A New Peptidic Vector for Molecular Imaging of Apoptosis, Identified by Phage Display Technology

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Phosphatidylserine (PS) exposure on the cell surface is an early marker of apoptosis. To select PS binding peptides as vectors of contrast agents to image apoptosis, a phage library has been exposed to perfused mouse livers. Phages not retained on control livers during the first perfusions were used for selections on apoptotic livers in a second series of perfusions. Four selected phages were further evaluated for binding to PS-coated enzyme-linked immunosorbent assay (ELISA) plates. They presented an apparent affinity constant (Ka ^{app}) for PS ranging from 6.08×10^{10} M to 1.62×10^{11} M. These phages did not bind to phosphatidylcholine, and competition with annexin V confirmed their specific interaction with PS. The phage with the highest affinity-bound PS in ELISA with a Ka $^{app} = (1.6 \pm 0.2) \times 10^{11}$ M. It carried the TLVSSL peptide that was synthesized. Specific competition with annexin V and with the synthetic peptide was performed and confirms the specificity of the interaction.

Key words: phage display, apoptosis, phosphatidylserine, MRI, contrast agent

INTRODUCTION

PROGRAMMED CELL DEATH, OR APOPTOSIS, leads to a progressive cell shrinking, accompanied by chromatin and cytoplasm condensation, followed by a characteristic DNA fragmentation. Cellular fragments, known as apoptotic bodies, are eventually formed and phagocyted by neighboring cells without triggering inflammatory reactions.[1,2] This is in contrast to necrotic cell death, during which the cell membrane breaks and releases its cytoplasmic content in the extracellular medium. Apoptosis is a spontaneous process of cell elimination, which begins during embryogenesis,[3] allowing cellular turnover. It mediates cell and tissue homeostasis[4] by maintaining an equilibrium with mitosis and by allowing pathological cells or cells in excess to be removed. Apoptosis is triggered by either intracellular or external signals. It is well known that DNA alterations,[5] the accumulation of unfolded proteins in the endoplasmic reticulum (ER),[6,7] inhibition of the ER-Golgi transport, and various cytotoxic compounds[8] often used in chemotherapy can initiate apoptosis. It can also be induced by the activation of plasma membrane receptors by anti-Fas antibodies or tumor necrosis factor alpha (TNFα). In contrast, growth factor deprivation can also lead to cell death. Apoptosis involves an active participation of the cells to their own death through the control of intracellular events led by sets of antagonistic genes. Whatever the initial trigger, once apoptosis has been initiated, a common pathway is activated involving mitochondria, the Bcl-2 protein family,[9,10] and the effector caspases.[11]

The lack or overactivation of apoptosis is responsible for a broad range of pathologies. A genetic misregulation of apoptosis[12,13] can lead to uncontrolled cell proliferation and contribute to carcinogenesis. Excess apoptosis contributes to neurodegenerative diseases[14,15] such as Alzheimer disease, Parkinson disease,[16] and amyotrophic lateral sclerosis.[17] Other pathologies (i.e., autoimmune diseases[18,19]), graft rejection,[20] and consequences of ischemia/reperfusion[21,22] also have an apoptotic component.

Considering that apoptosis is part of such diverse pathologies, the detection of apoptotic cells by noninvasive imaging would help in their diagnosis as well as in the follow-up of a therapy. In this context, phosphatidylserine seems to be an interesting marker.[23-25] The very first event that can be experimentally

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Received Oct 5, 2005, and in revised form Jan 12, 2006. Accepted for publication Jan 13, 2006.

Journal of Biomolecular Screening 11(X); 2006
DOI: 10.1177/1087057106288220

detected in an apoptotic cell is a disturbance of the plasma membrane leading to flipping of phosphatidylserine from the cytoplasmic side of the lipid bilayer toward the outer side. Targeting with annexin V, its natural ligand, has been successfully achieved both in vitro[26] and in vivo.[27,28] In the present work, the phage display technique was used with the aim of isolating peptides with high affinity for phosphatidylserine. Coupled to a magnetic, radioactive, or optical reporter, such peptides could produce specific contrast agents for the detection of apoptotic cells in various molecular imaging modalities (magnetic resonance imaging [MRI], optical imaging, SPECT).

Phage display[29-31] is an efficient tool to isolate peptides exhibiting affinity for a given target. In a classical phage display experiment, the bioactive peptides are selected by "panning" against purified receptors,[32] intact cells,[33,34] or organs. In the latter case, in vivo[35,36] or ex vivo[37] biopanning is carried out. In the present work, a library of 6-mer random peptides fused to the pIII minor coat protein of M13 phages was used. The selection of peptides with affinity for apoptotic structures was performed ex vivo on perfused apoptotic mouse livers. Subsequent evaluation of the selected peptides was performed against phosphatidylserine in vitro.

MATERIALS AND METHODS

Phage peptide library

A library (fUSE5 vector[38]) of M13 phages exposing a linear hexapeptide on all 5 copies of the pIII minor coat protein was used. This library is a mixture of fusion phages theoretically displaying approximately 4×10^7 different hexapeptide epitopes. This phage library contained 2×10^8 virions/mL. It was amplified in the TG1 *Escherichia coli* host strain.

Liver perfusion

All procedures related to animals fulfill the requirements of the institutional review board of our institution. Livers were isolated from 5- to 6-week-old mice BALB/cByJIco mice (B&K Universal Limited, Hull, UK) anesthetized with an i.p. injection of 6 mg/kg Nembutal (Sanofi Santé Animale Benelux, Brussels, Belgium) and injected with 500 U.I. of heparin (Leo Pharmaceutical Products, Ballerup, Denmark). The anterograde perfusion[39] was performed by catheterization (Angiocath 0.7×19 mm, Becton-Dickinson, Erembodegem-Aalst, Belgium) of the portal vein with 200 mL of Krebs-Henseleit solution saturated with carbogen (95%O_2/5%CO_2). A recirculating flow of 1.5 mL min^{-1} per g of liver was ensured by a Masterflex peristaltic pump (Cole-Palmer, Chicago, IL).

Apoptosis induction

Liver apoptosis was induced in the mice by intravenous injection of 10 µg of purified hamster anti-FAS antibody (Jo2, Becton-Dickinson).[40] Two hours after injection, the apoptotic livers were isolated and perfused with Krebs-Henseleit solution for 5 min to wash out the remaining blood. Subsequently, they were perfused with the phage display library as described below.

Histology

Liver slices were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Then, 5-µm tissue sections were stained by a modified terminal deoxynucleotidyl-transferase-mediated UTP end labeling (TUNEL) method, as described.[41,42] Briefly, after inhibition of endogenous peroxidases, the deparaffinized sections were incubated for 15 min at 37 ºC in terminal deoxynucleotidyltransferase (TdT) buffer (150 mM sodium cacodylate, 25 mM Tris-HCl, 0.25 mg/mL bovine serum albumin [BSA], and 1.5 mM $CoCl_2$, pH 7.2). The end-labeling reaction was performed for 2 h at 37 ºC in the TdT buffer containing 0.37 U/µL TdT, 10 µM dATP, and 2.5 µM biotin-16-dUTP (Boehringer, Mannheim, Germany). The incorporated biotin-16-dUTP was then detected with biotinylated peroxidase-avidin (ABC) complexes (Dakopatts, Glostrup, Denmark). The staining reaction was performed with 0.01% H_2O_2 and 0.02% DAB (3,3´-diaminobenzidine) in phosphate-buffered saline (PBS) buffer (0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.12 M NaCl, pH 7.4). Finally, the sections were counterstained with hemalun, dehydrated in alcohol and toluene, and examined under a Leica DC 300 F microscope (Leica Microsystems Semiconductor GmbH, Wetzlar, Germany).

Ex vivo biopanning of phage display libraries in perfused mice livers

An overnight culture of TG1 *E. coli* in LB (Luria-Bertoni) culture medium was diluted 100 times and was grown until its absorbance at a wavelength of 600 nm (A_{600}) reached a value equal to 0.6. To eliminate the phages interacting with healthy hepatic structures, the phage display library was first perfused through a nonapoptotic liver. For the first round of biopanning, 10^{12} virions of the phage display library were added to the perfusion medium of the nonapoptotic liver. Thirty minutes after phage administration, slices of liver were isolated for histological analysis. At that time, the liver perfusate was added to an equal volume of *E. coli* culture, prepared as described at the beginning of this paragraph, and incubated for 30 min at 37 ºC to allow phage amplification. After centrifugation (10 min, 76*g*), the bacteria were concentrated in 1 mL of LB culture medium supplemented with 10 mg/mL tetracycline. This suspension was plated on LB agar plates and incubated overnight at 37 ºC. Bacteria and phages were suspended in sterile LB medium added to the plates. The medium was cleared from cells and debris by centrifugation (30 min, 2264*g*, 4 ºC), and phages were precipitated from the supernatant by addition of 1:5 volume PEG 20% NaCl 2.5 M for 2 h on ice. The preparation

was centrifuged (20 min, 1145*g*, 4 ºC) and the phage pellet suspended in 1 mL of sterile water. Phages were precipitated again by addition of 200 µL PEG/NaCl. After 30 min on ice, the preparation was centrifuged (5 min, 11,336*g*, 4 ºC), and the phage pellet was suspended in 500 µl of TBS (15.2 mM Tris-HCl, 150 mM NaCl, pH 7.5). Phage concentration was calculated with the following formula: concentration (phages/mL) = A_{260} × dilution factor × 2.214 × 10^{11} (number of phages contained in 1 mL of a suspension with A_{260} = 1). This counterselection on nonapoptotic liver was repeated 3 times with the phages isolated from previous rounds. Then, the same protocol was used for the selection on apoptotic liver, but in this case, phages of interest were retained in the liver and had to be isolated. After perfusion, the liver was homogenized with an Ultra-turrax T25 homogenizer (IKA-Labortechnick, Janke & Kunkel, Staufen, Germany) in a volume of 40 mL TBS. The phages were recovered from the tissue homogenate by incubation with an equal volume of 0.1 M glycine, pH 2, at room temperature for 30 min. Cell fragments were removed by centrifugation (390*g*, 30 min), whereas the supernatant containing the phages was neutralized with 1:8 volume of 1 M Tris-HCl, pH 8. Phages of interest were amplified and precipitated as described above. This selection on apoptotic liver was repeated 3 times with the phages isolated during the previous rounds.

Phage clonal amplification

Bacterial colonies were seeded with sterile toothpicks into individual wells of microculture plates containing 200 µL of 2× YT medium (Sigma-Aldrich, Bornem, Belgium) with 10 mg/L tetracycline (Sigma-Aldrich). After overnight incubation at 37 ºC, microculture plates were centrifuged (170*g*, 4 ºC, 15 min). The supernatant containing the phages was used for enzyme-linked immunosorbent assay (ELISA) tests as described below.

In vitro phage-binding assay

Phosphatidylserine (1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine [DPPS], Genzyme Pharmaceuticals—Sygena Facility, Liestal, Switzerland) was immobilized on microplates (Greiner GmbH, Frickenhausen, Germany) after solubilization in ethanol at a concentration of 0.8 g/L. Wells were filled with 200 µL of this solution, and a film was formed after overnight evaporation of the ethanol at room temperature. The same procedure was used with phosphatidylcholine (1,2-distearoyl-sn-glycero-3-phosphocholine [DPPC], Avanti®, Avanti Polar Lipids, Alabaster, AL) as control. Subsequently, the plates were blocked with 4% milk powder solution in PBS (137 mM NaCl, 3.2 mM KCl, 6.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2–7.5) for 2 h at 4 ºC. DPPS- or DPPC-coated plates were incubated with 50 µL of phage suspension for 2 h at 37 ºC. The plates were washed 6 times with PBS containing 0.1% Tween-20. Bound phages were detected by ELISA, using the horseradish peroxidase (HRP)–conjugated anti-M13 antibody (HRP anti-M13, Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The peroxidase staining reaction was performed in HRP substrate solution containing 100 mM citric acid (pH 2), 200 mM Na_2HPO_4, 30% H_2O_2, and (OPD) o-phenylenediamine dihydrochloride (ICN, Asse-Relegem, Belgium). After incubation with the substrate for 30 min, the reaction was stopped by addition of 50 µL/well of 4 M H_2SO_4, and A_{450} values were determined with a Stat Fax–2100 ELISA plate reader (Awareness Technology, Palm City, FL).

Determination of the apparent affinity constant

The apparent affinity constant (Ka^{app}) of each phage library was determined by saturation experiments performed by the ELISA method described above. Serial phage dilutions ranging from ~10^{-8} M to ~10^{-16} M in phages were prepared in calcium buffer (2 mM $CaCl_2$, 150 mM NaCl, 10 mM HEPES, 3 mM NaN_3, pH 7.4). The quantity of bound phages obtained from the A_{450} values was plotted against the logarithmic concentration of total phage input, and the curve was fitted according to a sigmoidal profile. The phage concentration at half-saturation corresponds to the $1/Ka^{app}$.

Competition experiments

Competition ELISA was carried out as described above using phage concentration yielding half-maximal saturation in the saturation curve. Serial dilutions from 6 10^{-7} M to 10^{-10} M of annexin V (33 kDa from human placenta, Sigma-Aldrich) were prepared in calcium buffer. DPPS-coated plates were incubated with annexin V during 30 min at 37 ºC. Phages were then added and incubated for 1 h, 30 min at 37 ºC. The same procedure was followed for the competition ELISA with serial dilutions of the selected peptide (concentrations ranging from 3×10^{-6} M to 10^{-9} M in calcium buffer).

DNA sequencing

The ssDNA of each selected phage colony was isolated[43] and sequenced according to Sanger using the following primer: 3´GGAGTATGTCTTTTAAGT5´ (N.V. Invitrogen S.A., Merelbeke, Belgium), which maps into the M13 pIII gene with the CEQ DTCS Quick Start Mix and a CEQ 2000 XL DNA Analysis System (Beckman Coulter™, Fullerton, CA).

Peptide synthesis

The peptide TLVSSL was obtained by solid-phase synthesis (Merrifield method) according to the FMOC/t-butyl chemistry with Wang resin. The FMOC group (fluorenylmethyloxycarbonyl) was used to protect the α-NH_2 function of amino acids,

and DCC (dicyclohexylcarbodiimide)/HOBT (n-hydroxybenzotriazole) was used as a coupling agent. The hydroxyl functions of Thr and Ser were protected by a t-butyl ether. The total deprotection and the cleavage from the resin were performed by trifluoroacetic acid (TFA). Finally, the peptide was purified by chromatography (high-performance liquid chromatography [HPLC], C18 column, Merck-Hitachi, Darmstadt, Germany). Electrospray mass spectrometry confirmed the structure of the peptide (Q-TOF-2, Micromass, Manchester, UK). ES-MS for E3: $[M+H]^+$: 619, $[M+Na]^+$: 641 and $[M+H]^+$ for GG-E3: 733, $[M+Na]^+$: 755.

Peptide biotinylation

Total amounts of 15 µmol of biotin-NHS and 15 µmol of the chosen hexapeptide were solubilized in 1 mL of DMF (dimethylformamide), and the mixture was stirred during 24 h at room temperature. Then, 5 mL of demineralized water was added, and the solution was filtered and dialyzed during 48 h (cutoff of the membrane = 500, VWR International bvba, Leuven, Belgium). The biotinylated peptide was isolated as a powder after lyophilization. ES-MS: $[M+H]^+$: 875, $[M+Na]^+$: 867.

Fixation curve of biotinylated peptide

Plates were coated with DPPS as described above. After 3 washings with calcium buffer (2 mM $CaCl_2$, 150 mM NaCl, 10 mM HEPES, 3 mM NaN_3, pH 7.4), 100 µL of serial biotinylated peptide prepared in calcium buffer and ranging from ~4×10^{-5} M to ~2×10^{-12} M was added in the wells. After 1 h, 30 min at 37 ºC, the plates were washed 3 times with calcium buffer. Biotinylated peptide was detected by using the streptavidin–peroxidase polymer conjugate (Sigma-Aldrich). The peroxidase staining reaction was performed in an HRP substrate solution containing ABTS (2,2´–azino bis (3-ethylbenzthiazoline-6-sulfonic acid), Sigma-Aldrich). After incubation with 200 µL of the substrate for 10 min, A_{405} values were determined with a Stat Fax–2100 ELISA plate reader.

RESULTS

A filamentous bacteriophage M13 library displaying random hexapeptides was used to identify high-affinity phosphatidylserine (PS) binding sequences. We adapted the original biopanning method, and phage selections were performed by perfusion of isolated mouse livers. Phages not retained by nonapoptotic livers were perfused to apoptotic livers in a second biopanning step in order to select the phages adsorbed onto apoptotic structures. Apoptosis was induced in mouse livers by intravenous (IV) injection of the anti-Fas antibody (see Materials and Methods). The characteristic DNA fragmentation was detected in a large number of cells by the TUNEL staining on histological liver sections (**Fig. 1**).

Ex vivo biopanning of phage display libraries in perfused mice livers

Four and 3 consecutive panning rounds of the phage library were performed, respectively, on control livers and on apoptotic livers. The phage specificity for phosphatidylserine was then evaluated by perfusing a control liver with the library of phages selected on apoptotic livers. Only 2% of the injected phages were retained, a result that confirmed the efficiency of the depletion step on nonapoptotic cells (**Fig. 2A**).

In vitro target-binding assay

Amplified phage libraries obtained after each round of biopanning and individual phage clones isolated at the end of the whole biopanning cycle were assessed by ELISA to evaluate their affinity to phosphatidylserine. The affinity for PS decreased during the phage selection carried out on control liver (**Fig. 2B**). If the selection eliminates the phages exhibiting a high affinity for control hepatocytes, it can unfortunately also eliminate some phages interacting with PS. In the second part of the procedure, phages with high affinity for apoptotic hepatocytes, considered as PS carriers, were selected (**Fig. 2B**).

Selection of phages specific of phosphatidylserine

The affinity of the phage clone was tested on PS and phosphatidylcholine (PC) by ELISA. The A_{450} in the ELISA tests for PS and PC were measured at a phage concentration of 2×10^{11} phages/mL. An "efficiency" coefficient was defined by the following formula:

$$\text{Efficiency coefficient} = (A_{PS} - A_{PC}) \times (A_{PS} - A_{uncoated}),$$

where A_{PS} = absorbance to plates coated with DPPS, A_{PC} = absorbance to plates coated with DPPC, and $A_{uncoated}$ = absorbance of uncoated plates.

This "efficiency coefficient" was created to differentiate 3 sets of data (A_{ps}, A_{pc}, and $A_{uncoated}$) equally important to be characterized and to choose the clones. The best phages should be those exhibiting the largest affinity for PS ($A_{ps} - A_{uncoated}$) and simultaneously the largest difference of PS and PC affinity ($A_{ps} - A_{pc}$). $A_{uncoated}$ was taken into account to check that the phages do not interact with the blocking solution. According to these criteria, 22 of the 100 selected phage clones have been chosen for the following experiment. Eleven of the 22 selected clones presented an efficiency coefficient higher than 0.15, and 12 of them had an efficiency coefficient smaller than 0.15. All were sequenced to compare the sequences of clones with the high- and low-efficiency coefficient.

The PS saturation curves were determined for clones E3, F3, G5, and G7 (**Fig. 3**), which presented the highest efficiency

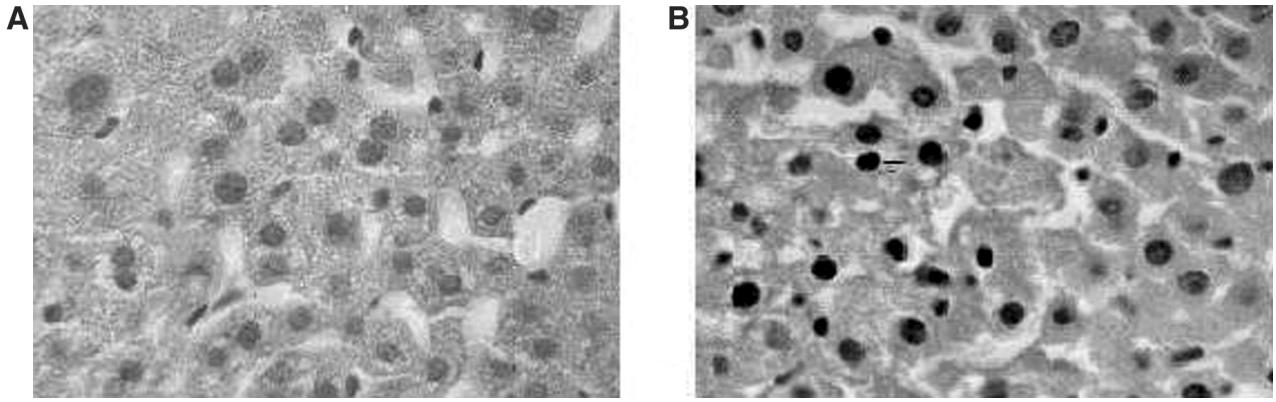

FIG. 1. Confirmation of apoptosis by the terminal deoxynucleotidyl-transferase-mediated UTP end labeling (TUNEL) assay. The livers of a control mouse (**A**) or a mouse injected intravenously (IV) with anti-Fas antibodies (see Materials and Methods) (**B**) were perfused with Krebs-Henseleit solution, fixed in 4% paraformaldehyde, and embedded in paraffin. Liver sections were stained with the TUNEL assay (see Materials and Methods) and counterstained with hemalun. Pictures were taken with a Leica DC 300 F microscope at a 540× magnification.

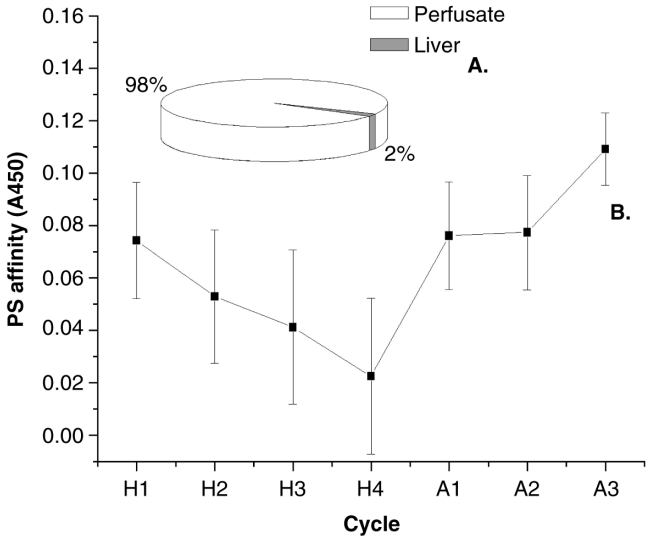

FIG. 2. (**A**) Amount of phages found in the perfusate and the healthy liver after the perfusion of the last library (A3). (**B**) Evolution of successive phage libraries affinity for phosphatidylserine (PS) during biopanning. Enzyme-linked immunosorbent assay (ELISA) plates were coated with PS and incubated with 10^{10} phages derived from libraries either not retained by successive perfusions through healthy liver (H1, H2, H3, and H4) or retained after successive perfusions through apoptotic liver (A1, A2, and A3). Phages bound to PS-coated plates were detected by incubation with horseradish peroxidase (HRP)–conjugated anti-M13 phage antibody followed by addition of the o-phenylenediamine dihydrochloride (OPD) substrate. Accumulation of the reaction product was detected by absorbance measured at 450 nm (A_{450} nm).

coefficient, to determine their apparent affinity constant (Ka^{app}) (**Fig. 4**). Clone E3 presented the highest apparent affinity constant ($(1.6 \pm 0.2) \times 10^{11}$ M).

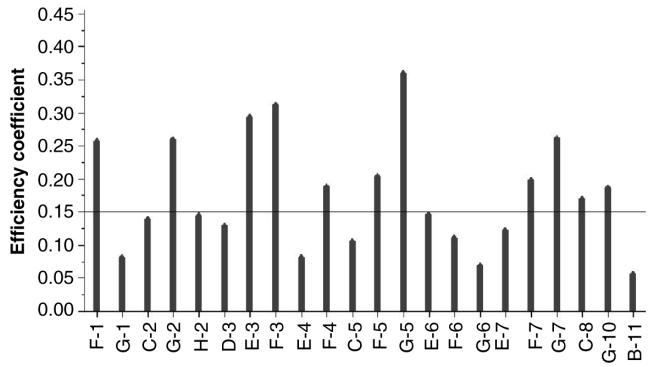

FIG. 3. Efficiency coefficient of individual selected phage clones. Enzyme-linked immunosorbent assay (ELISA) plates were coated with phosphatidylserine (PS) or phosphatidylcholine (PC), respectively, and incubated with the indicated phages (50 µL of 2×10^{11} phage particles/mL). Bound phages were detected by incubation with horseradish peroxidase (HRP)–conjugated anti-M13 phage antibody, as described in the legend to **Figure 2**.

Specificity of the interaction with phosphatidylserine

Annexin V, which is known to bind PS with a very high affinity ($Kd = 6\ 10^{-9}$ M),[44] was used to perform a competitive binding assay with phages E3, F3, G5, and G7. A progressive release of the phages was observed as the annexin V concentration increased (**Fig. 5**). These results confirmed that these clones selectively interacted with PS and not with the blocking buffer or with the polystyrene of ELISA plates. Another competitive binding assay was performed between phage E3 and its exposed peptide that was synthesized after identification of its sequence (see below). The half-maximal inhibition (IC_{50}) of phage E3 binding to PS by the E3 synthetic peptide (217.7 ± 92.3 nM) (**Fig. 6**) and the dissociation constant of biotinylated

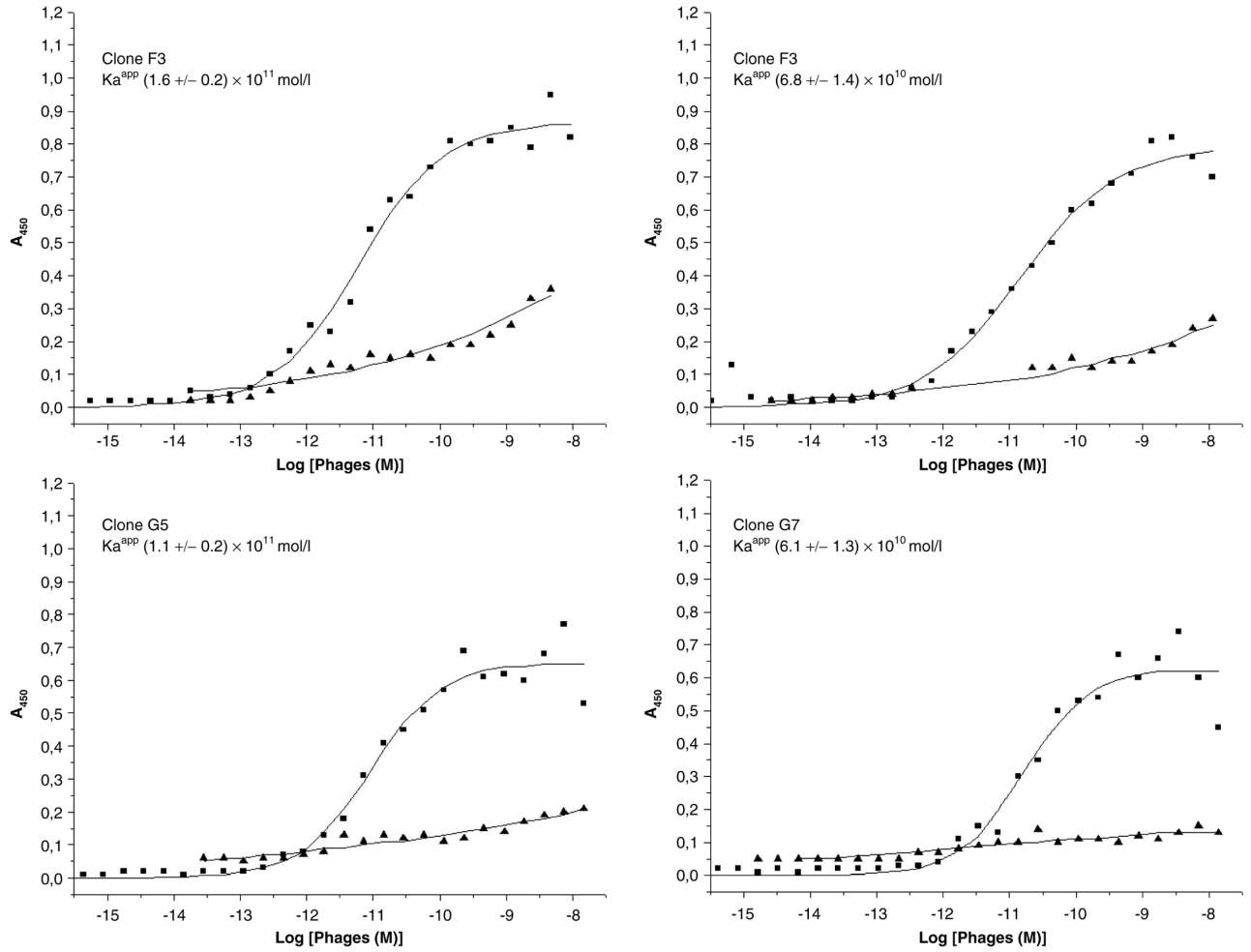

FIG. 4. Binding of the 4 selected phages (E3, F3, G5, and G7) to phosphatidylserine (PS; closed squares) and to phosphatidylcholine (PC; closed triangles). Enzyme-linked immunosorbent assay (ELISA) plates were coated with PS or PC and incubated with increasing amounts of each selected phage clone as indicated. To express the phage concentration, we have considered the whole phage as 1 molecule. Phages binding to the plates was detected by incubation with horseradish peroxidase (HRP)–conjugated anti-M13 phage antibody, as described in the legend of **Figure 2**.

E3 peptide obtained by the saturation curve (334 ± 84 nM) (**Fig. 7**) show that the peptide has less affinity than the whole-phage PS.

Peptidic sequences

The nucleotide sequence of the pIII minor protein gene was determined for the 23 phages selected after the last round of biopanning, and the encoded hexapeptide sequences were deduced (**Table 1**). Although no obvious consensus could be found, some sequences of 2 or 3 residues were conserved in several peptides. For example, low-efficiency coefficient clones F6 and B11 share the HPTxS motif. Most of the amino acid side chains are electrically neutral with polar and nonpolar residues present in similar proportions. However, the sequences from the phages whose efficiency coefficients are the highest show some similarities. These 4 sequences contain at least 1 leucine, isoleucine, or valine residue and 1 amino acid residue with a hydroxyl group in the lateral chain, such as threonine, serine, or tyrosine.

DISCUSSION

To identify phages displaying a hexapeptide with high affinity to PS, we have developed a biopanning strategy ex vivo on isolated and perfused mouse liver. The successive steps of selection carried out on control and apoptotic livers allow restricting the diversity of the initial phage library. Eventually, the library differed from its initial composition by an increased specificity toward PS. On the basis of their high and low affinities for PS and PC, respectively, some phages were selected for further study. Among them, the E3 phage presented an apparent

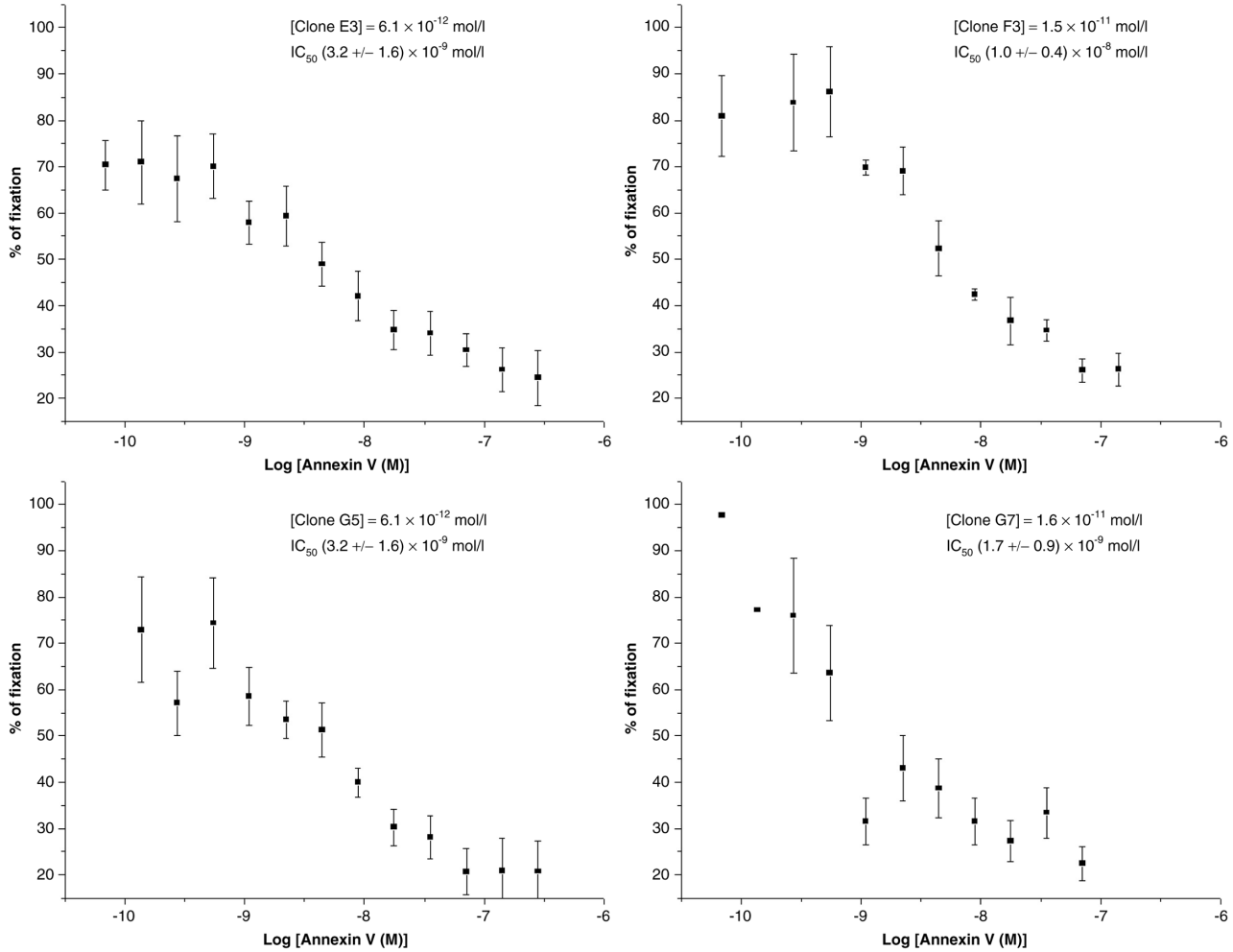

FIG. 5. Competitive binding between annexin V and the selected phages for phosphatidylserine (PS). Enzyme-linked immunosorbent assay (ELISA) plates coated with PS were incubated with the phage concentrations yielding half-saturation in the legend of **Figure 4** and increasing amounts of annexin V, as indicated (see Materials and Methods). Phages binding to the plates were quantified as described in the caption of **Figure 4**.

affinity constant of $(1.6 \pm 0.2) \times 10^{11}$ M. Its exposed peptide has been identified and synthesized. Competitive experiments confirmed that the specificity of the peptide toward PS was preserved. Nevertheless, the corresponding peptide has a lower affinity than E3 phage. With the IC_{50} obtained from the competition experiment between E3 phage and E3 peptide (IC_{50} = 217.7 ± 92.3 nM) and between E3 phage and annexin V (IC_{50} = 3.2 ± 1.6 nM), we can deduce that 69-fold higher concentration in the E3 peptide than annexin V was necessary to produce the same effect. Because the dissociation constant of annexin V is equal to 6×10^{-9} M, we can conclude that the dissociation constant of peptide is close to 4×10^{-7} M. Because the dissociation constant of the biotinylated peptide is equivalent to this value, we can conclude that the decrease of affinity cannot be attributed to the biotinylation. Two other explanations might be given to explain this lower apparent peptide affinity. First, a phage carries 5 copies of the hexapeptide, all being susceptible to interaction with PS on the plates. Second, the peptide presentation on the phage coat protein might bring a conformation with higher affinity for PS than the free peptide. In the future, the use of a library encoding peptides with 2 cysteines that block the conformation by disulfide bridges might circumvent this problem. In addition, the hexapeptide was much less potent than annexin V at competing with the corresponding phage from binding to PS-coated plates. The explanation might be that the overall dimensions of annexin V are considerably larger than those of the hexapeptide expressing phage and might sterically interfere with peptide binding.

In conclusion, for the first time, the ex vivo phage display technology has been used with the goal of selecting apoptospecific peptides. They will enrich the panel of contrast agent vectors useful in molecular imaging with the advantage of a better tissue

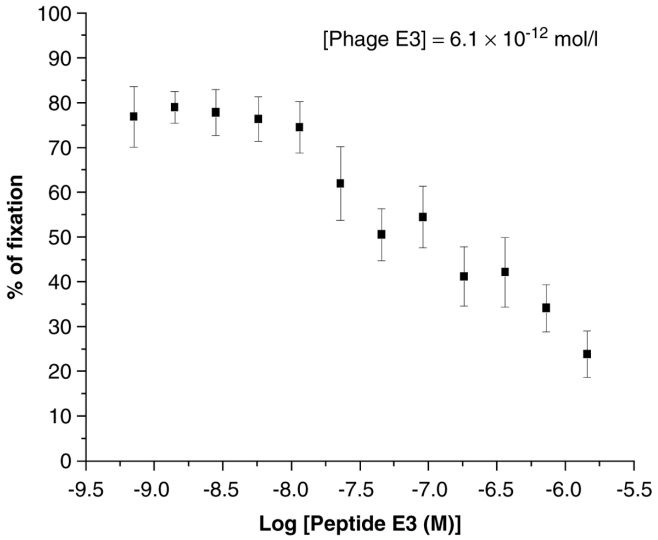

FIG. 6. Competitive binding for phosphatidylserine (PS) between the E3 synthetic peptide and the E3-displaying phage. Enzyme-linked immunosorbent assay (ELISA) plates coated with PS were incubated with phages and an increasing amount of the TLVSSL synthetic peptide as indicated. Phages binding to the plates were quantified, as described in the legend of **Figure 4**.

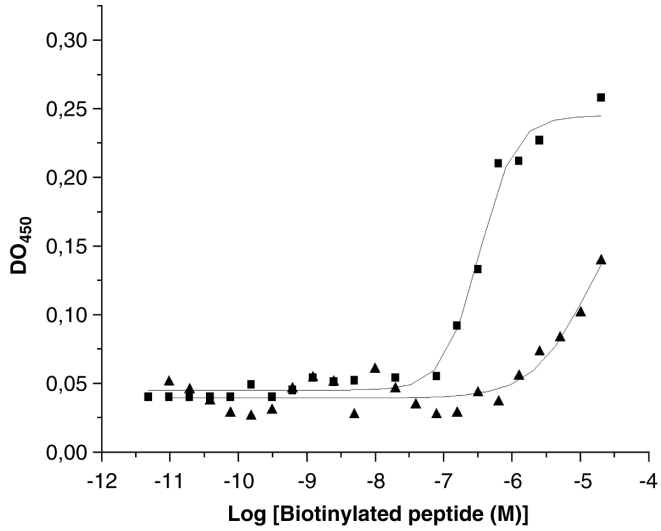

FIG. 7. Binding of the biotinylated-E3 peptide to phosphatidylserine (PS; closed squares) and to phosphatidylcholine (PC; closed triangles). Enzyme-linked immunosorbent assay (ELISA) plates were coated with PS or PC and incubated with increasing amounts of biotinylated peptide. Biotinylated peptide binding to the plates was detected by incubation with streptavidin–peroxidase polymer conjugate followed by addition of ABTS solution.

Table 1. Amino Acid Sequences of the Hexapeptides Displayed on the Phages Selected for Their High Affinity for PS versus PC and Classified by Decreasing Order of Efficiency Coefficient of the Corresponding Phages

Clones	*Sequences*
G5	**IGLTRY**
F3	**DGSSKL**
E3	**TLVSSL**
G7	**LPAKSP**
G2	ALGRFQ
F1	SIFPPK
F5	LASNRQ
F7	FRLIRS
F4	LDAMVS
G10	PWAWTS
C8	WYTAPT
E6	MVKWGT
H2	SLWRLS
C2	PLGVMR
D3	HGSTEV
E7	KFAGVN
F6	HPTQST
C5	AEKVLH
E4	NNGGLT
G1	SSRIGF
G6	AWALRF
B11	HPTGSM

The sequences beneath the black line correspond to phages with an efficiency coefficient smaller than 0.15, and the sequences in bold are carried by phages whose ka ^{app} for PS were measured by enzyme-linked immunosorbent assay (ELISA). PC, phosphatidylcholine; PS, phosphatidylserine.

penetrance, a nonimmunogenicity, and a lower cost than other vectorizing molecules of larger size (antibody, multi-subunit protein, etc.). Therefore, these peptides, coupled to suitable "reporters," should potentially yield contrast agents targeting apoptosis and allow for the visualization of concerned areas by various molecular imaging modalities (MRI, optical imaging, nuclear medicine, and ultrasound).

ACKNOWLEDGMENTS

The authors thank Professor D. Nonclercq (Histology, University of Mons-Hainaut) for his help with the TUNEL assay and Mrs. P. de Francisco for her help in preparing the manuscript. This work was supported by the ARC Program (contract number 95/00-194 and 00-05/258) of the French Community of Belgium. Professors Smith (University of Missouri, Columbia) and Ulrichts (Interdisciplinary Research Centre, University of Leuven, Campus Kortrijk, Kortrijk, Belgium) are thanked for their generous gifts of the phage library and the *E. coli* host strain.

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