ARTICLES

High-Throughput Method for Ranking the Affinity of Peptide Ligands Selected from Phage Display Libraries

A. González-Techera,[†] M. Umpiérrez-Failache,[†] S. Cardozo,[†] G. Obal,[‡] O. Pritsch,[‡,§] J. A. Last,[||] S. J. Gee,[⊥] B. D. Hammock,[⊥] and G. González-Sapienza*,[†]

Cátedra de Inmunología, Facultad de Química, Instituto de Higiene, and Departamento de Inmunobiología, Facultad de Medicina, UDELAR, Montevideo, Uruguay, Institut Pasteur de Montevideo, Uruguay, and Pulmonary/Critical Care Medicine, School of Medicine, and Department of Entomology and Cancer Research Center, University of California, Davis, California. Received July 23, 2007; Revised Manuscript Received February 18, 2008

The use of phage display peptide libraries allows rapid isolation of peptide ligands for any target selector molecule. However, due to differences in peptide expression and the heterogeneity of the phage preparations, there is no easy way to compare the binding properties of the selected clones, which operates as a major "bottleneck" of the technology. Here, we present the development of a new type of library that allows rapid comparison of the relative affinity of the selected peptides in a high-throughput screening format. As a model system, a phage display peptide library constructed on a phagemid vector that contains the bacterial alkaline phosphatase gene (BAP) was selected with an antiherbicide antibody. Due to the intrinsic switching capacity of the library, the selected peptides were transferred "en masse" from the phage coat protein to BAP. This was coupled to an optimized affinity ELISA where normalized amounts of the peptide−BAP fusion allow direct comparison of the binding properties of hundreds of peptide ligands. The system was validated by plasmon surface resonance experiments using synthetic peptides, showing that the method discriminates among the affinities of the peptides within 3 orders of magnitude. In addition, the peptide−BAP protein can find direct application as a tracer reagent.

INTRODUCTION

The development of phage-displayed peptide libraries (*1*) allows millions of peptides to be screened, simultaneously, for binding to a target of interest. In these libraries, any one bacteriophage displays a distinctive peptide sequence typically fused to either the pIII or pVIII viral coat protein. The peptide sequence can be encoded in the phage DNA, or most commonly in a phagemid system, consisting of a vector that codes for the peptide−viral coat protein fusion, and utilizes a "helper phage" to allow recombinant phage production. Since even a single peptide−target binding event can be detected and amplified, the technique has the potential to discover peptides that specifically bind to almost any target. For this reason, the technology has been applied in many fields (*2–5*).

Typically, the outcome of the selection procedure (panning) is analyzed by the so-called phage ELISA, but this test does not provide any information other than a negative/positive binding result. Further evaluation of positive clones requires DNA sequencing of the encoded peptide and the preparation of the encoded sequences as synthetic peptides, which must then be individually analyzed by competitive ELISA or surface

plasmon resonance experiments in order to identify the peptide sequences that bind with the highest affinities. For most purposes, not only the specificity, but very importantly, the affinity of the selected peptides is a major issue. This approach is a time-consuming and costly step that constitutes a major bottleneck for the technique, because it restricts the number of clones that can be assayed, and thereby contributes to the loss of low-abundancy but valuable clones.

In order to overcome these limitations, several works have been published in which model peptides selected from a phage library are transferred by different strategies to a partner molecule (i.e., maltose binding protein, bacterial alkaline phosphatase, etc.) to analyze the reactivity of the peptide out of the context of the phage particle (*6–9*). Due to its enzymatic activity and good expression in bacteria, BAP has been one of the most studied scaffolds. In this regard, Han et al. (*9*) devised a convenient method for the transference of the selected peptides to a highly active form of BAP using a ligation-independent cloning protocol, but the information provided by their procedure was only in terms of positive or negative binding. Other authors worked to obtain additional information on the peptide reactivity, particularly with regard to their relative affinity for the selector molecule (*6, 8*), but their approaches required one by one processing of the peptide−BAP candidates. Indeed, these methods require the purification of the peptide−BAP protein for each individual clone, followed by adjustment of the BAP activity of each clone in order to assay comparative amounts of individual peptides, which greatly restricts the number of clones that can be examined.

* Corresponding author. Av. A. Navarro 3051, piso 2, 11600 Montevideo, Uruguay; ggonzal@fq.edu.uy; tel (5982) 4874334.
† Facultad de Química, UDELAR.
‡ Institut Pasteur de Montevideo.
§ Facultad de Medicina, UDELAR.
|| School of Medicine, University of California, Davis.
⊥ Department of Entomology and Cancer Research Center, University of California, Davis.
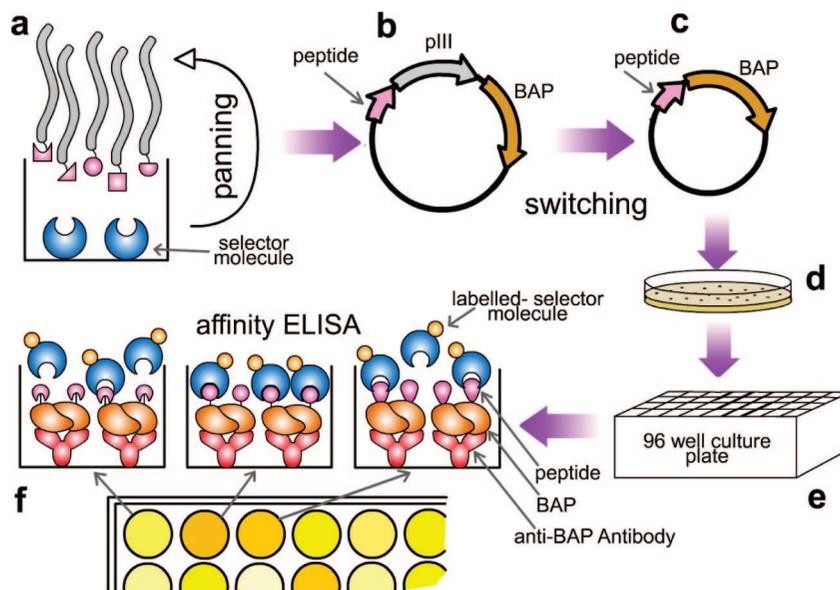
Figure 1. Schematic representation of the use of phage/mBAP switch libraries for the selection and high-throughput affinity evaluation of peptides. (a) The phage display paptide library containing the pAFF/mBAP vector is panned using standard protocols. (b) After 3–4 rounds of panning, the final phage output is amplified in *E. coli,* and the phagemid DNA is purified and digested with SpeI to delete pIII gene. (c) The digested pooled vectors are religated and used to transform *E. coli* to create a sublibrary of peptide−BAP fusion proteins. (d) The cells are plated and blue colonies (indicating BAP activity on the BCIP substrate) are individually transferred to 96-well block culture. (e) After overnight growth, the plates are centrifuged, the cell pellets are lysed with B-PER (Pierce), and the supernatants are analyzed by affinity ELISA. (f) Equal amounts of each peptide−mBAP protein are retained in the wells of a microtiter plate by capturing saturating concentrations of the cell lysates on wells coated with an anti-BAP antibody, and the fusion peptides are probed with the same concentration of the selector molecule (optionally labeled with biotin or a fluorescent tracer). Under these conditions, the readout of each well (amount of bound detector molecule) correlates with the affinity of the peptide under study.

To date, no practical procedure for high-throughput assessment of the relative affinities of the peptides selected from phage display libraries has been reported. Here, we present a novel method devised to solve the critical steps that limit this analysis. For this, we constructed a new type of phage display library with the intrinsic capacity of being easily "switched" into peptide−mBAP expression libraries. In this way, after initial selection of the phage library (usually three to four rounds of panning) the pool of selected phagemids is digested to remove the gene encoding the viral coat protein pIII, which allows fusion of the encoded peptides to the N-terminus of a highly active mutant bacterial alkaline phosphatase (mBAP). This switching process is coupled to a fast normalized selection procedure that does not require purification of the recombinant peptide−BAP fusions, and allows simultaneous direct investigation of hundreds of peptide candidates in terms of relative affinities and binding characteristics to the selector molecule. By this means, even if they are underrepresented, the most valuable peptide ligands can be rapidly identified. The method was developed and validated using a monoclonal antibody against the herbicide molinate as a model-selector molecule. In addition to providing information about the relative binding strengths of the peptides, the method yields ligand−enzyme recombinant proteins that can be used as tracer bioconjugates in different applications.

EXPERIMENTAL PROCEDURES

Cells and Plasmids. Vector pAFF2 (*10*) and *E. coli* cell strains ARI292 and ARI236 were kindly provided by Dr Peter Schatz; Affymax Research Institute, Palo Alto, CA. Vector pUY10 was a gift from Dr. Magela Laviña, Facultad de Ciencias, Montevideo, Uruguay. *E. coli* strain LMG194 (*11*), a strain derived from *E. coli* KS272 (*12*), a negative mutant for alkaline phosphatase, was a gift from Dr. Carina Gaggero, IIBCE, Montevideo, Uruguay.

Construction of pAFF/mBAP Phagemid Vector. Plasmid pUY10 encoding wild type BAP was used as template for PCR

amplification using primers (5′-TC*AGTCGAC*ACTAGTGCT-GCTCAGGGCGATAT TACT-3′) and (5′-CAG*AAGCTT*T-TATTAGTGATGGTGATGGTGATGTTTCAGCC CCAGA-GCGGCTTTCATGGTGT- 3′). *Sal*I and *Hin*dIII restriction sites (italics) were introduced for cloning, as well as a SpeI site (underlined) at the 5′ end of the BAP gene to allow later elimination of pIII phage coat protein gene. After PCR amplification using *pfu* DNA polymerase (Stratagene), the BAP product was digested with *Sal*I and *Hin*dIII (New England Biolabs), and cloned into the arabinose regulatable phagemid vector pAFF2. Quickchange Site-Directed Mutagenesis Kit (Stratagene) was used both for removing a BstXI restriction site within BAP, as well as for introduction of a Asp101→Ser mutation in the BAP gene cloned in pAFF2. This amino acid change has been reported to possess enhanced BAP catalytic activity (mBAP) (*13*). The obtained phagemid was named pAFF/mBAP (Figure 2).

Cloning of a Model Peptide on pAFF/mBAP and Transference of the Peptide from pIII to mBAP. pAFF/mBAP vector was digested with BstXI restriction enzyme (New England Biolabs). The digested vector was mixed with two "half sites" adaptor oligonucleotides, ON-28 and ON-29, and the long DNA oligo coding for the cyclic peptide CAREGYPARYC, which binds to EB7 mAb (*14*) followed by a SpeI site (Figure 2). The three oligonucleotides and the vector were annealed and later ligated with T4 DNA ligase (Invitrogen) using standard DNA procedures. ARI 236 competent cells were transformed with the ligation mix. Transformed cells were plated on LB agar ampicillin plates. DNA sequencing of clones confirmed the correct cloning of the model peptide fused to pIII phage coat protein. One of these clones was grown and a miniprep DNA extraction (Qiagen) was performed. Phagemid DNA was then digested with SpeI (New England Biolabs) restriction enzyme, removing pIII gene. Digestion was run on an agarose gel, and the band corresponding to the phagemid without the pIII gene was purified using QIAGEN Quiaquick gel extraction
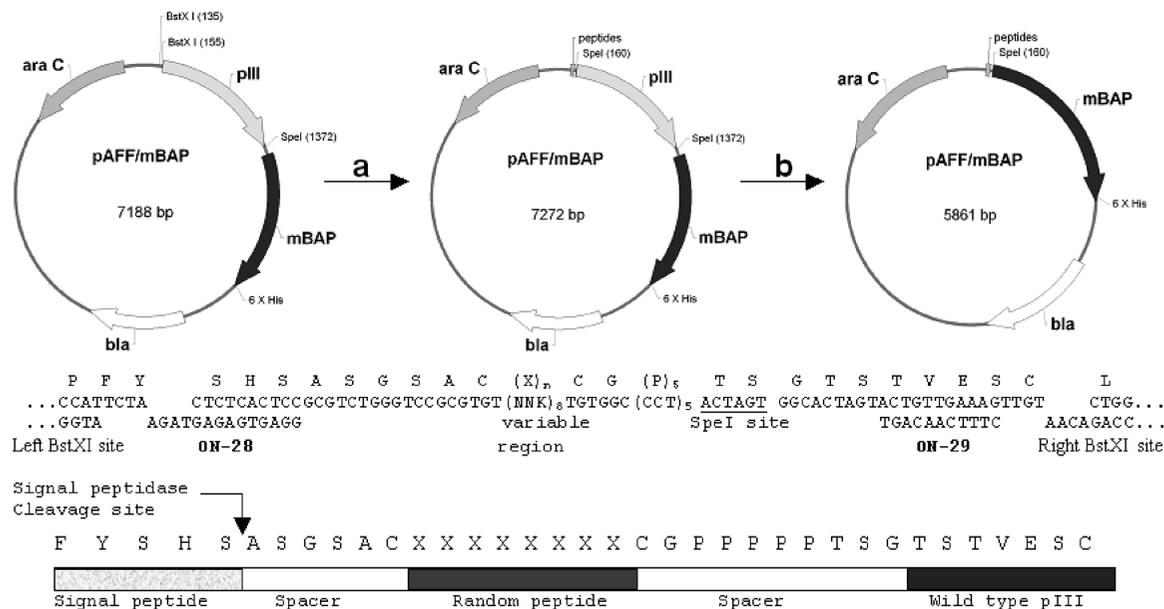
Figure 2. Representation of pAFF/mBAP vector, cloning of oligonucleotides in frame with pIII gene, and transfer of peptides from pIII to mBAP. pAFF/mBAP vector map: araC, arabinose represor gene; pIII, pIII phage coat protein gene; mBAP, mutated form of BAP gene with increased k_{cat}; bla, β-lactamase gene. (a) Library construction; the two BstXI restriction sites are used for cloning of the oligonucleotides encoding the peptides, as shown at the bottom. ON-28 and ON-29 adaptor oligonucleotides are used to form the cohesive termini required for ligation (bottom). The amino acid sequence of the assembled library is also shown at the bottom. (b) Library switching; SpeI digestion and ligation is used to switch from peptide−pIII to peptide−BAP expression. The SpeI restriction site required for this "switch" is shown underlined in the oligonucleotide sequence.

kit and ligated as described above. ARI 236 competent cells were transformed with the ligation mix. A direct screening for successful fusion of peptides−mBAP was performed by plating the transformed cells in LB plates with ampicillin (100 μg/mL), 0.2% arabinose, and BCIP (5-bromo, 4-chloro, 3-indolylphosphate (Pierce) at 40 μg/mL). After overnight incubation of the plates at 37 °C, bacterial colonies expressing peptide−BAP fusions could be clearly distinguished from colonies that were BAP by simply checking for blue colonies.

Capture of CAREGYPARYC-mBAP in ELISA Plate Wells. ARI 236 cells expressing the model fusion CAREGYPARYC-mBAP were cultured for 6 h at 37 °C after inducing with 0.2% arabinose. Cells were then pelleted by centrifugation (in aliquots of 1 mL) and the pellets were frozen at –80 °C. The cell pellet was lysed with 300 μL of B-PER reagent (Pierce) plus 1/25 volume of Complete EDTA-FREE Protease Inhibitor Cocktail (Roche Diagnostics, GmbH). Cell lysates were centrifuged and the supernatants were transferred to low-binding, nontreated, polystyrene plates (100 μL lysate + 100 uL AP wash buffer (50 mM Tris−Hcl pH = 8; 150 mM NaCl; 0.05% Tween-20)) and serial dilutions (twofold) were then performed in AP wash buffer. Dilutions were transferred to a 96-well microtiter plate (Nunc-Immuno Plate Maxi-Sorp) precoated with anti-BAP monoclonal antibody (mAb) (Sigma) at 1.25 μg/mL of IgG1 in 1× PBS (phosphate-buffered saline), polyclonal anti-BAP antibody (Polysciences, Inc.) at 2.5 μg/mL of total IgG in PBS 1×, both of them preblocked with BSA 1%. These serial dilutions were also transferred to a microtiter plate coated with Ni-NTA (Qiagen) and blocked with 1% bovine serum albumin (Sigma) in PBS buffer. Wells were washed 5 times with AP wash buffer and mBAP activity was detected by adding PNPP (*p*-nitrophenyl phosphate at 1 mg/mL) substrate (Pierce) in 50 mM Tris pH = 10, 200 mM NaCl, 1 mM MgCl₂. Plates were read at 405 nm in a microtiter plate reader (Multiskan MS, Labsystems).

Peptide Library Construction on pAFF-mBAP and Panning Procedure. A peptide phage library was constructed on pAFF/mBAP vector and harvested as described by Cwirla et

Table 1. Peptide Sequences of Clones Isolated in the Third Round of Panning with mAb 14D7[a]

clone	peptide sequence
clone 8	C K G L H M W F N C
clone 4	C A N P W L L K Y C
clones 1, 9, 10	C N N P W L A K Y C
clones 2, 5	C A A M G G W S K C
clones 6, 7	C A G F G G W A K C
clone 3	C H L L G G W A K C

[a] Peptides are grouped on the basis of their consensus sequence; conserved amino acids are shown in bold.

al. (*15*). Library diversity was estimated to be around 2×10^8 independent clones. This library was panned against an anti-molinate (herbicide) (*16*) mAb 14D7. Microtiter plates (Nunc-Immuno Plate Maxi-Sorp) were coated with mAb 14D7 at 5 μg/mL in PBS by incubating overnight at 4 °C (100 μL per well). Blocking was performed by completely filling the wells with 1% BSA in PBS and incubating at 37 °C for 1 h. After blocking, each well was washed 5 times with PBS−0.5% Tween-20 (PBST) and peptide library in PBS 1×−BSA1% was added to 6 precoated wells (approximately a total of 10^{11} phage particles). The plate was incubated for 2 h at 4 °C; wells were washed 10 times with cold PBST to remove unbound phages. Bound phages were eluted by adding 100 μL per well of elution buffer (0.1 N glycine, pH = 2.2 adjusted with HCl) and incubating at room temperature for 10 min. Neutralization of pH was done by adding 35 μL of 2 M Tris (pH unadjusted). The eluted phage (300 μL) were added to 10 mL of log-phase *E. coli* ARI 292 cells and amplified in LB media containing 0.25% K₂HPO₄, 0.1% MgSO₄, 0.1% glucose, and 100 μg/mL ampicillin to an $OD_{600} = 0.4$. Helper phage M13KO7 at a multiplicity of infection 10:1 was added. After a period of 30 min at 37 °C without shaking, arabinose and kanamycin were added at a final concentration of 0.02% and 40 μg/mL, respectively, and the cultures incubated overnight at 37 °C with vigorous shaking. This panning protocol was then repeated twice, and after three rounds of panning, individual amplified
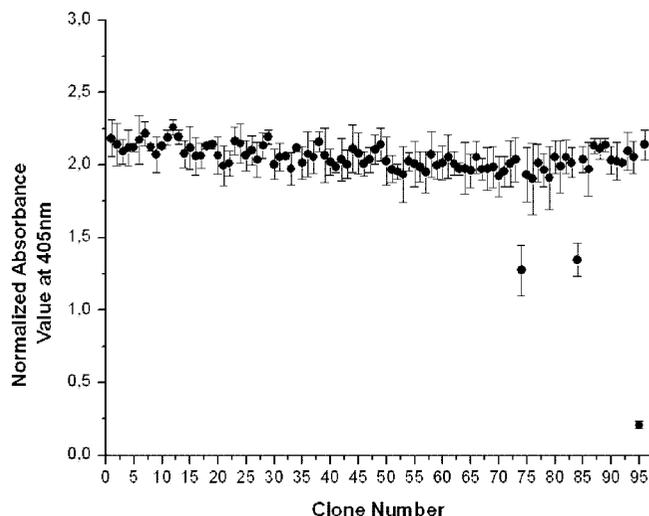
Figure 3. Comparative captured BAP activity of different peptide−mBAP fusion clones. Cell lysates from peptide−mBAP fusion expressing clones were used to saturate the binding capacity of microtiter plate wells coated with anti-BAP polyclonal antibodies. The retained BAP activity was detected with PNPP substrate, under conditions of initial velocity. Each value represents the average of three independent experiments (starting from the culture). The readout of replicates was normalized as described in the Methods section. Clone 95 corresponds to a negative control clone (LMG194 cells transformed with pAFF2 vector).

phage clones were tested for their ability to bind 14D7 coated wells. Phage ELISAs were performed as reported before (*5*).

High-Throughput Screening of the Peptide-mBAP Sublibrary Enriched in Clones Specific for mAb 14D7. ARI 292 cells were infected with approximately 5×10^7 phage particles from the third output of panning and grown in LB−ampicillin as described. Phagemid DNA was obtained using the Qiagen miniprep kit (Qiagen). The encoded peptides were fused to mBAP by switching with SpeI as described above. Competent LMG194 was transformed with the ligation mix and plated as described above. Blue colonies were individually inoculated in 0.5 mL of LB−ampicillin with 0.1% glucose in wells of a 2 mL 96-well block (Qiagen). The plate was sealed with airpore tapesheets (Qiagen) and grown with shaking at 37 °C. The next day, 80 μL of glycerol were added, and the block was frozen at −80 °C (master plate).

Replicates of this plate were established by touching the surface of the master plate frozen cultures with a multichannel micropipette using sterile tips. The tips were then used to inoculate 1 mL cultures of LB−ampicillin, 0.1% glucose, that were grown at 37 °C in a 96-well block. The next day, 5 μL was used to inoculate wells of a fresh block containing 1 mL of LB−ampicillin and 0.2% arabinose. The block plate was incubated for 6 h at 37 °C with vigorous shaking and was then centrifuged for 30 min at 1200 $\times$ *g* at 4 °C. The supernatant was discarded, and the pelleted cells were frozen at −80 °C. Each cell pellet was lysed with 300 μl of B-PER reagent (Pierce) plus 1/25 volume of Complete EDTA-FREE Protease Inhibitor Cocktail (Roche Diagnostics, GmbH) by gently pipetting up and down and using a multichannel micropipette. Cell lysates were centrifuged as previously, and 100 μL of each clone supernatant were transferred with a multichannel micropipette to each of two ELISA plates precoated with polyclonal anti-BAP antibody, and preblocked with 1% BSA. After 1 h incubation, one of the plates was washed 10 times with AP wash buffer, followed by addition of 100 μL of PNPP in substrate buffer (50 mM Tris, 200 mM NaCl, 1 mM MgCl$_2$, pH 10), and the reaction was incubated at 37 °C. The plate was read at 405 nm in a microtiter plate reader (Multiskan MS, Labsystems).

The second plate was washed with PBS, 0.05% Tween 20 (PBST), and probed with 100 μL of biotinylated mAb 14D7 (0.6 μg/mL) in PBST for 1 h at room temperature. After washing, streptavidin−peroxidase (Roche Diagnostics) conjugate was added (1:1000 dilution) and incubation was continued for 30 min at room temperature. The plate was washed with PBST, and 100 μL of the peroxidase substrate (0.4 mL of a 6 mg/mL DMSO solution of 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.1 mL of 1% H$_2$O$_2$ in water in a total of 25 mL of 0.1 M citrate acetate buffer, pH 5.5) was dispensed into each well. The enzymatic reaction was stopped by the addition of 50 μL of 2 M H$_2$SO$_4$, and the absorbance was read at 450 nm. Three replicates were prepared in this way to perform three independent experiments for plates A and B. Values obtained for clone number 1 were used as an internal control for normalization of the ELISA values.

Affinity Measurement by Surface Plasmon Resonance. Interaction analyses were performed using a Biacore 3000 optical biosensor equipped with research-grade CM5 sensor chips (Biacore AB, Uppsala, Sweden). Data were collected at the highest collection rate, with the biosensor instrument thermostatted to 25 °C. HBS-P (10 mM Hepes, 0.15 M NaCl, 0.005% P20 surfactant, pH 7.4) running buffer, amine coupling reagents (*N*-ethyl-(3- dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine HCl) were purchased from Biacore AB. mAb14D7 was immobilized on a CM5 sensor chip using standard amine-coupling chemistry. The carboxymethyl dextran surface was activated with a 7 min injection of a 1:1 ratio of 0.4 M EDC/0.1 M NHS. The antibody was coupled to the surface with a 7 min injection of 14D7 mAb diluted in 10 mM sodium acetate (pH = 5.0). Remaining activated groups were blocked with a 7 min injection of 1 M ethanolamine (pH = 8.5). The immobilization procedure was performed at 25 °C and a 14D7 surface density of approximately 12 000 RU was obtained. Peptides were assayed on the sensor chip in the 20 nM to 100 μM range.

Purification of Peptide−mBAP. Selected clones expressing different fusions were grown expressing the recombinant protein. Recombinant proteins were purified by standard Ni-NTA agarose (Qiagen) following manufacturers' instructions. An aliquot of the purified proteins was run on a 12% SDS-PAGE and stained with Coomasie blue.

Competitive Molinate Standard Curves Performed with Peptide−mBAP as Tracers. Nunc Maxisorp ELISA plates were coated with 0.6 ug/mL of 14D7 mAb and blocked as described above. After that, a fixed amount of peptide−mBAP previously assessed by checkerboard titration was mixed with different amounts of molinate and transferred to the 14D7 mAb coated wells, to allow competition for binding to the antibody. After 1 h incubation, plates were washed with AP Wash buffer and PNPP substrate was added. Plates were read as described above.

RESULTS

Overall View of the Concept of Phage/mBAP Switch Libraries and Vector Construction. The basic principles underlying the use of phage/mBAP switch libraries for the selection and evaluation of peptides are schematized in Figure 1. As shown in the figure, the system relies on two critical steps: first, it must allow massive transfer of the individual peptides selected on the phages to create a large peptide−mBAP library. Second, the peptide−mBAP expression library must be easily examined for the desired binding properties of the peptides. The developments required to accomplish these two aims are described in the following sections.

Vector Construction. Vector pAFF/mBAP (Figure 2), which contains a truncated version of the mutated *E. coli* BAP, was
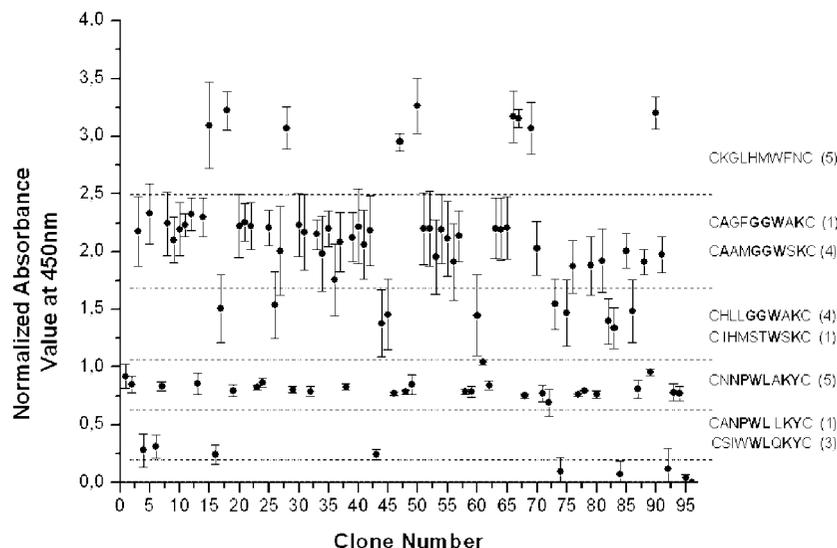
Figure 4. Affinity ELISA performed on the peptide−BAP fusion proteins probed with mAb 14D7. Saturating amounts of peptide−BAP fusion proteins captured on anti-BAP polyclonal antibody-coated wells were reacted with the same low amount of biotinylated mAb 14D7, and developed with streptavidin−horseradish peroxidase conjugate. According to the readouts, the peptides were divided into five groups (dotted lines). The sequences of five representative clones of each group are shown on the right side (consensus residues in bold), with their frequency indicated in parentheses. Each value represents the average of three independent experiments, each of them starting from a new culture from the "master plate". The readout of replicates was normalized as described in the Methods section. Clone 95 corresponds to a negative control clone (LMG194 cells transformed with pAFF2 vector).

Table 2. Kinetic Parameters of the Peptide−Antibody Interaction Measured by Surface Plasmon Resonance

peptide sequence	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (M)
CKGLHMWFNC	$3.1 \pm 0.1 \times 10^5$	$1.6 \pm 0.1 \times 10^{-2}$	$5.1 \pm 0.1 \times 10^{-8}$
CAAMGGWSKC	$8.3 \pm 0.1 \times 10^3$	$7.3 \pm 0.1 \times 10^{-3}$	$8.8 \pm 0.2 \times 10^{-7}$
CHLLGGWAKC	$2.8 \pm 0.1 \times 10^4$	$6.4 \pm 0.1 \times 10^{-2}$	$2.3 \pm 0.1 \times 10^{-6}$
CNNPWLAKYC	–	–	1×10^{-4} [a]

[a] No binding was observed for this peptide at the highest concentration (100 μM) assayed, indicating that its K_d is higher than 10^{-4} M, the detection limit of the Biacore 3000 instrument.

constructed as described in the Methods section. A shortened version of BAP (lacking residues 1–10) was used because it has been reported to be more resistant to proteolysis (*17*) with no loss of catalytic activity (*18*). A 6 × His tag was appended to its C-terminus to facilitate the purification of the recombinant protein. The vector contains two noncomplementary BstXI sites that are used to insert the random oligonucleotide sequences encoding the peptides. Oligonucleotide cloning introduces an additional SpeI site that allows later switching of peptide fusions from pIII to BAP.

Switch from pIII to m-BAP Using a Model Peptide. The system was initially explored using the model cyclic peptide CAREGYPARYC, which binds to mAb EB7 (*14*). The DNA sequence encoding for the peptide was cloned in frame with pIII in the pAFF-mBAP vector. Later, this construct was digested with SpeI (Figure 2), then ligated and used for transformation of *E. coli* ARI236. Peptides correctly fused to mBAP could be identified as blue colonies on LB ampicillin agar plates after addition of arabinose and the chromogenic substrate for alkaline phosphatase, BCIP (Figure 1d). Although the cells used for transformation (ARI236) have the wild-type alkaline phosphatase gene in their chromosome, streaking of this strain in control plates produced white colonies suggesting that endogenous expression of alkaline phosphatase is negligible. Fusion of peptides to mBAP through SpeI switching proved to be highly efficient, as nearly 99% of the colonies were positive by the BCIP assay.

Analysis of Peptide−mBAP Expression and Immobilization in ELISA Plate Wells. The expression of the peptide−mBAP fusion was studied using cell cultures of ARI 236 expressing

the CAREGYPARYC−mBAP protein. After 6 h induction with arabinose, the expression of the recombinant peptide−BAP protein was analyzed by Western blot. The immunoreactivity of the peptide was easily detectable with mAb EB7 (*14*) and comparable to that of BAP with the anti-BAP antibody (Supporting Information, Figure A). Since BAP spontaneously forms dimers by self-association, two copies of the peptide are expected to be displayed when the enzyme is used as scaffold. Further analysis of the CAREGYPARYC−mBAP expression was tested by growing the clone in 1 mL cultures in 96-well culture blocks (supplementary methods). The cell pellet was lysed with B-PER reagent, and the supernatant was incubated into microtiter wells precoated with different antibodies or Ni-NTA. Saturation was directly examined by measuring BAP activity (Supporting Information, Figure B). The best capture efficiency was obtained with polyclonal anti-BAP antibodies. The supernatant could be diluted over 100 times without significant change in signal, showing that the amount of recombinant CAREGYPARYC−mBAP obtained from 1 mL culture was in large excess of the amount required to saturate the wells. This result proved to be valid for other peptide sequences as shown in the following section. The endogenous alkaline phosphatase activity, although detectable, was negligible and about 500-fold less than that of the recombinant protein. Nevertheless, to rule out any possible interference of this activity, we adopted the strain LMG194 for all of the following expression experiments. This strain is mutant for alkaline phosphatase, and also for the catabolic arabinose genes, and hence induction of gene expression can be done without arabinose being consumed by *E. coli*.

Panning a Library Constructed in pAFF-mBAP, Output Diversity and Switch of Selected Peptides from pIII to mBAP. A library coding for 8-mer cyclic peptides with an estimated diversity of 2 × 10^8 independent clones was constructed on phagemid pAFF-mBAP. As model selector molecule we used mAb 14D7 (Figure 1a), which is specific for the herbicide molinate, and is the basis for a competitive assay for this herbicide (*16*). Since competitive elution with the analyte would not warrant elution of the highest-affinity clones, we used acid conditions in order to ensure that all low- and high-affinity
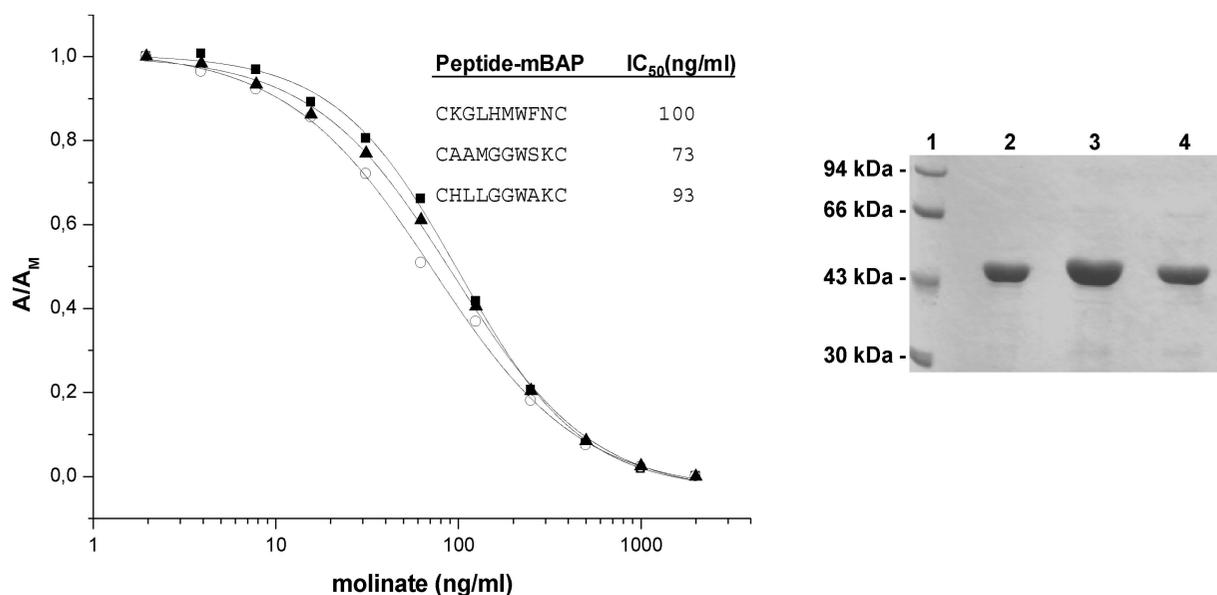
Figure 5. Purified peptide–mBAPs used as tracers in molinate competitive standard curves. Left panel: Peptides belonging to three groups with different affinities for mAb 14D7 (used for coating in the ELISA wells) were used as tracers to obtain competitive molinate standard curves. The peptide sequences and the corresponding amounts of molinate necessary to produce a 50% loss in signal (IC_{50}) are shown in the insert. Right panel: SDS-PAGE Coomassie blue stained: lane (1) molecular weight marker, (2) commercial affinity purified native BAP (Sigma), (3) Ni-NTA-agarose purified CAAMGGWSKC-mBAP, (4) Ni-NTA-agarose purified CHLLGGWAKC-mBAP.

clones were selected. After three rounds of panning, individual phage clones were tested for binding to the antibody by ELISA on plates coated with either mAb 14D7 or BSA (negative control). Ten out of ten clones examined were specific for the antibody, and six different sequences were identified (Table 1). Thus, at this point of the selection process most clones were specific and diverse, which was very convenient for the purpose of developing a method for ranking the affinities of a high number of clones. DNA from this pool of phage was "switched" with SpeI as described in the methods section (Figure 1b,c) and electroporated into LMG194. Blue colonies were picked from an arabinose-BCIP plate (Figure 1d) and were used to inoculate the "master plate" described in the Methods section.

High-Throughput Screening of the Relative Affinities of mAb 14D7 Specific Peptides. As mentioned before, the intrinsic affinity of the peptides for the selector molecule can not be estimated by phage ELISA (6). To overcome this limitation, we devised a method that rapidly normalizes the amount of peptide ligand to be tested by capturing saturating amounts of peptide–mBAP fusion on each of the microtiter wells to be analyzed. The method was applied to screen the third output of the panning experiment. Individual clones expressing peptide–mBAP fusions were picked from the "master plate", grown, and later lysed in 96-well square culture plates as described in the Methods section. Cell lysates were transferred to two identical plates coated with polyclonal anti-BAP antibody. One of the plates was used to verify that the same molar amount of each peptide–mBAP fusion (same BAP activity under conditions of initial velocity) was retained in each well, Figure 3. This was valid for all but two of the clones examined, clones 74 and 84. Once it was verified that the same amount of the peptides were retained in each well, mAb 14D7 (0.6 μg/mL) was used to examine the reactivity of the peptides. Under these conditions, the signal of each well is expected to be proportional to the affinity of the peptide for the antibody (Figure 1f). On the basis of the ELISA signal, the clones were grouped in five categories (Figure 4). Excellent reproducibility was attained for individual clones analyzed in three independent experiments. Sequence analysis of five clones from each category revealed peptide sequences characteristic of each group, supporting the assumption that the method sorts the peptides

according to their reactivity with the antibody. Notice that, in none of the cases, was a peptide sequence belonging to a particular affinity group found in another group. To confirm this, four representative peptides belonging to each of the different affinity groups were synthesized (including disulfide formation). Binding of the soluble peptides to mAb 14D7 was studied using surface plasmon resonance. The results confirmed that the method properly sorts the peptides in a range of up to 3 orders of magnitude as shown in Table 2. Peptide CNNPW-LAKYC, which was the most abundant after selection, exhibited immeasurable interaction on the Biacore chip, showing that the avidity effect due to the two peptide copies expressed in the peptide–mBAP protein may be the cause of the moderate binding observed in the affinity ELISA. In spite of its low affinity for the antibody, this peptide was most probably preferentially selected due to a favorable expression in *E. coli*, which resulted in an over-representation of this phage clone in the amplified phage stock. This may also promote a multivalent display of the peptide in the phage particle reinforcing the avidity of the interaction with the antibody. Supporting that, experimental evidence of the preferential expression of this peptide was obtained during the preparation of the recombinant peptide–mBAP fusion proteins (not shown).

Finally, notice that, in addition to favor the selection of the desired peptides, the method produces peptide–mBAP fusions that have the potential of being used directly as probe or tracer reagents in different applications. Supporting that, the peptide–mBAP fusions of three of the peptides selected here could be used to set up sensitive competitive assays for molinate as shown in Figure 5. Similar IC_{50} values were obtained with the three peptide–mBAP tracers, and these values were similar to that obtained for the competitive assay set up using the chemically synthesized hapten (16) (69 ng/mL).

DISCUSSION

In the past decades, phage display has been consolidated as a reliable and rapid technology for the identification of bioactive peptides and antibody fragments selected from high-diversity libraries. For most purposes, not only the specificity, but very importantly, the affinity of the selected peptides is a major issue.

This is particularly true for bioactive peptides that must show agonist or antagonist activity at the low concentration reached at the place of action. For this reason, the affinity of the peptides obtained after the initial selection from fully random peptide libraries is often improved through the construction of a secondary phage display library (mutagenesis library). In the mutagenesis libraries, the consensus residues of the initial phage pool are over-represented to favor the optimization of their flanking residues along the selection process (*10, 19*). In both cases (random and mutagenesis libraries), the outcome of the panning experiments should be easily examined to identify the higher-affinity clones. However, the identification of high-affinity binders among the pool of selected phage-borne peptides is not an easy task, and there is currently no systematic, yet practical, method to do this.

To deal with this problem, several alternative methods have been developed to facilitate the evaluation of the specificity and relative affinity of the isolated peptides; some of these methods use BAP as a partner fusion protein to study the peptide out of the context of the phage (*69*), but they are not compatible with high-throughput analysis. Here, we present a simpler method for the generation of large collections of clones expressing peptide−BAP fusions, coupled with a new method for rapid and simultaneous evaluation of the relative affinity of a large numbers of clones. In this method (affinity ELISA), the BAP activity works as an internal control to verify that equal amounts of peptides are assayed in each well of the affinity ELISA. Notice that the use of a much simpler affinity ELISA based on the capture of the phage particle bearing the peptide with an antiphage antibody would not produce reliable results due to the differential expression of individual peptides, which, as discussed above, would affect the average copy number of the displayed peptides.

The method showed excellent reproducibility and allowed us to sort the peptides within a wide range of affinities (up to 3 orders of magnitude) as shown by surface plasmon resonance analysis. Although we cannot rule out that, depending on their sequences, the peptides may show occasional interactions with the fusion partner that affect their binding to the target, in the four cases examined here there was a complete agreement between the affinity ELISA and the surface plasmon resonance analysis. By modification of the affinity ELISA conditions, it may be possible to shift this range to the target affinity (i.e., using stringent washing, lower amount of selector molecule, lowering the incubation time with the substrate, etc.). Interestingly, the highest-affinity clone (CKGLHMWFNC) which happened to be a low-abundance clone (about one in ten in the third output) could be easily spotted. This shows the potential of the method as a tool to identify valuable clones that are underrepresented in the selected phage pool. These "rare" nonconsensus fitting and low-frequency clones would be probably lost by the standard procedure of DNA sequencing and chemical peptide synthesis based on consensus or single sequences.

An additional benefit of the method is that the selected peptides can be directly produced as recombinant peptide−protein fusions. In our model, the recombinant peptide is linked to a high-turnover mutated form of BAP, in a fixed stoichiometric relationship, constituting a highly defined bioconjugate reagent that can be promptly used, for instance, as an immunoassay reagent. The applications of the concept of switch phage libraries can be extended to fusion partners others than BAP. In principle, any gene that is properly expressed in the periplasmic compartment of *E. coli* could be used to substitute the BAP gene in the pAFF/mBAP-PhoA vector, allowing the creation of sublibraries carrying the desired gene. This may include a different tracer activity such as firefly luciferase or green fluorescent protein to monitor intracellular binding of the peptides, or intein genes, that may help to produce the free recombinant peptide.

LITERATURE CITED

(1) Smith, G. P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science 228*, 1315–1317.

(2) Szardenings, M. (2003) Phage display of random peptide libraries: applications, limits, and potential. *J. Recept. Signal Transduction Res. 23*, 307–349.

(3) Weber, P. C., Pantoliano, M. W., and Thompson, L. D. (1992) Crystal structure and ligand-binding studies of a screened peptide complexed with streptavidin. *Biochemistry 31*, 9350–9354.

(4) Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G., and Gallop, M. A. (1992) Peptide ligands for a sugar-binding protein isolated from a random peptide library. *Proc. Natl. Acad. Sci. U.S.A. 89*, 5393–5397.

(5) Cardozo, S., Gonzalez-Techera, A., Last, J. A., Hammock, B. D., Kramer, K., and Gonzalez-Sapienza, G. G. (2005) Analyte peptidomimetics selected from phage display peptide libraries: a systematic strategy for the development of environmental immunoassays. *Environ. Sci. Technol. 39*, 4234–4241.

(6) Wright, R. M., Dudas, D., Gavin, B., Dottavio, D., Hexham, J. M., and Lake, P. (2001) A high-capacity alkaline phosphatase reporter system for the rapid analysis of specificity and relative affinity of peptides from phage-display libraries. *J. Immunol. Methods 253*, 223–232.

(7) Zwick, M. B., Bonnycastle, L. L., Noren, K. A., Venturini, S., Leong, E., Barbas, C. F., 3rd, Noren, C. J., and Scott, J. K. (1998) The maltose-binding protein as a scaffold for monovalent display of peptides derived from phage libraries. *Anal. Biochem. 264*, 87–97.

(8) Huang, X., Zhang, X. E., Zhou, Y. F., Zhang, Z. P., and Cass, A. E. (2007) Construction of a high sensitive Escherichia coli alkaline phosphatase reporter system for screening affinity peptides. *J. Biochem. Biophys. Methods 70*, 435–439.

(9) Han, Z., Karatan, E., Scholle, M. D., McCafferty, J., and Kay, B. K. (2004) Accelerated screening of phage-display output with alkaline phosphatase fusions. *Comb. Chem. High Throughput Screen. 7*, 55–62.

(10) Martens, C. L., Cwirla, S. E., Lee, R. Y., Whitehorn, E., Chen, E. Y., Bakker, A., Martin, E. L., Wagstrom, C., Gopalan, P., Smith, C. W., et al. (1995) Peptides which bind to E-selectin and block neutrophil adhesion. *J. Biol. Chem. 270*, 21129–21136.

(11) Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol. 177*, 4121–4130.

(12) Strauch, K. L., and Beckwith, J. (1988) An Escherichia coli mutation preventing degradation of abnormal periplasmic proteins. *Proc. Natl. Acad. Sci. U.S.A. 85*, 1576–1580.

(13) Mandecki, W., Shallcross, M. A., Sowadski, J., and Tomazic-Allen, S. (1991) Mutagenesis of conserved residues within the active site of *Escherichia coli* alkaline phosphatase yields enzymes with increased kcat. *Protein Eng. 4*, 801–804.

(14) Gonzalez-Sapienza, G., and Cachau, R. E. (2003) Identification of critical residues of an immunodominant region of Echinococcus granulosus antigen B. *J. Biol. Chem. 278*, 20179–20184.

(15) Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) Peptides on phage: a vast library of peptides for identifying ligands. *Proc. Natl. Acad. Sci. U.S.A. 87*, 6378–6382.

(16) Rufo, C., Hammock, B. D., Gee, S. J., Last, J. A., and Gonzalez-Sapienza, G. (2004) Robust and sensitive monoclonal enzyme-linked immunosorbent assay for the herbicide molinate. *J. Agric. Food. Chem. 52*, 182–187.

(17) Nakata, A., Shinagawa, H., Rothman, F. G. (1987) *Phosphate Methods*, Torriani, Ed., p 139, American Society, Washington, DC.

(18) Hoffman, C. S., and Wright, A. (1985) Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. *Proc. Natl. Acad. Sci. U.S.A. 82*, 5107–5111.

(19) Wrighton, N. C., Farrell, F. X., Chang, R., Kashyap, A. K., Barbone, F. P., Mulcahy, L. S., Johnson, D. L., Barrett, R. W., Jolliffe, L. K., and Dower, W. J. (1996) Small peptides as potent mimetics of the protein hormone erythropoietin. *Science 273*, 458–464.

González-Techera A., Cardozo S., Umpiérrez-Failache M., Obal G., Pritsch O., Last JA,. Gee SJ., Hammock BD and González-Sapienza G.

A high throughput method for ranking the affinity of peptide ligands selected from phage display libraries
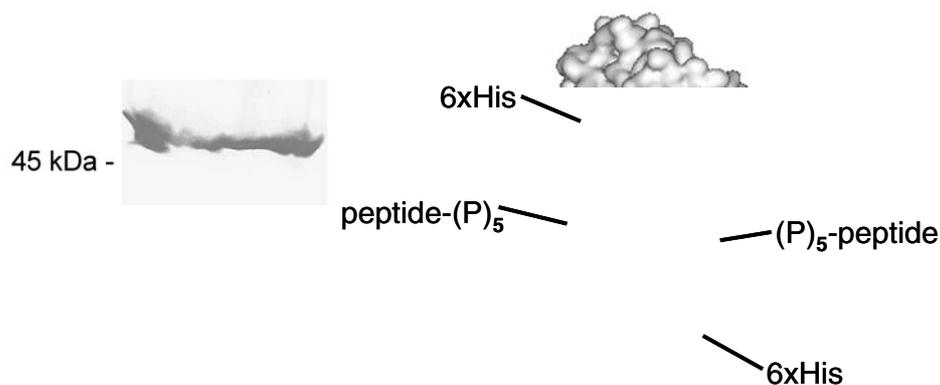
Supporting Figure A. Reactivity of the recombinant peptide-mBAP fusion protein evaluated by Western Blot. Left panel: Binding of MoAb EB7 to recombinant CAREGYPARYC-mBAP fusion assessed by western blot. A 5 µl pellet of ARI 236 cells expressing the peptide-BAP fusion was mixed with Laemli loading buffer (without reducing agent), heated at 100ºC for 5 min and run on a 10% SDS-PAGE. The blotted bands were probed with anti-BAP antibody or MoAb EB7, panels A and B respectively. Right panel: 3D structure of BAP. Two molecules of BAP (white and gray) self associate to form a dimmeric structure. Two black spheres are used to represent the N-terminal Ala residues that are bound to the penta Pro spacer that holds the peptide. The 6 x His tag is fused to the C-terminal Lys residue, represented as a light gray sphere. The active sites of both monomers are on the opposite site of the figure.
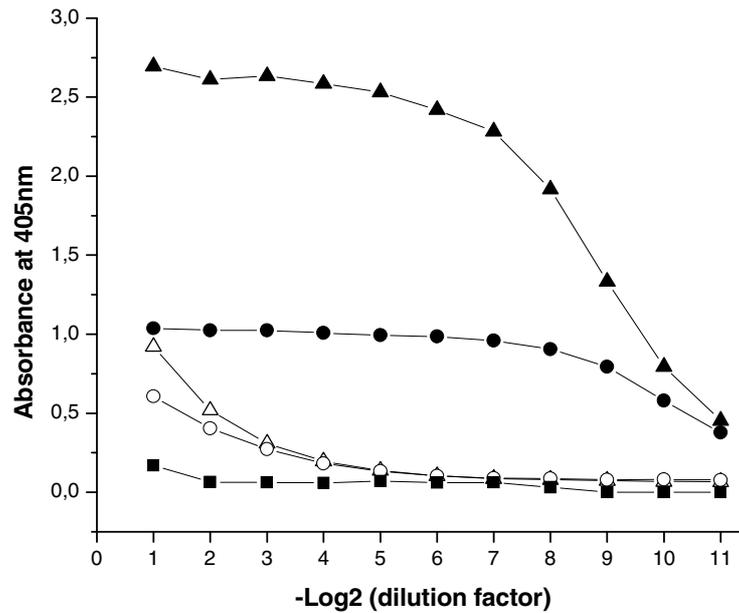
Supporting Figure B. **Capture of CAREGYPARYC-mBAP on ELISA plates using different coating reagents.**
Cell lysate dilutions of ARI 236 expressing CAREGYPARYC-mBAP (black) or a control (ARI 236 transformed with pAFF2 vector (same vector without BAP gene, white)) were incubated in ELISA wells coated with rabbit polyclonal anti-BAP antibodies (triangles), anti-BAP MoAb (circles), or Nickel-NTA (HisSorb, QIAGEN) (squares). BAP activity was developed with PNPP substrate. The first value of the plot on the X axis corresponds to 100 μl of B-PER (Pierce) cell lysate plus 100 μl of AP wash buffer. Signal was obtained, after washing, by adding PNPP substrate and read as described in the Methods section.