidase conjugate (Pharmacia Biotech, Freiburg, Germany) diluted 1:5000 in MPBS. After incubation for 60 min at room temperature on a rocker platform, wells were rinsed again three times each with PBS and TPBS, and bound phages were detected by the addition of 75 µl of an ABTS substrate solution (Sigma, Taufenstein, Germany) to each well. Absorbance was determined at 405 nm after 20 min of incubation.

Periplasmic expression of soluble antibody fragments

E. coli HB2151 cells were infected with phages for 30 min at 37 °C and plated. Subsequently, single colonies were grown at 37 °C until OD₆₀₀ of 1.0 was reached. Thereafter, the incubation was continued in the presence of 1 mM isopropyl thiogalactoside at 30 °C overnight. After centrifugation at 3000g, cell pellets obtained from 1000-ml culture volumes were resuspended in 20 ml ice-cold 0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0, followed by the addition of further 30 ml ice-cold

² Abbreviations used: NB, neuroblastoma; PBS, phosphate-buffered saline; MPBS, PBS-milk powder (2% w/v); TPBS, PBS-Tween (0.1% v/v); TBS, Tris-buffered saline; CDR, complementarity-determining region; cfu, colony-forming unit; ABP, actin-binding protein; PVDF, polyvinylidene difluoride; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; NBT, nitroterazolum blue chloride; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; ELISA, enzyme-linked immunosorbent assay; NTA, nitrilotriacetic acid.

0.2 M Tris–HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0 (diluted 1:4 with water). The suspension was incubated for 90 min on ice, then centrifuged at 10,000g, and the supernatant was dialyzed against PBS. Purification of the recombinant antibody fragments was performed using Ni–NTA–agarose (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

Selection of the peptide phage display library

Immunotubes were coated with 1 ml of purified scFv (50 µg in 0.1 M NaCO₃, pH 8.6) at 4 °C overnight. Blocking was performed with TPBS for 2 h at room temperature. After removal of the blocking buffer, 2×10^{11} phages (10 µl of the Ph.D.-7 Peptide 7-mer Library, NEB, Frankfurt, Germany) were added, followed by incubation for 60 min at room temperature under continuous rotation. The phage-containing supernatant was discharged and the tube was washed 10 times with TBS–Tween (0.1% w/v) and another 10 times with TBS. Bound phages were eluted by adding 1 ml 0.2 M glycine–HCl, pH 2.2, and incubating for 9 min at room temperature (continuously rotating). Eluted phages were neutralized immediately by adding 0.15 ml 1 M Tris, pH 9.1, and amplified by reinfection of 20 ml of an exponentially growing culture of *E. coli* ER2738. After incubation for 4.5 h at 37 °C (constant shaking), cells were centrifuged at 10,000g for 10 min and phages were precipitated from the supernatant at 4 °C overnight by the addition of 3.6 ml of 20% w/v polyethylene glycol, 2.5 M NaCl. The precipitate was harvested by centrifugation at 10,000g for 20 min, resuspended in 200 µl TBS, and applied for the next round of selection. After three rounds of selection single plaques were randomly picked and further characterized.

Generation of antibodies with specificity for ABP278

For assessment of immunoreactivity of the NB-p260, an ABP278-specific domain was amplified from cDNA of LA-N-1 NB cells using the following oligonucleotides: ABP7300, gatcgcccccagccgcatgctcctgtaac and ABPback, gatcgccgcgcagcactgtgacatgaaaag.

The amplified DNA was introduced into the vector of the Griffin-1 library pHEN2 via *Sfi*I and *Not*I restriction sites and used to electroporate *E. coli* HB2151 cells. Single colonies were grown until OD₆₀₀ of 0.9 and expression was induced with isopropyl thiogalactoside (final concentration: 1 mM). After 12 h at 30 °C the cells were harvested at 2800g for 10 min. For solubilization of the recombinant protein the cells were resuspended in PBS–8 M urea and incubated overnight at room temperature. Debris were removed by centrifugation at 10,000g and the supernatant was applied to Ni–NTA–agarose. Eluted fractions were analyzed by SDS–PAGE and dialyzed against PBS to remove imidazole and urea.

The purified protein was used to raise polyclonal antibodies from rabbits according to standard immunization protocols.

Other methods

SDS–PAGE and Western blotting were performed according to established protocols [20].

Results

NB-p260 was purified from crude extracts of LA-N-1 neuroblastoma cells according to a published protocol with slight modifications [19]. Analytical SDS–PAGE demonstrated homogeneity of the purified protein (Fig. 1, lane 2) and the identity of the NB-p260 was assessed by Western blotting using human antineuroblastoma IgM antibodies [19] (Fig. 1, lane 3). NB-p260 was obtained in yields of approximately 10 µg/10⁹ LA-N-1 cells.

For the generation of monoclonal antibody fragments the human synthetic antibody library Griffin-1 [3] was subjected to selection against the purified NB-p260 immobilized on a polystyrene support. This library is composed of scFv-formatted variable regions of human origin, providing a diversity of approximately 2×10^9 . The phagemid-based vector system contains his and myc tags for purification and detection of selected antibody fragments. After iterative panning against NB-p260, significant enrichment in the fourth round of selection was assessed by the increase in phage output from selection and the amplification constants (Table 1). Subsequent analyses by ELISA demonstrated significant

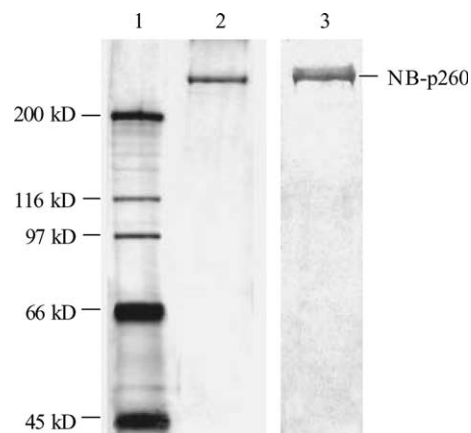


Fig. 1. Analysis of purified NB-p260 (50 ng) by gel electrophoresis and Western blotting. Gel electrophoresis was performed on a 7.5% polyacrylamide gel under reducing conditions, followed by silver staining (standard proteins, lane 1; NB-p260, lane 2). Identity of NB-p260 was verified by Western blotting according to standard protocols utilizing anti-NB-p260 IgM containing serum [19] and an anti-human IgM–alkaline phosphatase conjugate (diluted 1:20,000 in MPBS) (lane 3).

Table 1
Enrichment of the antibody phage display library

Selection round	Control		NB-p260	
	Output of phages (10^4 cfu)	Amplification constant	Output of phages (10^4 cfu)	Amplification constant
1	3	3	3	3
2	2	4	2	5
3	2	3	5	10
4	2	9	150	600

Amplification constants were determined from titers of phages resulting from each selection round by taking into account the individual input of the round and the relation of input and output of the former round. As control a completely analogous selection against milk powder was performed. For details see Materials and methods.

reactivity of the majority of randomly picked clones (Fig. 2C). The diversity of the enriched sublibrary was assessed by fingerprinting via *MvaI* restriction of amplified DNA of individual scFv. The presence of a dominant subset of restriction pattern fingerprinting suggested identity of most of the analyzed clones (Figs. 2A and B), an assumption that was confirmed by DNA sequencing. Analysis of the CDR of the dominant clone revealed a CDR3 of the heavy chain consisting of only three residues in length while the CDR3 of the light chain was composed of nine residues in length (Fig. 3).

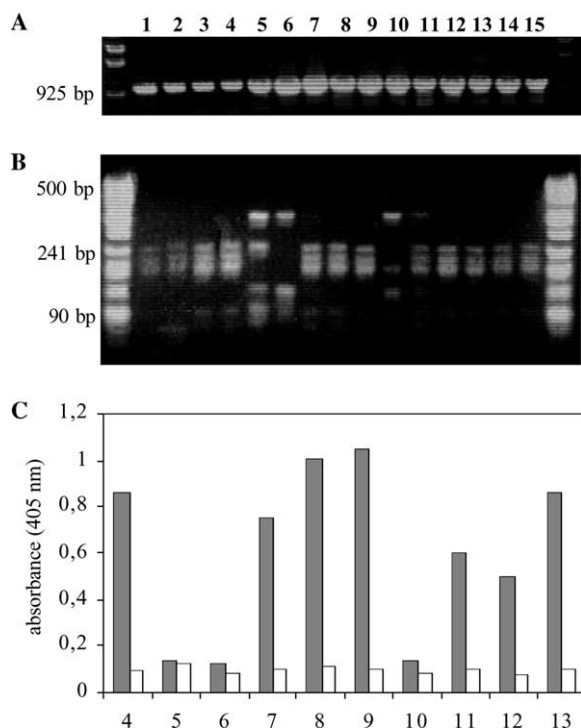


Fig. 2. Restriction pattern and immunoreactivity of selected phages. (A) Shown are gel electrophoretical analyses of PCR-amplified scFv DNA using vector-derived primers including the pelB sequence, promoter region, and parts of gIII. Colonies were randomly picked. (B) The scFv DNA of selected phages was extracted from agarose, digested with *MvaI* for 3 h, and analyzed by gel electrophoresis (lanes 2–16 represent the clones 1–15). (C) The immunoreactivity of selected clones against NB-p260 (filled bars, controls by omission of NB-p260, open bars) was analyzed by ELISA as described under Materials and methods.

Phages of the selected clone were used for infection of the nonsuppressor *E. coli* strain HB2151 and expression of antibody fragments was directed into the periplasmic space. After purification of soluble antibody fragments by Ni-NTA-agarose analytical SDS-PAGE demonstrated homogeneity of the antibody fragments (Fig. 4A). Yield of expression was found to be in the range of 400 μ g/l. The reactivity against purified NB-p260 (Fig. 4B, lane 1) of both the phage displayed and the soluble scFv was confirmed by Western blotting (Fig. 4B, lanes 2 and 3). Optimal results were obtained with a phage titer in the range of 1×10^{11} – 5×10^{11} cfu and soluble scFv at a concentration of approximately 1 μ g/ml. In Western blot analysis of crude extracts of LA-N-1 cells (Fig. 4B, lane 4) the soluble scFv demonstrated no crossreactivity indicating the specificity of the antibody fragments.

To obtain information about the identity of NB-p260, we analyzed the specificity of the paratope of the anti-NB-p260 scFv by subjecting a peptide phage display library to selection against these antibody fragments. After three rounds of panning, enrichment of the library was demonstrated by polyclonal ELISA and individual clones were characterized by DNA sequencing. Analysis of the obtained peptide sequences revealed a conserved motif in a broad subset of the clones. The sequence motif LPPNPTK was found in 5 of 13 clones and derived motifs based on the $LP_n(X)_n P(X)_n$ structure ($n = 1$ – 2) were found in another 3 clones. Therefore, the sequence motif LPPNPTK was used to perform a retrieval of information from protein databases. The most successful algorithm was found to be that provided by the Protein Prospector at the University of California Los Angeles, specialized in peptide mass fingerprinting. As the apparent molecular weight of the target protein was known, the search was limited to proteins of human origin with a molecular weight in the range of 250–290 kDa. Additionally, the number of possible mismatches was limited to two positions. Due to these limitations only nine proteins were found to display the needed homology (Table 2). Six of the nine belong to the ABP family, two represent a subunit of a calcium channel, and one is a receptor associated mediator. Two of the nine proteins could be excluded since

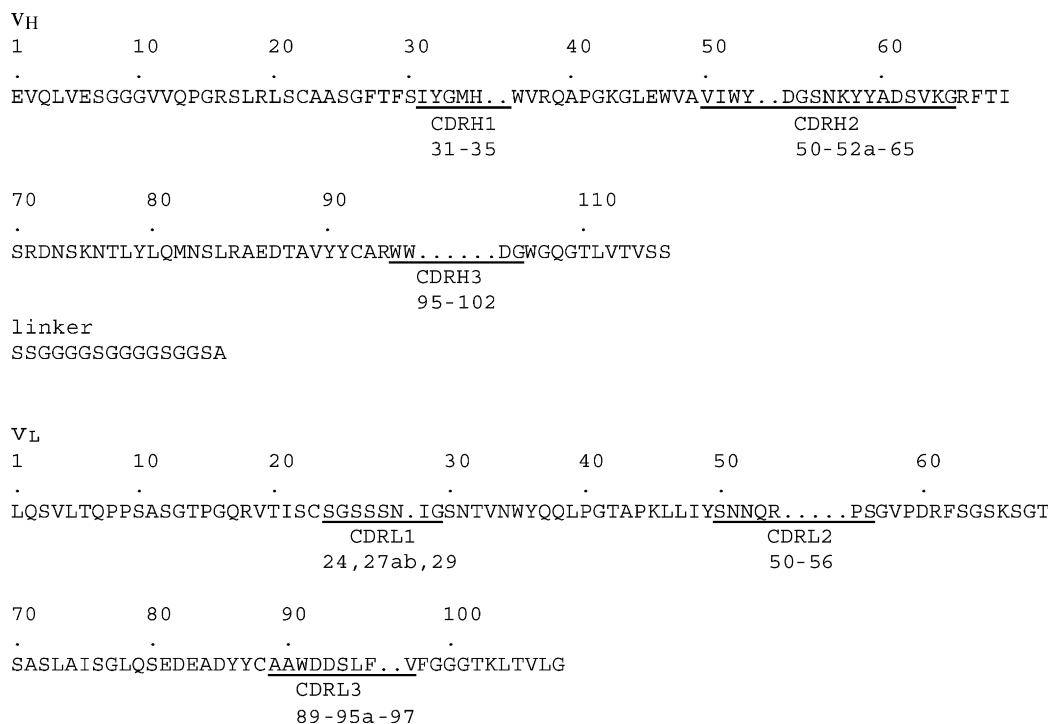


Fig. 3. Sequence analysis of the dominating anti-NB-p260 scFv. Numbering and determination of the CDR was performed according to Kabat et al. [21]. Alignments to V-Base (MRC, Cambridge) revealed usage of VH3 and VL1 segments.

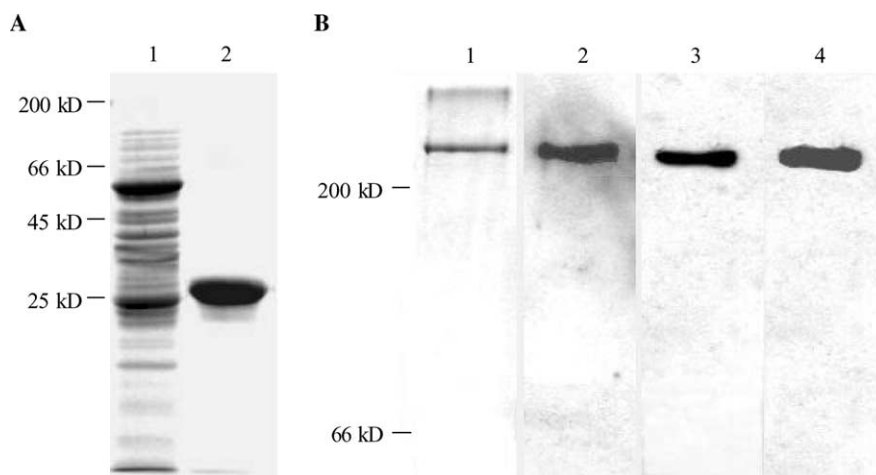


Fig. 4. Purification and characterization of the immunoreactivity of the predominant scFv. (A) The predominant scFv was purified from periplasmic extracts (lane 1) as described under Materials and methods and analyzed by SDS-PAGE under reducing conditions, followed by staining with Coomassie blue (lane 2, 2 µg protein). (B) Purified NB-p260 (lanes 1–3, 50 ng protein) and an extract of LA-N-1 NB cells (lane 4) prepared as described [19] were separated by 5% SDS-PAGE under reducing conditions and stained with silver (lane 1) or transferred to a PVDF membrane (lanes 2–4). Recombinant anti-NB-p260 phages were applied at a concentration of 5×10^{11} cfu in MPBS. After incubation overnight at room temperature bound phages were detected by chemiluminescence using an anti-M13-horseradish peroxidase conjugate (diluted 1:5000) (lane 2). Soluble scFv, preincubated for 30 min with stoichiometric amounts of a 9E10 anti-myc-horseradish peroxidase conjugate (Invitrogen Life Technologies, Karlsruhe, Germany) were applied at a concentration of 1 µg/ml in MPBS, incubated for 2 h at room temperature, and detected by chemiluminescence (lanes 3 and 4).

their isoelectrical points are in the mild basic range, whereas ion-exchange chromatography data indicated an isoelectrical point of NB-p260 in the acidic range. From the remaining seven candidates, six belong to the ABP family.

To verify the identification we assessed the immunoreactivity of NB-p260 using polyclonal sera with specificity for the N and C termini of ABP278. The N terminus is conserved among the ABP family, but the C-terminal fragment provides ABP278-specific sequences.

Table 2
Restricted homology search for proteins of human origin

Epitope homologous to LPPNPTK	Identified proteins	m.w. (Da)	i.p.
LPPVPPK	Voltage-operated calcium channel alpha-1 subunit	261,730	8.5
LPINPTI	T calcium channel alpha 1G subunit	262,473	6.1
LAPNPTY	Silencing mediator of retinoic acid and thyroid hormone receptor	274,035	8.0
LPPDPSK	ABP-278 (beta-filamin)	278,193	5.4
LPPDPSK	Filamin (beta-filamin)	278,196	5.4
LPPDPSK	Filamin 2 (gamma-filamin)	288,902	5.7
LPPDPSK	Filamin, muscle isoform (gamma-filamin)	287,357	5.6
LPPDPSK	Gamma-filamin	291,023	5.6
LPPDPSK	Gamma-filamin	292,469	5.6

Listed are the proteins from homology searches for the LPPNPTK sequence motif using the Protein Prospector at the University of California at Los Angeles. As limitations for database entries the molecular weight was restricted to a range between 255 and 295 kDa and only two mismatches were allowed.

C-terminus-specific antisera, however, were not commercially available. Therefore, amplification by PCR and cloning of the C-terminal region (aa 2445–2602) of the ABP278 from cDNA of LA-N-1 NB cells were performed and the ABP278 cDNA fragment was expressed in *E. coli* HB2151 cells. Recombinant protein was solubilized in 8 M urea, purified by Ni-NTA affinity chromatography from crude cell lysates, and employed to raise polyclonal antibodies. Using Western blot analysis, both the polyclonal sera directed against the conserved N terminus of filamin and the polyclonal antibodies raised against the C terminus of ABP278

recognized NB-p260 (Fig. 5, lanes 1 and 2), thus confirming the identity of NB-p260 with ABP278.

Discussion

In this study we have developed a procedure for protein identification that takes advantage of the unique specificity of antibodies and their fragments. The method is based on the sequential combination of antibody phage display technology to generate target-specific antibodies and peptide phage display to utilize the paratopes of these antibodies for the selection of a randomized peptide library. Employing phage display of synthetic or semisynthetic antibody fragment libraries, there is no need for large amounts of antigen, and time-consuming procedures including immunization and hybridoma technology are avoided. Additionally, synthetic libraries in general are applicable to a broad range of targets including origins other than human. These types of libraries nowadays can be built according to common protocols or can be obtained from different institutions.

Epitope mapping of the target-specific antibodies with a random peptide library was performed with phage-displayed peptides, since solid-phase synthesis, although utilized successfully, still remains intense in effort and is of limited diversity [22,23]. Based on the assumption that the length of linear epitopes is in the range of four to six amino acid residues, the peptide length was restricted to seven residues, covering the needs of sufficient length.

Since the increase of peptide length is reciprocally accompanied by a reduction of copy number of one peptide species in a given population of defined size the use of libraries of longer peptides may result in loss of peptide species and therefore decrease in specificity.

In consequence, libraries of heptapeptides have been shown to be selected more efficiently than those of dodecapeptides [24], which may result from the full diversity of heptapeptide libraries.

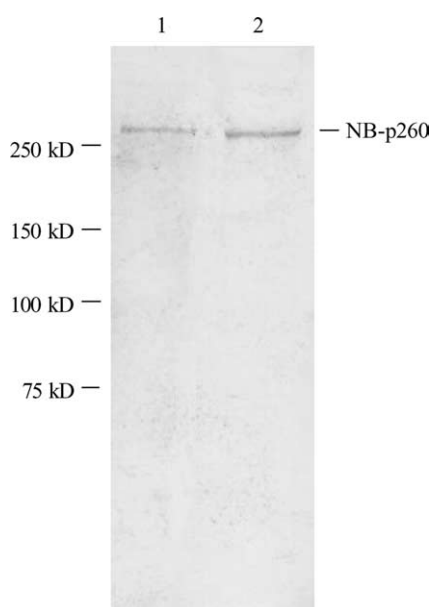


Fig. 5. Detection of NB-p260 with anti-ABP-antibodies. Purified NB-p260 (20 ng) was separated by 5% SDS-PAGE under reducing conditions, transferred to a PVDF membrane, and incubated overnight with polyclonal antibodies with specificity to the N terminus (goat antibodies, diluted 1:200) (lane 1) and to the C terminus of ABP-278 (rabbit antibodies, diluted 1:1000 in MPBS) (lane 2). Bound antibodies were detected using an anti-goat-alkaline phosphatase conjugate or anti-rabbit-alkaline phosphatase conjugate (both diluted 1:10,000 in MPBS) using NBT/BCIP as substrate.

One important implication of random peptide library-derived sequences is the requirement of a linear nature of the antibody epitope for successful alignment. To ensure linearity of the epitopes we included preparative SDS-PAGE as the final purification step of the target protein. Although bound SDS may cause loss of some epitopes and, therefore, may limit the selection process, it is essential for linearization of the protein. In contrast to random peptide libraries, cDNA expression libraries may provide more detailed molecular information [25], but especially in the case of high-molecular-weight proteins, such as the NB-p260, this approach often is hindered by the need for high-diversity cDNA libraries without 5'-bias-based loss of N termini. Additionally, structural information obtained from conformational binding sites may be misleading due to misfolding of protein structures in lytic or secretorial library systems.

Proof of principle of our protein identification strategy is demonstrated by the successful analysis of NB-p260, a neuroblastoma-derived tumor antigen involved in apoptotic induction in human neuroblastoma cells by natural IgM antibodies [19]. Several factors including the low concentration of NB-p260, its high molecular weight in the range of 260–280 kDa, and its degradational tendencies in established purification procedures have contributed to hinder its identification by classical N-terminal sequencing. Applying our protein identification strategy, the first crucial step was the generation of target-specific antibody fragments. While this step was accomplished successfully, only a limited diversity of resulting antibody fragments was obtained. Whether this is due to the existence of dominant epitopes or to advantages in selection of phages as a result of secretion or assembly efficiency remains elusive. The latter, however, is supported by the observation that the predominant clone showed a high expression level after transfer in HB2151 cells. Most important for the identification of NB-p260 was the fact that the dominant scFv clone proved to be highly specific for SDS-denatured NB-p260.

Using the purified scFv for selection of a heptapeptide phage display library, analysis of the derived peptide sequences exhibited a conserved motif in the majority of clones after three rounds of selection. Based on this motif, a homology search in databases of proteins resulted in the identification of nine candidates. The vast amount of database entries could be excluded based on the restriction of maximal two mismatches and on known properties of NB-p260 including its human origin and its molecular weight. Further consideration of the isoelectrical point of NB-p260 and the probability of tissue-specific expression restricted the number of candidates to three members of the filamin family, the actin binding proteins ABP278/276, ABP280, and ABPL. Both ABP278 and ABPL exhibit identical primary structures in the putative epitope region, whereas ABP280 displays significantly lower homology and,

therefore, was excluded. From the remaining candidates ABP278 represented the most probable one for NB-p260 since its molecular weight is similar to the apparent molecular weight of NB-p260 estimated by SDS-PAGE [26]. This result could be verified by the immunoreactivity of NB-p260 with two types of antisera directed against the N and C termini of ABP278. Most important was the reactivity of NB-p260 with antibodies raised against the C terminus of the ABP278 since this fragment distinguishes ABP278 from other ABPs of the filamin family. It should be mentioned that in a later study the identity of NB-p260 with ABP278 was also confirmed by mass spectrometric sequencing [27].

Since ABP of the family of filamins are known to interact with cytoplasmic domains of a broad range of membrane proteins [28–37], how ABP278 may serve as apoptosis-inducing receptor for human IgM antibodies remains to be determined. Human ABP278 has been identified recently in two-hybrid screens of platelet glycoprotein Ib and presenilin [38,39] and cloned subsequently from placenta cDNA [40]. The C-terminal domain employed for raising polyclonal antibodies is known to be immunoreactive with Graves disease immunoglobulins [41]. While additional information is not available currently, the successful cloning of the C terminus of ABP278 from a cDNA library of LA-N-1 cells provides further evidence for transcription and expression of this protein in NB cells.

In summary, the successful identification of NB-p260 demonstrates the potential of the combination of two phage display techniques for obtaining sequence information about proteins of unknown identity. The diversity of possible primary structures hampering the identification process can be reduced significantly by starting with a critical mass of information about the protein of interest. One major advantage of our strategy is the potential usefulness of selected monoclonal antibody fragments since they represent valuable tools for purification and interaction studies with the particular target protein.

Acknowledgments

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