Direct production and purification of T7 phage display cloned proteins selected and analyzed on microarrays

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Phage display technology has emerged into a powerful tool for identifying proteins with specific binding properties. This technology adds amino acid sequences to the carboxy terminus of a phage capsid protein, thus generating a fusion protein displayed on the surface of the phage. Here, we have developed a high-throughput strategy to synthesize purified protein that solves many of the problems associated with crude phage lysates. Phage DNA was used as a template for a nested PCR that added the T7 promoter, ribosome binding site, and a His_6-tag. The PCR product was then used as a template for in vitro transcription/translation. The resulting His_6-tagged recombinant protein was then purified by nickel affinity chromatography. The functionality of the purified protein was verified using protein microarray analysis.

INTRODUCTION

The search for antibodies that can distinguish diseased cells from normal cells is of paramount importance. Serological identification of recombinantly expressed genes (SEREX), based on the detection of antigens within recombinantly expressed tumor cDNA phage libraries by autologous antibodies (1), has been used to isolate tumor or autoimmune antigens. SEREX combines serological analysis with antigen cloning techniques to identify human tumor antigens eliciting high-titer immunoglobulin G (IgG) antibodies (www.cancerimmunity.org/SEREX/introduction.htm) (2). This method has identified new tumor antigens that have subsequently been shown to contain epitopes for humoral immune responses (3,4). However, for efficient SEREX cloning, the serum titer against the antigen of interest needs to be high, the serum needs to be relatively specific, and large quantities of serum at the right point in time are necessary because the serum response may change over time (5). Furthermore, because B cells are present in the tissue used to construct the cDNA library, IgG cDNA may be incorporated into the library. Secondary anti-IgG antibodies could detect these protein fragments as false positives (5).

Typical phage display technology involves expression of recombinant proteins or peptides that are fused to a phage coat protein. Pertinent proteins, such as fused peptides, antibodies, and enzymes, may be synthesized and selected to acquire certain binding affinity and specificity (6–9). DNA fragments encoding millions of variants of protein fragments are cloned into the phage genome and expressed as a fusion protein as part of one of the phage coat proteins (10). Phage that display a certain protein ligand may be bound to an immobilized target and retained. Non-adherent phage are washed away, and the bound phage are recovered from the surface and subsequently used to re-infect bacteria for further enrichment.

To develop a system amenable to high-throughput to synthesize and produce purified recombinant antigens, we used agarose beads bound with an antibody to human SIRT2 as bait to screen a T7 phage human brain cDNA library. Human SIRT2 was used for these experiments since it is known to be expressed in brain. The protein is involved in the G2/M checkpoint in mitosis, and highly specific polyclonal antibody to SIRT2 was available (11). Four rounds of phage screening, referred to as biopanning, were found to produce an adequate number of reactive clones (approximately 8%). A problem with the use of phage lysates in immunological studies is that the majority of the proteins in the lysate are of host bacterial origin. Therefore, we developed a system in which the phage DNA in lysates were used as a template for a nested PCR that in turn generated a template for in vitro transcription/translation of the cloned epitope of interest. Utilizing the binding affinity of a His_6-tag that was incorporated via the PCR steps, the recombinant

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protein was then purified by nickel affinity chromatography. This process can be performed in high-throughput to generate pure protein from the clones identified by phage display technology. Moreover, microarray chips containing purified proteins may be generated using clones that have been identified as reactive with disease versus normal human sera.

Functional analysis of crude and pure recombinant fusion protein was performed using peptide competition immunoassay on protein microarrays. The goal of this study was to develop a strategy for producing purified recombinant phage protein that can act as a superior antigen for future studies of serum antibodies as disease markers on protein microarrays. A flow chart of our technique is shown in Figure 1.

MATERIALS AND METHODS

Isolation of a SIRT2 Phage Recombinant Clone by Differential Biopanning of T7 Phage cDNA Brain Library

The isolation of a SIRT2 phage clone was achieved by differential biopanning technology. The first step in this strategy was to negatively immunoselect a T7 bacteriophage human brain cDNA library (Novagen, Madison, WI, USA) with pre-immune rabbit serum to remove the nontargeted proteins that react with pre-immune serum. The pre-adsorbed T7 phage library was then positively immunoselected with SIRT2 rabbit antisera, which acted as bait for selection of T7 bacteriophage bearing the SIRT2 insert. The biopanning procedure and phage propagation were done as per the manufacturer's instructions.

We performed differential biopanning with negative and positive selection using pre-immune rabbit serum and rabbit SIRT2 polyclonal antibody as per the manufacturer's suggestions (TB178 T7Select® System; Novagen). Briefly, 25 µL of protein G+ agarose beads were washed in 1× phosphate-buffered saline (PBS) and were blocked in 1% bovine serum albumin (BSA) for 1h. The beads were next incubated with pre-immune rabbit serum at a dilution of 1:20 for 2 h, washed three times with 1× PBS, and incubated with the T7 phage display human brain cDNA library for 2 h. This subtractive biopanning step is crucial for removal of proteins other than SIRT2 in the T7 phage display library that react with IgGs present in pre-immune rabbit serum. The beads were centrifuged at 2040× *g*, and the supernatant T7 phage cDNA library was then incubated with SIRT2 polyclonal antibodies immobilized on protein G+ agarose beads as discussed in the previous section. The incubation was done at 4°C overnight. The beads were washed three times with 1× PBS, and the bound T7 phage cDNA library was eluted with 1% sodium dodecyl sulfate (SDS). The eluant was next amplified using *Escherichia coli* strain BLT5616 for the next round of biopanning. Four rounds of biopanning were performed, and the selected phage library was used for immunoscreening. Immunoscreening was performed using standard plaque-lift methods. Approximately 1000 plaques from each biopanning (1–4) were transferred onto individual Hybond™-N+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). Southern blots were performed using SIRT2 cDNA as the probe. Among the clones evaluated at biopannings 3 and 4, approximately 1.5% and 8% hybridized to a SIRT2 probe. Numerous phage were isolated, and two independent SIRT2 phage clones were employed in this study (SIRT2_A and SIRT2_B).

In Vitro Transcription/Translation of Phage Lysate

Template generation: first-stage PCR amplification. DNA from the amplified phage was used as a template for the first stage PCR amplification. The forward PCR primer contained the ribosome binding site (Shine del Garno sequence), a His_6-tag, and the ATG start codon to the gene of interest. PCR amplification was carried out in a reaction volume of 25 µL containing 0.5 µL of the appropriate phage lysate, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM PCR nucleotide mix (Promega, Madison, WI, USA), 2.5 U *Taq* DNA polymerase (Promega), 1 pmol forward

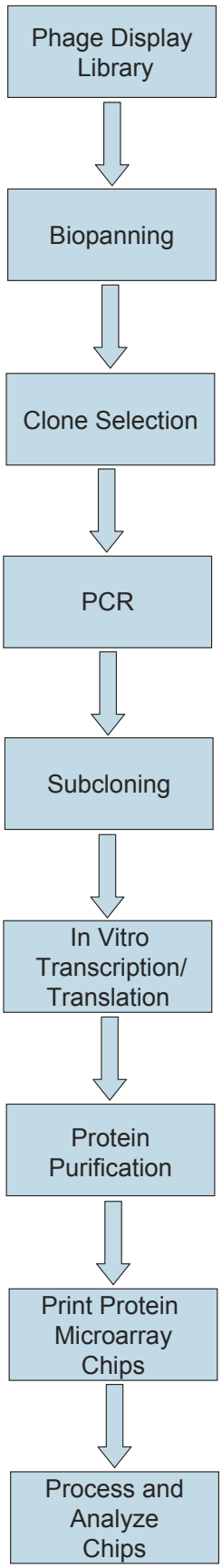

Figure 1. Description of the methods used for production and purification of cloned proteins used for microarray analysis.

primer (5′-AACGGTTTCCCTCTA GAAATAATTTTGTTTAACTTTA AGAAGGAGATAAATG**CATCAT CATCATCATCATCAT**ATGGCTAGC ATGACTGGTGGACAGCAAATG -3′; His_6-tag bolded and underlined), and 10 pmol reverse primer (5′-GGGGTTTTTTGCTGAAAGGAGG-3′) (all primers were synthesized by Sigma-Genosys, The Woodlands, TX, USA). A Mastercycler® thermal cycler (Eppendorf, Hamburg, Germany) was programmed for 94°C for 3 min, then 10 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 10 cycles of 94°C for 1 min and 72°C for 1 min.

Template generation: second-stage PCR amplification. The second PCR added a T7 promoter at the 5′ end of the PCR product obtained from first-stage PCR amplification. PCR amplification was carried out in a reaction volume of 50 µL containing 1.0 µL of the first stage product, 3 mM $MgCl_2$, 200 µM PCR nucleotide mix, 2.5 U *Taq* DNA polymerase, 10 pmol second forward primer (5′-AGATCTCGATCC CGCGAAATTAATACGACTCACTA TAGGGAGACCACAACGGTTTCCC TCTAGAAA-3′), and 10 pmol of the reverse primer described above. The thermal cycler was programmed for 5 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C 1 min, then 25 cycles of 94°C for 1 min and 72°C 1 min, and then followed by 72°C for 7 min, and finally 4°C hold. Ethanol precipitation was performed with Pellet Paint Co-Precipitant (Novagen) according to the manufacturer's protocol. One 50-µL PCR provided approximately 1 µg DNA after precipitation.

EcoPro™ transcription/translation. Coupled transcription/translation (12,13) was performed with EcoPro T7 System (www.emdbiosciences.com/docs/NDIS/inno16-001.pdf; Novagen) using a 50-µL reaction in 0.2-mL thin-walled RNase- and DNase-free tubes (Molecular BioProducts, San Diego, CA, USA). Thirty-five microliters EcoPro extract were added to 11 µL (2–4 µg) of the resuspended template. Next, methionine (supplied with the EcoPro kit) and rifampicin (Sigma-Aldrich, St. Louis, Mo, USA) were added to final concentrations of 0.2 and 0.061 mM, respectively. The tube was then incubated for 1 h at 37°C.

Nickel-Nitrilotriacetic Acid Magnetic Agarose Bead Purification

The recombinant protein produced in the EcoPro reaction contained bacterial protein inherent to the *E. coli* extract. Purification was carried out exploiting the His_6-tag using nickel affinity purification. This procedure used nickel molecules bound to agarose beads to purify the protein. Fifty microliters of each protein were added to 930 µL lysis buffer in a 1.5-mL microfuge tube. The lysis buffer consisted of 10 mM imidazole (Qiagen, Valencia, CA, USA), 50 mM NaH_2PO_4, 200 mM NaCl, 0.05% Tween®20 (Sigma-Aldrich) with a final pH of 8.0. Thirty microliters nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen) were then added to the protein, and the tubes were gently agitated at 4°C for 3 h. The tubes were centrifuged at 15,300× *g* briefly and placed on a 12-Tube magnet magnetic separator (Qiagen) for 1 min. The supernatant was removed, and the beads were washed three times with 20 mM imidazole in the buffer described above. The beads were again collected by the magnet and eluted two times with 250 mM imidazole in the same buffer. For each elution, the beads were resuspended in 30 µL elution buffer and incubated for 1 min at room temperature. The two 30-µL eluates were then pooled together. Because imidazole increases the background during fluorescent scanning of microarrays, a buffer exchange was performed. The sample was concentrated through a 30-kDa centrifugal device (Micron YM-30; Millipore, Bedford, MA, USA) along with two 400-µL aliquots of 1× SSC buffer (150 mM sodium chloride, 15 mM sodium citrate). The final concentrated volume was approximately 20 µL.

Western Blot/Silver Stain Analysis

Total protein from phage lysate, EcoPro product, and nickel-purified EcoPro product were separated by electrophoresis on 10% SDS polyacrylamide gels. Proteins were transferred to Protran® nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH, USA). The membrane was blocked with 5% nonfat milk for 1 h at room temperature, followed by incubation with mouse monoclonal antibody to the T7 gene 10 protein (anti-T7 Tag antibody; Novagen) at a dilution of 1:10,000 or a rabbit antiserum to the SIRT2 protein (11) at a 1:1000 dilution for 1 h at room temperature. The T7 Tag monoclonal antibody binds to the first 11 amino acids of the N-terminal region of the gene 10 protein of each phage. A horseradish peroxidase-conjugated goat anti-rabbit or sheep anti-mouse antibody was used as a secondary antibody (Pierce, Rockford, IL, USA). Silver staining was performed to demonstrate the purity of the protein according to standard protocols (14). BSA was loaded at 10 and 50 µg/lane on gels destined for silver staining in order to estimate the amount of purified proteins.

Peptide Competition

The SIRT2 antibody was incubated for 3 h at 4°C with either a SIRT2 peptide (DEARTTEREKPQ) (11), a negative control peptide (VFQSGVMLGDPNSS; Sigma-Genosys), or no peptide. The negative control peptide was derived from the T7Select 10-3B Vector upstream from the multiple cloning region. The peptides were used at a final concentration of 0.05 mg/mL for microarrays and at 0.01 and 0.001 mg/mL for Western blot analyses.

Preparation and Processing of Protein Microarrays

Five replicates of each protein sample were arrayed onto nitrocellulose-coated FAST® Slides (Schleicher & Schuell) using the ArrayIt® SpotBot® (Telechem, Sunnyvale, CA, USA). Microarrays were blocked with a 4% milk-PBS solution. The microarrays were then washed three times with 1× PBS. The primary antibody was allowed to bind to the arrayed proteins for 1 h. The antibody was blocked with either the SIRT2 peptide, the control peptide, or no peptide. The SIRT2 antibody was used at a dilution of 1:1000, and the T7 Tag monoclonal antibody was used at

a dilution of 1:3333 (0.2 µg/µL). The microarrays were then washed three times for 4 min in PBS with 0.1% Tween-20 and then incubated for 1 h with Alexa Fluor® 532 green-labeled goat anti-mouse (Molecular Probes, Eugene, OR, USA) at a dilution of 1:40,000 and Alexa Fluor 647 red-labeled goat anti-rabbit (Molecular Probes) at a dilution of 1:2000. The arrays were washed three times for 4 min in PBS with 0.1% Tween-20, followed by two washes of 2 min each in PBS. The arrays were then dried and scanned with a GenePix® 4100A using GenePix Pro 5.0 software (both from Axon Instruments, Union City, CA, USA) at wavelengths of 532 nm (green) and 635 nm (red). The images were quantified with ImaGene software (BioDiscovery, El Segundo, CA, USA) and analyzed using Microsoft® Excel®. The local background for both the green and the red channels were subtracted from the intensity of each spot. A dye ratio of red:green was then calculated for each spot, and the five replicates were averaged and then used to calculate a standard deviation. For the SIRT2 antibody slides, the average of the red channel of the control empty phage was subtracted from each individual sample (pre-immune or SIRT2 antibody) after background subtraction and prior to calculating the dye ratios. Because the green channel of the SIRT2 and control samples were very similar, this did not impact the shape of the graphs performed. Two-tailed, two-sample unequal variance *t*-tests were used to compare samples with no peptide block to samples blocked with the control peptide. One-tailed, two-sample unequal variance *t*-tests were used to compare samples with either no peptide block or the control peptide block with samples blocked with the SIRT2 peptide.

RESULTS AND DISCUSSION

Western blotting analyses or microarrays using nitrocellulose-coated slides can reveal antigens in phage lysates that are detectable with pure antibodies and antisera. Because high background signals can be a problem in such assays, we decided to refine the recombinant antigen preparations by synthesizing and purifying the proteins using the phage DNA contained in the crude bacterial lysate as a template for in vitro transcription/translation and nickel affinity purification. This approach has the advantage of eliminating other phage and bacterial proteins that would result in nonspecific binding of the immunoglobulins to the protein of interest.

In Vitro Transcription/Translation of T7 Phage Displayed Antigens

Our strategy for producing and purifying recombinant fusion proteins utilized a nested PCR design that generated linear DNA templates compatible with EcoPro in vitro transcription/translation system. Crude phage lysates were used as the template for the first-stage PCR. While the same reverse primer was used in both the first- and second-stage PCR, a different forward primer was used in each stage. The first-stage PCR added a ribosome binding site, a His_6-tag, and the ATG start codon in-frame with the N terminus of the gene 10 capsid protein. At the end of the first stage, 1 µL first-stage product was removed and used as the template for the second-stage PCR. The second stage of the nested PCR added the T7 promoter from the second forward primer. The second-stage forward primer overlapped the first forward primer and added 46 bases to the first-stage product. As expected, the product of the second PCR (data not shown) appeared approximately 0.05 kb larger than the product from the first-stage PCR product (data not shown). The DNA template generated by nested PCR was then ethanol-precipitated to remove salts that could inhibit RNA and protein synthesis. The PCR products were used as templates in the EcoPro coupled transcription/translation reactions.

Characterization of Recombinant Phage Fusion Proteins and In Vitro Transcribed/Translated T7 Gene 10 Recombinant Fusion Proteins on Polyacrylamide Gels

Western blot analysis was performed on the SIRT2_A phage and on the vector control phage, as well as the corresponding SIRT2 and control in vitro transcribed/translated recombinant proteins (Figure 2, A, the SIRT2 antibody and B, the T7 Tag antibody). As expected, the T7 antibody detected all the SIRT2 and control phage proteins (Figure 2B), while the antibody to SIRT2 detected only the SIRT2_A phage and SIRT2 in vitro transcribed/translated recombinant protein (Figure 2A, lanes 2, 4, and 6). The SIRT2 antibody exhibited some background bands that were not seen with the T7 antibody. These

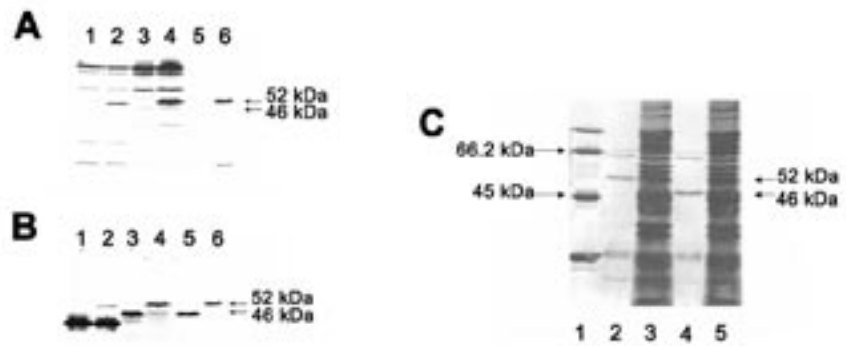

Figure 2. Analysis of in vitro transcription/translation products. (A and B) Western blot analyses of phage lysates and corresponding recombinant in vitro transcription/translation proteins. SIRT2 (A) and T7 (B) antibodies were used to probe replicate blots. Lane 1, control phage; lane 2, SIRT2_A phage; lane 3, crude control in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 4, crude SIRT2 in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 5, control nickel-purified in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 6, SIRT2 nickel-purified in vitro transcribed/translated T7 gene 10 recombinant fusion protein. (C) Silver stain of recombinant in vitro transcription/translation protein. Lane 1, silver stain marker ; lane 2, nickel-purified SIRT2 T7 gene 10 recombinant fusion protein; lane 3, crude SIRT2 T7 gene 10 recombinant fusion protein; lane 4, nickel-purified control T7 gene 10 recombinant fusion protein; lane 5, crude control T7 gene 10 recombinant fusion protein.

background bands could have been a consequence of the polyclonal nature of the SIRT2 antibody, whereas the T7 antibody is monoclonal. The recombinant fusion protein of the SIRT2_A phage appeared at 51 kDa (Figure 2, A and B, lane 2), and the control phage protein was present at 45 kDa (Figure 2B, lane 1). In addition to the unique band of interest, the T7 Western blot analysis also revealed a more intense band at 40 kDa for each phage sample, which corresponded to the wild-type gene 10 capsid protein present in all phage lysates because they were synthesized from a bacterial episome. The in vitro transcribed/translated SIRT2 recombinant protein was apparent at 52 kDa (Figure 2A and 2B, lane 4) and the control appeared at 46 kDa (Figure 2B, lane 3). The sizes of these proteins were slightly larger than the natural phage protein because of the additional amino acids that were added during the nested PCR.

The relative purity of in vitro transcribed/translated recombinant proteins was investigated through silver stain analysis. While the proteins were clearly visible by Western blot analysis (Figure 2, A and B, lanes 3 and 4), silver stain analysis (Figure 2C, lanes 3 and 5) did not allow the bands of interest to be discerned due to the high level of background *E. coli* proteins inherent in the EcoPro extracts. Phage protein samples were also not distinguishable by silver stain due to the high level of background protein (data not shown). Further purification of the in vitro transcribed/translated recombinant proteins was performed using nickel affinity chromatography exploiting the N-terminal His_6-tag. The nickel-purified protein was analyzed by Western blot analysis (Figure 2, A and B, lanes 5 and 6) and silver stain (Figure 2C, lanes 2 and 4). The nickel-purified protein had less background proteins with minimal loss of the proteins of interest. The difference in background between the purified and unpurified protein was clearly evident by silver stain analysis (Figure 2C, lanes 2 versus 3 and lanes 4 versus 5). Based on known amounts of BSA loaded adjacent to the samples (data not shown), we were able to estimate that, after nickel purification, one 50-µL EcoPro reaction provided 150 ng of the protein of interest.

Functional Analysis of Recombinant Phage Fusion Proteins and In Vitro Transcribed/Translated T7 Gene 10 Recombinant Fusion Proteins Using Polyacrylamide Gels

The fidelity of the binding to the rabbit antibody by the SIRT2_A phage and SIRT2 in vitro transcribed/translated recombinant protein was demonstrated by inhibiting the antibody binding of the protein by preincubation of the antibody with the C-terminal SIRT2 peptide antigen used to generate the SIRT2 antibody (Figure 3). Three replicate Western blot analyses were probed with the unblocked SIRT2 antibody (Figure 3A), SIRT2 antibody blocked with 0.01 mg/mL SIRT2 C-terminal peptide (Figure 3B), or SIRT2 antibody blocked with 0.001 mg/mL SIRT2 C-terminal peptide (Figure 3C). In a comparison of the phage samples, there was a SIRT2 band apparent on the blot probed with the unblocked SIRT2 antibody (Figure 3A, lane 5), but no band was detected when the SIRT2 antibody was blocked with 0.01 mg/mL peptide (Figure 3B, lane 5). The SIRT2 band was incompletely competed when the peptide concentration was reduced to 0.001 mg/mL (Figure 3C, lane 5). The fact that the SIRT2 peptide competed with the SIRT2 antibody for the binding site on the phage demonstrated the functionality of the cloned phage protein as isolated by our biopanning procedures. As with the phage protein, SIRT2 antibody binding to the unpurified and nickel-purified in vitro transcribed/translated SIRT2 recombinant proteins were similarly sensitive to competition by the SIRT2 peptide using Western blot analysis. We found that these recombinant proteins exhibited the expected bands using unblocked SIRT2 antibody (Figure 3A, lanes 1 and 3). Detection of both the purified and unpurified in vitro transcribed/translated recombinant SIRT2 protein was completely inhibited when the SIRT2 antibody had been previously blocked with 0.01 mg/mL SIRT2 peptide (Figure 3B, lanes 1 and 3) but not when the peptide concentration was lowered to 0.001 mg/mL (Figure 3C, lanes 1 and 3).

Functional Analysis of Recombinant Phage Fusion Proteins and In Vitro Transcribed/Translated T7 Gene 10 Recombinant Fusion Proteins Using Microarray Technology

The ability to compete for the binding epitope on recombinant phage proteins and on in vitro transcribed/translated recombinant proteins with

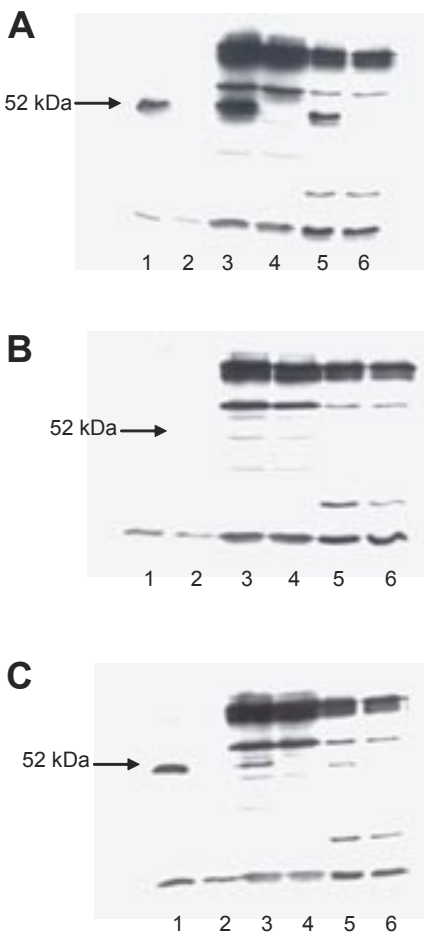

Figure 3. SIRT2 peptide competition as demonstrated on Western blot analyses. Identical blots were treated with (A) unblocked SIRT2 antibody, (B) SIRT2 antibody blocked with 0.01 mg/mL SIRT2 peptide or (C) SIRT2 antibody blocked with 0.001 mg/mL SIRT2 peptide. Lane 1, nickel-purified SIRT2 in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 2, nickel-purified control in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 3, crude SIRT2 in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 4, crude control in vitro transcribed/translated T7 gene 10 recombinant fusion protein; lane 5, SIRT2 phage lysate; lane 6, control phage lysate.

denaturing gels led us to analyze these proteins on nondenaturing microarrays. Two independent SIRT2 phage (SIRT2_A and SIRT2_B) and the control phage, as well as the nickel-purified in vitro transcribed/translated SIRT2 and control recombinant proteins, were robotically spotted onto nitrocellulose-coated slides. The microarrays were processed with the T7 antibody and either the SIRT2 antibody or the corresponding pre-immune serum with no peptide block, a SIRT2 peptide block, or blocking with a negative control peptide (Figure 4). The signal in the green (532 nm) channel resulted from the Alexa Fluor 532-labeled secondary IgG to the mouse monoclonal antibody bound to the T7 backbone gene 10 protein and serves to normalize the amount of protein in each spot on the microarray. The signal in the red (635 nm) channel resulted from the reactivity of the SIRT2 rabbit antibody with the spotted samples using an Alexa Fluor 647-labeled secondary antibody against rabbit IgG.

The microarray spots for the phage proteins can be seen in Figure 4A. The red channel signals for both SIRT2 phage were significantly more intense than that of the control phage. This was true when the microarrays were processed with the unblocked SIRT2 antibody or with the SIRT2 antibody preincubated with the negative control peptide. When the SIRT2 antibody was preincubated with the SIRT2 peptide, the red channel intensity for both SIRT2 phage dropped to a level comparable to the vector control. This indicated that the specific peptide had blocked the SIRT2 antibody such that the red channel signal had decreased to a level that could be considered background. These qualitative visual results were confirmed by quantitation of the spots on the microarray. The antibody binding, as indicated by the signal ratio of the red:green channels, was approximately 0.5 for the SIRT2 phage using unblocked SIRT2 antibody or SIRT2 antibody preincubated with the control peptide (Figure 4B). When the microarrays were processed with SIRT2 antibody preincubated with the SIRT2 peptide, the ratio approached zero. *t*-tests for both the SIRT2_A phage and the SIRT2_B phage showed no difference between the control peptide and no peptide (P value = 0.79 and 0.41, respectfully). When the control or no peptide treated samples were compared with the SIRT2 peptide treated samples, the difference was significant (both P values < 0.001).

When microarrays were processed with pre-immune serum blocked or not with the SIRT2 or the control peptide, the SIRT2 phage, and the control phage exhibited low red channel intensities consistent with the expected level of background (Figure 4C). The ratio was <0.05 for all phage samples, regardless of whether the serum was unblocked or blocked with the SIRT2 or control peptide (Figure 4D).

Similar results were seen with the purified in vitro transcribed/translated T7 gene10 recombinant fusion proteins. Microarray analysis confirmed the retention of the SIRT2 epitope on the nickel-purified recombinant SIRT2 protein (actual microarray spots shown in Figure 4E). The purified SIRT2 in vitro-synthesized recombinant protein provided a considerably more intense signal with the SIRT2 rabbit antibody in the red channel than the vector control protein as demonstrated by the microarray spot intensities. When the SIRT2 antibody was preincubated with the SIRT2 peptide before incubation with the microarray, the signal dropped to a level equivalent to the vector control protein. As anticipated, treatment of the SIRT2 antibody with the control peptide did not affect the signal intensity (Figure 4E). The quantitated ratio of red:green was between 1.5 and 2.0 for the SIRT2 nickel-purified recombinant protein that was processed with either the unblocked SIRT2 antibody or the SIRT2 antibody preincubated with the negative control peptide (Figure 4F). Blocking with the SIRT2 peptide decreased the ratio of the SIRT2 protein to 0.5. A *t*-test of the dye ratios for SIRT2 in vitro transcribed/translated protein blocked with the SIRT2 peptide compared with SIRT2 in vitro transcribed/translated protein blocked with either the control peptide or with the no peptide treatment revealed a significant difference ($P < 0.0001$). While this difference was highly significant, the red:green ratio does not drop to the level of the control protein (0.5 versus 0.1), thus suggesting that the peptide competition may have been incomplete. The experiment was also performed with the pre-immune serum, and the red channel for all samples exhibited only background levels of intensity (Figure 4G). Irrespective of the peptide competition, the red:green ratios for all samples processed with the pre-immune serum were <0.1 (Figure 4H).

It is of interest to note that the red:green ratio for the nickel-purified in vitro transcribed/translated recombinant protein processed with the unblocked SIRT2 antibody was approximately four times greater than that of the corresponding recombinant phage fusion proteins. Within the phage, there was a 40:1 ratio of gene 10 protein to recombinant fusion protein. This was based on Novagen's statement on page 3 of their TB178 T7Select System Manual (www.novagen.com) that there are 415 capsid wild-type proteins per phage in comparison to only 10 fusion proteins per phage head. The ratio of gene 10 protein to recombinant fusion protein changed to 1:1 in the vitro transcribed/translated proteins. Thus, the unblocked SIRT2 red:green ratio was expected to be higher. The purity of the samples can be assessed based on the ratio of the red-to-green signal. For both the phage and the nickel-purified recombinant fusion proteins, the green channel served to normalize the amount of protein spotted. The SIRT2 nickel-purified recombinant fusion proteins give a higher red channel signal relative to the green channel than the corresponding phage. Moreover, the increased purity of the in vitro transcribed/translated protein of interest improved the binding specificity to the antibodies and lowered the resulting background signal.

In summary, we have demonstrated that protein antigens isolated by phage display cloning using a polyclonal antibody as bait can be screened on protein microarrays. These antigens were purified in large quantities from in vitro transcription/translation reactions using PCR products as templates, by adding a strong promoter, and affinity tags. The resulting proteins were suitable for high-throughput screening on protein microarrays in which

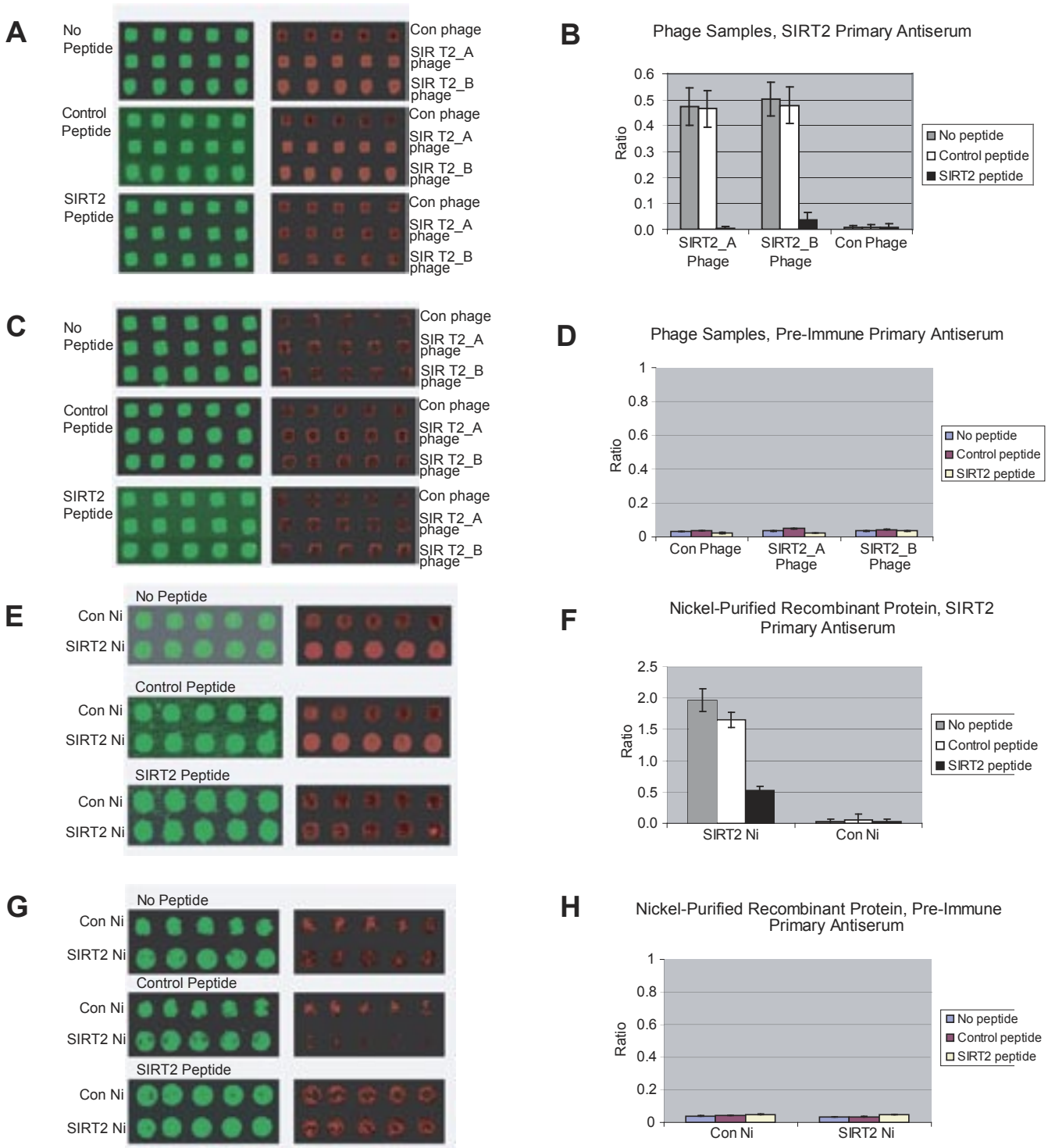

Figure 4. Analysis of protein microarrays. (A) Protein microarray of phage lysates detected with the SIRT2 antibody (red channel, right three panels) and the T7 antibody (green channel, left three panels). The green channel served to normalize the red channel for variations in robotic spotting. (B) Graphical analysis of phage lysates detected with the SIRT2 primary antibody. The mean of the control phage red channel was subtracted from both the SIRT2 and control samples prior to calculating the dye ratio. (C) Protein microarray of phage lysates detected with the pre-immune rabbit serum. The panel layout is the same as in panel (A). (D) Graphical analysis of phage lysates detected with the pre-immune serum. (E) Protein microarray of nickel-purified recombinant protein detected with the SIRT2 antibody (red channel, right three panels) and the T7 antibody (green channel, left three panels). The top panel was detected with unblocked SIRT2 antibody, the middle panel was detected with SIRT2 antibody blocked with the SIRT2 peptide, and the bottom panel was detected with SIRT2 antibody blocked with the control peptide. (F) Graphical analysis of nickel-purified recombinant protein detected with the SIRT2 antibody. The mean of the control phage red channel was subtracted from both the SIRT2 and control samples prior to calculating the dye ratio. (G) Protein microarray of nickel-purified recombinant protein detected with the pre-immune serum. The panel layout is the same as in panel (E). (H) Graphical analysis of nickel-purified recombinant protein detected with the pre-immune serum.

the epitopes retained full antigenic functionality. Microarray chips containing purified proteins will allow for the development of assays that are more sensitive than assays using crude phage lysates.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Tureci, O., U. Sahin, and M. Pfreundschuh.** 1997. Serological analysis of human tumor antigens: molecular definition and implications. Mol. Med. Today *3*:342-349.
2. **Eichmuller, S., D. Usener, R. Dummer, A. Stein, D. Thiel, and D. Schadendorf.** 2001. Serological detection of cutaneous T-cell lymphoma-associated antigens. Proc. Natl. Acad. Sci. USA *98*:629-634.
3. **Jager, D., C. Taverna, A. Zippelius, and A. Knuth.** 2004. Identification of tumor antigens as potential target antigens for immunotherapy by serological expression cloning. Cancer Immunol. Immunother. *53*:144-147.
4. **Jager, E., D. Jager, J. Karbach, Y.T. Chen, G. Ritter, Y. Nagata, S. Gnjatic, E. Stockert, et al.** 2000. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. J. Exp. Med. *191*:625-630.
5. **Ditzel, H.J., Y. Masaki, H. Nielsen, L. Farnaes, and D.R. Burton.** 2000. Cloning and expression of a novel human antibody-antigen pair associated with Felty's syndrome. Proc. Natl. Acad. Sci. USA *97*:9234-9239.
6. **Benhar, I.** 2001. Biotechnological applications of phage and cell display. Biotechnol. Adv. *19*:1-33.
7. **Griffiths, A.D. and A.R. Duncan.** 1998. Strategies for selection of antibodies by phage display. Curr. Opin. Biotechnol. *9*:102-108.
8. **Kay, B., J. Winter, and J. McCaferty.** 1996. Phage display of peptides and proteins. *In* A Laboratory Manual. Academic Press, San Diego.
9. **Winter, G., A.D. Griffiths, R.E. Hawkins, and H.R. Hoogenboom.** 1994. Making antibodies by phage display technology. Annu. Rev. Immunol. *12*:433-455.
10. **Smith, G.P.** 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science *228*:1315-1317.
11. **Dryden, S.C., F.A. Nahhas, J.E. Nowak, A.S. Goustin, and M.A. Tainsky.** 2003. Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol. Cell Biol. *23*:3173-3185.
12. **Martemyanov, K.A., A.S. Spirin, and A.T. Gudkov.** 1997. Direct expression of PCR products in a cell-free transcription/translation system: synthesis of antibacterial peptide cecropin. FEBS Lett. *414*:268-270.
13. **Spirin, A.S.** 1991. Frontiers in Bioprocessing II. American Chemical Society, Washington, DC.
14. **Rabilloud, T.** 1990. Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. Electrophoresis *11*:785-794.

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