Hunting Interactomes of a Membrane Protein

OBTAINING THE LARGEST SET OF VOLTAGE-DEPENDENT ANION CHANNEL-INTERACTING PROTEIN EPITOPES*†

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The identification of epitopes involved in protein-protein interactions is essential for understanding protein structure and function. Large scale efforts, although identifying the interactions, did not always yield these epitopes, could not confirm most of the known interactions, and seemed particularly unsuccessful for native intrinsic membrane proteins. We have developed a fluids-based approach (non-steady-state kinetics) to obtain the broadest set of the epitopes interacting with a given target and applied it to a phage display methodology optimized for membrane proteins. Phages expressing a liver cDNA library were screened against a membrane protein (voltage-dependent anion channel) reconstituted into liposomes and captured on a chip surface. The controlled fluidics was obtained by a surface plasmon resonance (SPR) device that combined the advantages of working with minute reaction volumes and non-equilibrium conditions. We demonstrated selective enrichment of binders and could even select for different binding affinities by fractionation of the selected outputs at various elution times. With voltage-dependent anion channel as bait (a mitochondrial channel critical for cellular metabolism and apoptosis) we found at least 40% of its already reported ligands and independently confirmed 55 novel functional interactions, some of which fully blocked the channel. This highly efficient approach is generally applicable for any protein and could be automated and scaled up even without the use of a SPR device. The epitopes directly identified by this method are useful not only for unraveling interactomes but also for drug design and therapeutics. 

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The sequencing of genomes led to the study of interactomes, i.e. the context-dependent sets of interacting proteins. Finding these interaction partners and mostly the interacting epitopes is crucial to the study of pathophysiological conditions and to the future of therapeutics. Because of the magnitude of such a task, several novel high throughput approaches have been developed including theoretical ab initio predictions, computer-aided in silico design, and large scale efforts based on various combinatorial chemistry strategies. Regardless of the approaches, recurrent problems in large scale methods often include their poor signal-to-noise ratio due to important numbers of false positive and negative hits, their lack of reproducibility, and the poor results obtained when searching for epitopes against membrane proteins (for a review, see Supplemental Discussion). More recently, efforts were centered on in vivo tagging systems coupled with mass spectroscopy and improved combinatorial display techniques (for reviews, see Refs. 1 and 2). The latter methodologies allow the selection of putatively relevant epitopes from a large molecular library by a screening process, and over the years, the yeast two-hybrid (3) and phage display (4) methods have been complemented by various types of cellular or ribosomal display with larger or more specific libraries.

In this context, because of its simplicity and highly reductionist in vitro approach, the phage display offers real advantages in dealing with pure membrane proteins if reconstituted in phospholipids. However identifying native interacting partners with the phage display still remained problematic. Over the years, the use of synthetic peptide libraries whether randomized (5), coupled to antibodies (6), coupled to portions of a receptor (7), or even affinity-selected for a known domain (8, 9) led in most cases to results that mimic but do not match the native peptide epitopes (5, 10, 11). Hence to identify such epitopes, the use of cDNA or genomic libraries would presumably be better. But it is fair to say that M13-derived phages never allowed a thorough screening of protein-protein interactions outside of the antigen-antibody realm (10–15). Here using the lytic T7 phage system, we report the successful application of a selection strategy tailored for membrane proteins.

Our strategy combined (i) exploring the full set of the protein epitopes expressed in a human liver cell line, (ii) using the native fold of the membrane protein achieved by its successful functional incorporation into liposomes, and (iii) the high stability, small surface, and (mostly) controlled fluidics obtained by using a surface plasmon resonance (SPR) detector device. This represents a serious shift in the phage display and any affinity selection paradigm. First, it enabled us to

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* The abbreviations used are: SPR, surface plasmon resonance; VDAC, voltage-dependent anion channel; LUV, large unilamellar vesicle; RU, response unit(s); IPTG, isopropyl-β-D-galactopyranoside; pfu, plaque-forming units; PEG, polyethylene glycol; COX, cytochrome c oxidase; Y2H, yeast two-hybrid.
work with very small reaction volumes (SPR measurement chamber). Having a small reacting surface (1 mm²) means fewer target sites, which is an advantage in terms of specificity. Usually the reacting surface is quite large as titer plates or target-coated beads are used. Second, the use of controlled fluidics enabled us to perform the selection in non-steady-state conditions. These are conditions where the mass transport, i.e. the availability of the potential ligands, is limited on the chip surface (16) thus presumably biasing the system toward high affinity multivalent ligands (17). In such a regime, where binding is influenced by transport, enhanced competition between ligand molecules is expected (18). Such conditions are generally avoided. The SPR device provided the added advantages of the stability and fractionation during the selection, both of which are not inherent to such a device. By fractionation under continuous flow of the output phages and iterations of the procedure, we demonstrated selection of phages and thus cellular epitopes with altered binding time constants.

With this method, we selected a set of 375 putative epitopes targeting the mitochondrial membrane protein voltage-dependent anion channel (VDAC). This protein is a key integration point in the control of metabolism and the apoptotic processes (19–21), and it is involved in numerous protein-protein interactions including mitochondrial contact sites between both membranes (reviewed in this work; see references cited in Table IV). Independent functional testing narrowed these epitopes to a subset of 75 functional sequences among which 55 concern novel proteins interacting with the target. The validity of such an approach is further evidenced by the fact that we were able to confirm at least 40% of the independently known interactions for our chosen target protein. This compares more than favorably with competing approaches with confirmation rates of already known interactions at or below 10%.

**EXPERIMENTAL PROCEDURES**

**Reagents—**All reagents were analytical grade. VDAC from yeast was purified to near homogeneity according to the method of De Pinto and co-workers (22, 23). Phospholipids were 1,2-di-lyso-phosphatidylcholine Type II-S from soybean (Sigma) supplemented with 20% pure cholesterol (Sigma). Running buffer HBS-N (0.15 M NaCl, 10 mM HEPES, pH 7.4) was filtered and degassed (Biacore®). Crystal grade cytochrome c oxidase was kindly provided by Dr. Verkhovsky, Helsinki, Finland.

**Liposome Preparation—**Phospholipids (1.52 mg) with 20% cholesterol (0.3 mg) were dissolved in 1 ml of hexane in a 10-ml round bottom flask. A thin lipid film was deposited by evaporation of hexane under a filtered N₂ stream (0.22-μm filter). One milliliter of experimental buffer (1 mM KCl, 5 mM CaCl₂, 10 mM HEPES, pH 7.2, filtered through 0.22-μm filters) was added, and multilamellar vesicles were formed by extensive vortexing. The lipid suspension was then submitted to four freeze-thaw cycles and to a 10-s sonication period to yield large unilamellar vesicles (LUVs). Finally the size of these vesicles was homogenized by pushing the suspension 25–30 times through a 100-μm polycarbonate filter in a mini extruder (Avanti® Polar Lipids, Inc.).

**VDAC Liposomes—**VDAC liposomes were obtained by diluting (1:3; v/v) the previous liposome (LUV) suspension with 50% experimental buffer (as above) and by adding twice separately 50 μl of a purified VDAC-containing solution (1 mg/ml VDAC in 5 mM Tris, pH 7.0, 0.5 mM EDTA, 2.5 mM KH₂PO₄, 25 mM KCl, 1% Triton) to 400 μl of liposomes, waiting each time 10 min for VDAC to insert. The VDAC proteoliposomes were then diluted (1:3; v/v) in an appropriate buffer (30 mM KCl, 10 mM HEPES, pH 7.4, filtered through 0.22-μm filters) to obtain a final VDAC LUV suspension with a very low final Triton concentration (0.05%), a physiological osmolarity (300 mosM), and a lipid concentration of 7.6 μg/ml. Blank liposomes (without any protein) were made and diluted in the same fashion.

**Liposome Immobilization on an L1 Sensor Chip—**Vesicles were captured on the L1 sensor chip as described previously (24). After a short time the surface of an L1 sensor chip was cleaned by a 2-min injection of 20 mM CHAPS at a flow rate of 20 μl/min followed by the “extra clean” rinsing routine. HBS-N was used as running buffer after filtration and degassing. Liposomes (80 μl, 1 mlx lipids) were then immediately injected at a flow rate of 2 μl/min and captured. This fixed lipid layer was then washed at a flow rate of 100 μl/min with sodium hydroxide (10 mM, 150 μl). In the case of VDAC liposomes, 120 μl of proteoliposome solution (1 mg phospholipids, flow rate identical) were used. A 12-h-long control period (running buffer at 5 μl/min) was needed to ascertain the stability of the chip surface. For the proteoliposomes, the SPR signal values showed a relatively important drift after 2 h of stabilization (around 3 resonance units (RU)/min for typical experiments); this drift reached its lowest possible values after waiting overnight and fell at the reported intrinsic drift range of the device (-0.3 RU/min) (technical handbook). For the blank liposomes, such low values were already reached after 2 h. The degree of coverage of the surface of the chip was determined by the ratios of the background reading signals (in relative RU) in the presence or absence of liposomes. The uncovered chip surface was then blocked with BSA (0.1 mg/ml, injection of 25 μl at 5 μl/min) (25).

**Biopanning of the Phage Library against VDAC Liposomes—**The T7-Select™ Liver library (Novagen, Madison, WI) was used. This cDNA library expresses its inserts fused to the C terminus of the T7 gene10B major capsid protein with an average of 10 copies displayed per virion, and inserts range from 300 to 3000 bp. The biopanning rounds were performed in a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) using an L1 sensor chip and HBS-N (above) as running buffer.

**Phage Selection—**To select for phages that bind to VDAC, the initial library was amplified and injected at very low flow rate (1 μl/min) over a chip covered with VDAC liposomes (~5000-RU level). Such a low flow rate allows better competition between the viral particles for the binding sites. To filter out the phages bearing plain lipophilic epitopes or epitopes targeted to BSA, i.e. to increase the specific signal to noise ratio, the phage solution is first flushed at the same flow rate over three flow cells covered with blank liposomes (covered at ~7000-RU level) and blocked with BSA before being allowed to reach the fourth flow cell with proteoliposomes. A titer of 10⁶ phages/100 μl was used to cover the whole range of the library sequence space (10⁵ variants/ml, manufacturer’s manual and our own control). Such panning rounds were repeated three times.

**Phage Collection—**Following the competition and binding phase, the reaction chamber was briefly washed with buffer (5 min at 1 μl/min) to remove any unbound or poorly bound material. To recover VDAC-binding phages based on different release rates, fraction collection was performed from flow cell 4. An amount of buffer was injected at 1 μl/min and recovered in full after 10 min for the first eluted fraction, after 30 min for the second eluted fraction, and after 40 min each for the third and fourth fractions. This was consecutively performed thus yielding four phage fractions. As a control for the
selective enrichment the same procedure was used over flow cell 3 (liposome only-binding phages). As prior to the next panning round the chip surface had to be regenerated, the phages with extremely low dissociation rate constants (near “irreversible” binders) were collected during a regeneration procedure. To that effect the sensor chip was first washed thoroughly by injection of buffer at high flow rate (90 μl at 30 μl/min) followed by an injection of 1% SDS (6 μl at 2 μl/min) to remove phages and liposomes from the sensor surface. T7 phages are resistant to a brief exposure (3–5 min) to 1% SDS and if directly diluted after recovery (SDS samples are brought to a volume of 30 μl with HBS-N) can reliably be titrated and propagated.

Phage Amplification and Titration—Phage titers were determined by infection of 250 μl of BLT5615 cells, harvested in the presence of carbenicillin (50 μg/ml), with 100 μl of a 10-fold dilution of the eluted phages. The cell-phage samples were added to 3 ml of H-top agar containing 4 mM IPTG and plated on LB agar supplemented with carbenicillin. IPTG is required to induce the production of the 10A capsid protein of the T7 phages. The plates were left overnight at room temperature; the number of plaque-forming units (pfu) was counted for all samples.

To amplify the phages in between each selection round, 10 ml of BLT5615 cells (harvested at log phase in the presence of carbenicillin and 1 mM IPTG) were infected with 20 μl of phage eluate. Incubation for a maximum of 1.5 h at 37 °C in a sterile chamber caused complete lyses of the cells. Cell debris was removed by centrifugation for 10 min at 8000 × g, and 15% chloroform was added to the phage-containing supernatant to clear the sample from unprecipitated debris. This step was found to be essential to decrease microfluidics (chip) and macrofluidics (catheters) problems within the Biacore machine. The catheters of the machine were changed in between rounds of testing. The phage samples were further purified by PEG precipitation (phages: PEG 6000, 50% solution, 1:6, v/v) and finally dissolved in HBS-N. Phage titers following amplification were determined as described above.

Affinity Tests of the Selected Phage Populations—To evaluate the binding of phage populations selected from a surface-displayed library, affinity tests were performed within the SPR device (26). The L1 sensor chip was covered either with VDAC liposomes or liposomes and blocked (see above). Coverage of the chip surface was kept high, typically around 7000 RU for VDAC liposomes and 8000 RU for liposomes. Amplified phage populations at a concentration of 3.3 × 10^{10} pfu/ml were injected separately over the two surfaces at a high flow rate (30 μl/min) for 2 min after which dissociation curves were followed for 10 min. Prior to each injection of phages, a buffer-only control run was performed.

Single Clone Amplification—A portion of the top agarose of individual plaques was scraped up and used to infect 10 ml of BLT5615 cells as described above.

PCR Amplification of cDNA Inserts and Sequencing—The T7 library protocol (Novagen) was strictly followed. Finally the samples were purified (Qiaquick purification kit), and purity was controlled by electrophoresis on a 1% agarose gel. The PCR amplified DNA was submitted for DNA sequencing with the T7Forward primer.

Epitope Identification—Using the BLAST Network Client “blastcl3” program, all DNA inserts of the 375 clones were compared with the non-redundant (nr) nucleotide sequence databases, i.e. all of GenBank™ + EMBL + DDBJ sequences. For the 547 non-redundant identifiers in the database (894 with redundancy), the homologies were mostly greater than 90% but at least 75% and a minimum length of 30 bp (with the exception of 16 hits around 20 bp). The difference between the numbers of identifiers and sequences is presumably due to similar DNA sequences present in different genes, i.e. encoding similar domains in markedly different proteins. This was specifically controlled for four randomly chosen clones (data not shown). 481 proteins were obtained by subtracting irrelevant data (pseudogenes, non-protein DNA, RNA, and clusters) from the 547 identifiers.

Cytchrome c Oxidase Assay—Functional effects of VDAC on cytochrome c oxidase (COX) were assayed by following the oxidation of reduced cytochrome c at 550 nm by COX according to Roman et al. (27). Spontaneous oxidation of cytochrome c was controlled and found to be less than 1% in the absence of the enzyme.

VDAC Permeability Assay—VDAC liposomes (LUVs) were prepared as described above except for an additional dilution step (1:2, v/v) in 0.75 M KCl and stabilized on ice for 30 min. Fixed volumes of phages (2 × 10^{10}–2 × 10^{11} phage particles, controlled by titration) were added to 200 μl of proteoliposomes solution, the mixture was stabilized for 10 min, and 10 μl of a 200 mM stock of PEG 800 (final concentration, 10 mM) was added right before reading. Volume changes were recorded as variations in light scattering with a Bio-Rad microplate reader at 400 nm. Slopes during the reswelling phase of the experiments were computed by linear fits.

RESULTS

Protein Reconstitution into Proteoliposomes and Immobilization—To select for binders, pure, correctly folded, and stable bait is needed. The purified target protein was embedded into LUVs, and to obtain the “native” conformation of the protein, we used conditions known for many years to allow the functional reconstitution of the VDAC protein (28–30). In such conditions, there are essentially no differences between patch clamp experiments made with mitochondrial phospholipids (31) and measurements made in planar phospholipid bilayers (29). Although not strictly binding the protein into its native state, this is as close as possible with the pure protein used in a cell-free system. Using an SPR device, three flow cells of the chip were covered with control liposomes, i.e. devoid of target protein; only the fourth cell was covered with VDAC proteoliposomes. Prior to each experiment, the stability of the surface of the chip was ensured by letting it settle under running buffer conditions until the drift in the SPR signal fell within the range of the electronic drift of the device (usually after 12–14 h).

Screening cDNA Libraries—The biopanning selection was performed within the biosensor device (Fig. 1a, black arrows). This selection phase comprised three steps. In the first step, the naïve phage library is first amplified and injected at the lowest possible flow rate into the machine, and the phages are forced to pass sequentially over the three blank flow cells before competing for target binding. Such a low flow rate would theoretically allow a better competition between the viral particles for the target binding sites. Forcing the phages on the first three flow cells allowed us to filter out the phages bearing plain lipophilic epitopes or epitopes targeted to the blocking agent (BSA). In an ensuing step (Fig. 1a, red arrows), the phages that bound to both proteoliposomes or blank liposomes would be eluted and fractionated by perfusing a mild running buffer into the respective chambers. This would collect the positive binders based on their release rates from the bait protein. At this stage, a third step (not shown on the figure) was to disrupt the chip surface with detergent. This enabled us to collect undissociated phage particles in a last
Fraction termed the regenerate. All these fractions were then analyzed for bulk affinity in separate sets of experiments (Fig. 1b). An example of such affinity measurement is given in Fig. 1c. For each fraction, specific versus aspecific bulk binding varies. In our example, fraction 2 yielded the best signal. This does not imply that this fraction contains the best ligands but that this fraction contains more phages with fairly good binding epitopes. Based on the need (high or low affinity), each of

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**Fig. 1. Experimental flow chart, fractionation, and biopanning strategies.**

a, SPR biopanning setup. After the immobilization and the stabilization of either plain liposomes (on the first three flow cells of an L1 sensor chip) or proteoliposomes (on the fourth flow cell), $1 \times 10^9$ phages particles ( naïve for first round or postselection amplification) were injected into the SPR device and forced onto the flow cells sequentially at the slowest possible rate (1 μl/min) (black arrows, ➀). After 100 min of perfusion, the elution buffer was used to collect independently bound phages from the third and fourth flow cells (red arrows, ➁). The phages from the third flow cell were used as control; phages from the fourth flow cell were eluted and collected in four fractions corresponding to four periods of 40 min. b, setup for the quality controls of either the various eluted phage populations or single clones. Flow cells 1 and 3 were covered with plain liposomes; flow cells 2 and 4 were covered with proteoliposomes. The binding of the phages was measured in the biosensor as relative RU. c, affinities measured by SPR of the four eluted fractions from the second round of a panning aimed at a late strategy (see d). The bulk affinities of VDAC-selected phages for VDAC liposomes (gray bars) are compared with those for plain liposomes (white bars). d, flow chart outlining the three selection strategies performed during this work (early, late, and regenerate); other paths are possible. For each round, we used either the early elutable fraction (more reversible binders, white path), the last fraction (less reversible or higher affinity binders, light gray path), or the regenerate fraction (irreversible binders, dark gray path). Before reinitiating a biopanning round, the output phages from each fractionation were amplified and subsequently repurified before injection to ensure normalization of the input titer of the phages into the SPR device and to minimize the loss of low copy phages. Such panning rounds were repeated three to four times for each of our strategies. (L, three flow cells chip surfaces covered with liposomes; VL, fourth flow cell covered with VDAC liposomes). lipos, liposomes; Fra, fraction.
these fractions can be used as the seeding stock for the next phage generation, enabling a wide range of different biopanning strategies (see Fig. 1d).

**Affinity of the Selected Pools**—Biopanning allowed us to select for specific phages. A first evidence of positive selection lies in the differential binding characteristics of each collected fraction (Fig. 1c). The selective enrichments were also consistent with the panning strategies. During panning, for a given input titer of phages, there is a decrease in output titer following the first round of selection followed by a slight increase in output titers with the subsequent rounds. These dynamics are taken as an evidence of the selection processes. Fig. 2 shows that the titers of the phage population for each of the fractions are decreasing/increasing and stabilizing according to the selection strategy that was performed. This presumably leads to different types of phage populations. Such differences can be assessed both between the different panning rounds and within a single round.

An increase in selective binding can be evidenced with the panning rounds. In Fig. 3a, the sensorgrams, i.e. binding measured as RU, of bulk phage populations are shown. With each passing round (i) the signal increases, consistent with an increased binding for proteoliposomes, and (ii) the shape of the signals is altered in a manner consistent with a more efficient on-rate of binding. There is also a marked decrease in signal between the naïve, i.e. unselected, library and the output of round 1; this would be consistent with the clearing from the library of the phages binding to plain phospholipid. The comparison of the phages from the first and third rounds with respect to binding on plain or on proteoliposomes demonstrates that their average selectivity improved as evidenced by the decrease in aspecific binding of those phages on pure liposomes. It is worth noting that the shapes of the binding curves obtained from plain liposomes are consistent with less aspecific binding as panning rounds increased (Fig. 3c).

Within a single round, the fractionation procedures yielded phage populations differing in affinities for the target protein. A comparison of the sensorgrams at the end of round 3 (Table I) shows that the time constants for both the association and dissociation processes of the binders markedly varied when the selected populations were tested on VDAC proteoliposomes. Alternatively these parameters remained roughly constant when the populations were tested on plain liposomes. Such poor discrimination for liposomes is expected if the lipids have only a minor role in the selection process. The ratios of the time constants between proteoliposomes and liposomes increase with the fraction number for both the association (from 0.064 to 0.18) and the dissociation processes (from 0.31 to 0.54). This is consistent with the fractions being enriched in phages that bind less rapidly but remain bound longer. The net result in terms of bulk affinities will depend on the balance between the population-averaged off and on time constants (ratios given in Table I, last line). These data evidence that the fractionation procedure can select phage populations (i.e. epitopes) based on a mix of their on and off rate constants.

**Characterization and Identification of the Individual Clone Sequences**—After three or four biopanning rounds, the output
phages of several fractions were plated on their host cells and titered. Of those plates, 375 phage single clones from different fractions were randomly picked up according to Table II, amplified, and sequenced for further characterization. From the 375 sequences, 67 yielded either no identifier or a homology with a still unidentified DNA sequence in the database. From the remaining 308 sequences, we obtained 547 non-redundant identifiers with a 90% homology and a minimum length of 30 bp. Of these 547 identifiers, 481 were real proteins. We chose to scan the nucleotide database instead of the protein database to alleviate eventual frameshift problems following clone sequencing. Incidentally we encountered this problem when running a control of a subset of 10 epitope sequences by either polling the nucleotide database or by first translating the epitope and then polling the protein database. Our identified set is thus larger than the actual expected set of protein epitopes but is likely to include the latter. A basic statistical comparison of the set of proteins obtained by our selection (481 entries) with the full set of human proteins available in the database (7304 entries) allowed us to gain some insight in our selection process (Table III). Globally we found no localization bias except for soluble cytoplasmic and plasma membrane proteins. The statistical distribution of the sequences that were organelle-specific matched the distribution found in the database. The split between transmembrane and membrane-associated proteins was again identical between the whole human protein database and our identified hits. When analyzing the fractions, however, it is worth noting that we did find a sizable enrichment in mitochondrial protein sequences in the late fraction of the third round (27% of 69 sequences) combined with a marked decrease in cytoplasmic and plasma membrane epitopes (Table III, last column), the latter epitopes being found more abundantly within the early fraction. This would indicate a bias toward a more stable association for epitopes originating from the mitochondria and would confirm that fractionation also yielded qualitatively different binders.

Relevance of the Insert Sequences—The first way to assess the efficacy of the approach is to compare our output set with the already reported VDAC-interacting proteins (Table IV). Of 24 proteins already reported, nine were confirmed, 11 were not found, and in five instances a related protein could be identified. Two non-eukaryotic proteins, a bacterial porin and a viral protein, and a plant-specific protein were not expected to be found. Given the variation number within the library ($10^7$), the chances of re-identifying one VDAC-interacting epitope fortuitously are exceedingly small (probability in the order of $(1/10^7)^n$ where $n$ is the number of reconfirmed interactions).

In addition to such a basic comparison, a more systematic screening of the eventual functional effects of the selected epitopes was warranted to further validate a portion of our dataset and thus our method. This was performed based on the ion channel function of the target molecule rather than on...
plain binding. VDAC function can be assayed by measuring volume changes in proteoliposomes. This is easily arrayed in microwell titer plates. In Fig. 4, following appropriate functional and permeability controls (Fig. 4, a and b), several permeability curves obtained with those clones are shown for illustrative purposes. The slopes during the reswelling phase are proportional to the channel permeability. Based on this assay and by comparison with the response obtained with the naïve library (used as negative control; slope values, 6 ± 1), we could classify our epitope sequences as either neutral (slope values, 5–8), channel openers (slope values, >10), or channel inhibitors (slope values, <3). We chose such marked cutoffs to focus on reliable major effects. Of all our 375 tested epitopes, 116 were neutral, 24 were openers, and 51 were inhibitors. Of major interest is that five of the latter clones either fully or near fully blocked the channel. The possibility to record a full block is also additional evidence that our proteoliposomes were not leaky. The remainder (184 clones) gave unreliable results. As some of the clones bore fragments from the same protein, we identified a total of 55 different epitopes able to functionally interact with our target in a permeability assay (listed in Supplemental Results).

**Table I**

| Time constant | Fra 1 | Fra 2 | Fra 3 | Fra 4 |
|---------------|-------|-------|-------|-------|
| \( \tau_{on} \) VDAC (s) | 0.9 ± 0.1 | 0.9 ± 0.1 | 1.3 ± 0.2 | 2.5 ± 0.2 |
| \( \tau_{on} \) Lipos (s) | 14 ± 0.2 | 14 ± 0.2 | 12 ± 0.05 | 14 ± 0.1 |
| Ratio | 0.064 | 0.064 | 0.108 | 0.180 |
| \( \tau_{off} \) VDAC (s) | 8.0 ± 1.8 | 13.4 ± 2.0 | 15.1 ± 2.4 | 14.1 ± 2.3 |
| \( \tau_{off} \) Lipos (s) | 25.6 ± 1.9 | 32.5 ± 2.0 | 30.0 ± 2.0 | 26.1 ± 2.7 |
| Ratio | 0.31 | 0.41 | 0.50 | 0.54 |
| \( \tau_{off}/\tau_{on} \) ratio VDAC | 8.9 | 14.9 | 11.6 | 5.6 |

* Time constants derived from the hyperbolic fits of the sensorgrams recorded from the four eluted phage fractions collected at the end of the third round of biopanning following the late strategy and tested for binding in the SPR machine either on VDAC proteoliposomes or plain liposomes. Because we are dealing with a heterogeneous population of binders, the measured time constants and not their derived rate constants are given in the table.

**Table II**

**Individual clone repartition**

Shown are the number of clones sequenced according to panning round and fraction.

| Fractions | Fraction 1, early strategy | Fraction 2, early strategy | Fraction 3, early strategy | Fraction 4, late strategy | Regenerate, late strategy |
|-----------|---------------------------|---------------------------|---------------------------|---------------------------|--------------------------|
| Third round | 50 | 25 | 25 | 100 | 25 |
| Fourth round | 50 | |

**Table III**

**Cellular localizations of putative interacting proteins and number of all human proteins available in the database**

ECM, extracellular matrix; ER, endoplasmic reticulum; DB, database.

| Protein localization | Human DB* | Output DB* | Output third round, b fraction 4 |
|----------------------|-----------|------------|----------------------------------|
| Cytoplasm            | 1562 (23%)| 128 (30%)  | 9 (15%)                          |
| ECM                  | 940 (14%) | 63 (15%)   | 19 (31%)                         |
| ER + Golgi           | 161 (2%)  | 10 (2%)    | 2 (3%)                           |
| Mitochondrion        | 325 (5%)  | 32 (7%)    | 17 (27%)                         |
| Nucleus              | 1352 (20%)| 77 (18%)   | 6 (10%)                          |
| Plasma membrane      | 176 (3%)  | 81 (19%)   | 2 (3%)                           |
| More than one compartment | 2349 (34%) | 41 (9%) | 7 (11%) |
| Total                | 6865 (100%)| 432 (100%)| 62 (100%)                        |
| Unknown              | 439       | 49         | 7                                |
| Transmembrane        | 1814 (24%)| 134 (28%)  | 18 (28%)                         |
| Membrane             | 530 (7%)  | 21 (4%)    | 3 (5%)                           |

* Human entries were extracted from the list of subcellular locations for Swiss-Prot entries (release 40); results of meta-analysis of the annotation of Swiss-Prot by Eisenhaber and Bork (50, 51).

b Selection output, BLAST-identified sequences that were verified for subcellular localizations using the SOURCE database (source.stanford.edu).
### TABLE IV

**Reported VDAC-interacting proteins versus this study output**

| Protein | Method used | Ref. | DB hit | Clone no. | GenBank™ accession no. |
|---------|-------------|------|--------|-----------|------------------------|
| Actin   | Bilayer, enzymology | 52 | Actin, γ 1 | 3RL007 (173/180) | BC023204 |
| Plasminogen (kringle 5) | Binding studies | 53 | Plasminogen | 3L012 (104/105) | BC060513 |
| Gelsolin | Functional, co-immunoprecipitation | 54 | Gelsolin precursor | 4L083 (218/219) | S65738 |
| C-Raf | Bilayer co-immunoprecipitation | 55 | C-Raf | 4L044 (98/106) | AY271661 |
| ADP/ATP carrier | Affinity matrix, MALDI-MS | 56, 57 | ADP/ATP carrier (ANT-2) | 3E007 (246/273) | L78810 |
| Mt HSP70 | Two-hybrid, co-localization, liposomes | 58 | HSP70 1A and 1B | 3E2E004 (234/270) | NM_005346 |
| Voltage-gated Ca²⁺ channel (Torpedo electric organ) | Co-localization (immunogold), immunoblotting | 59 | Voltage-gated Ca²⁺ channel (Cav2.3 α subunit) | 3E036 (31/34) | AF223391 |
| NO synthase (endothelial) | Co-immunoprecipitation, MS | 60 | NO synthase (neuronal, NOS1) | 3E3E007 (100/105) | AY445095 |
| Bcl-2 family proteins (Bcl-xL, Bax, Bak, Bim, etc.) | Functional, co-immunoprecipitation, antibodies, etc. | 41–44 | Bcl-2 | 3E048 (227/253) | AY220759 |
| Cytoplasmic dynein light chain (Tctex-1) | Two-hybrid, co-localization, bilayer | 58 | Cytoplasmic dynein heavy polypeptide 1 (DNCH1) | 3E028 (248/274) | AF097732 |
| Cyclophilin D | Affinity matrix, liposomes | 56 | NKTR (natural killer cells cyclophilin-related protein) | 4E036 (53/53) | BC007658 |
| Myosin (Drosophila melanogaster) | Two-hybrid | 61 | Myosin light chain kinase (MLCK) | 4L044 (99/105) | BC039103 |
| Fau protein (D. melanogaster) | Two-hybrid | 61 | Ubiquitin B | 4L091 (283/266) | BC009301 |
| GABA<sub>₄</sub> receptor | Co-purification, photoaffinity labeling co-immunoprecipitation | 62, 63 | GABARAPL1 (GABA-A receptor-associated protein-like 1) | 3E009 (506/509) | NM_031412 |

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Another way to test for relevance would be to measure the VDAC protein on the function of this enzyme and found VDAC to be markedly effective at nanomolar concentrations (27).

**DISCUSSION**

In this work we present a novel strategy combining the advantages of combinatorial chemistry with controlled fluidics and apply it to a mitochondrial membrane protein as a target. We functionally reconstituted the target protein into proteoliposomes to maximize the likelihood of finding ligands to the native folds of our target and to avoid a selection based on interfering proteins. We used this strategy to screen a T7 phage-expressed cDNA library from liver cells.

The control of the fluidics was obtained by SPR. As far as SPR is concerned, some reports exist of selection against soluble proteins or for antibody engineering (32); panning was performed inside the device but without reamplification between rounds. So far SPR machines have mostly been used for evaluating panning results and finding which single clones to sequence. By eluting and fractionating the bound phage populations, we could perform various selection strategies based on the relative affinities of the phage for the target protein. At first glance, this could also be achieved from panning performed in test tube, but there is still a major difference. In the test tube the binding molecules are allowed to reach steady-state conditions, whereas by design, in our experiment this will never be the case because of the presence of a competition between the diffusion and the perfusion processes during the selection. Indeed the real advantages of the SPR device for us lay with its microfluidics: the epitope-bearing viruses were very slowly perfused (1 \( \mu l/\text{min} \)) in the small volume and minute binding surface of a reaction chamber (0.06 \( \mu l \), 1 mm²) for 100 min. This unusual geometry has various useful consequences for ligand selection. First, working with fewer binding sites theoretically enhances selectivity. These target sites could even be fewer due to the probable presence of incorrectly reconstituted protein. Even with such a caveat, it remains that ligands with better selectivity would be selected, albeit against irrelevant protein epitopes. Then kinetic analysis requires working in conditions where the binding molecules are not mass-limited but diffusion-limited. In such conditions the collision rate is maximal around \( 10^{9} \text{M}^{-1} \text{s}^{-1} \); the effective collision rate yielding actual binding is smaller. Alternatively if one is not interested in kinetic parameters but in binding, then limitations in analyte/substrate availability may become useful. It is known that, at low flow rates (5 \( \mu l/\text{min} \)), mass transport of the perfused molecules becomes limiting in the SPR device that we used (16). Because we used even slower flow rates (1 \( \mu l/\text{min} \)) and because our viral particles have a diffusion coefficient that is 2 orders of magnitude lower than those of proteins (\( D = 0.6 \times 10^{-7} \text{cm}^2/\text{s} \) for the T7 icosahedral particles (33)), it is quite certain that we are in such a mass-limited regime in the panning chamber during the selection process. It has been predicted that in such conditions, i.e. binding influenced by transport and heterogeneity in the free analyte, the binding will likely be due to the

| Protein                  | Method used                              | Ref.  | DB hit                          | Clone no. (bp similarity) | GenBank™ accession no. |
|--------------------------|------------------------------------------|-------|---------------------------------|----------------------------|------------------------|
| Hexokinase               | Functional, binding studies              | 64, 65| N/F                             | 3E034 (22/22)             | NM_172890              |
| Glycerol kinase          | Functional, binding studies              | 64, 65| N/F                             |                            |                        |
| Creatine kinase          | Co-sedimentation, gel permeation, SPR    | 66    | N/F                             |                            |                        |
| Aldolase                 | Affinity purification, two-hybrid, overlay assay | 67, 68| N/F                             |                            |                        |
| GAPDH                    | Affinity purification                     | 68    | N/F                             |                            |                        |
| MAP2                     | Affinity purification                     | 69    | N/F; no explanation             |                            |                        |
| Tubulin                  | Co-immunoprecipitation                   | 70    | N/F; no explanation             |                            |                        |
| mBPR                     | Co-purification                          | 71    | N/F                             |                            |                        |
| TOM20                    | Binding studies, co-immunoprecipitation  | 38, 72| N/F                             |                            |                        |
| Capping protein (D. melanogaster) | Two-hybrid                                   | 61    | N/F; no explanation             |                            |                        |
| VDAC modulator           | Bilayer                                   | 73    | N/A; not yet identified         |                            |                        |
| Sucrose synthase 3       | Two-hybrid                               | 67    | N/A; plants and Cyanobacteria   |                            |                        |
| PorB                     | Immunoprecipitation                       | 74    | N/A; prokaryotic                |                            |                        |
| HBx                      | Co-localization                           | 75    | N/A; prokaryotic                |                            |                        |
population of molecules having the higher association rate constants and/or lower diffusion coefficients (17). In short we are working in conditions mostly avoided when using SPR but in which likely better epitopes against the target ought to be selected. In those limiting conditions, the receptors (our target VDAC) would compete for binding during the association phase, and epitope rebinding is likely to occur during the dissociation phase (18). Because a longer time is needed to reach a given binding level, multivalent binders, needing more time to bind, would be favored. This should presumably work well with the T7 phages (low copy multivalent epitope display). For these reasons, performing biopanning rounds on bead-fixed proteoliposomes (e.g. see Ref. 34) cannot be considered a comparable method.

No golden standard exists to massively screen for ligands/epitopes. Numerous methods have been developed and used, and each seemingly imposes its own technical biases upon the selection process (for a review, see Ref. 2). No method exists to identify and validate putative ligands at the same time. A more detailed and critical review of the relevant literature (see Supplemental Discussion) evidences that no large scale method can be considered complete, that each generates more than its fair share of false positive as well as negative results, that combining independent methods will offer the most promising and accurate results, and that, regardless of what some wording may suggest, reliably screening for epitopes against membrane proteins still remains out of reach for nearly all of the newly developed techniques. In

Fig. 4. Functional testing of positive clones. a, the integrity of the liposomes is evidenced by the swelling response of blank liposomes (circles), and the functional reconstitution of VDAC is evidenced by the nearly instantaneous isovolumetric response of VDAC proteoliposomes following an identical hypo-osmotic shock (closed squares). The latter response does not differ much from the measurement performed in the absence of any osmotic challenge (open squares). b, VDAC liposomes shrinking in response to a hyperosmotic shock brought about by a VDAC-impermeable osmotant (PEG 1450, open circles). Alternatively VDAC liposomes first shrink and then reswell when a VDAC-permeable osmotant was added (PEG 800, closed squares). PEG was 10 mM in both instances. c, the proteoliposome responses as in b in the presence of phages (naïve library used as a negative control or the individual clones tested). The osmotant molecule was added after preincubation of the proteoliposomes with identical concentrations of phage single clones. Note the various slopes proportional to the VDAC permeability to the osmotant. This allows the classification of the phages according to their effect on the permeability (more permeable than naïve in green, less permeable in orange, and fully impermeable in red). Note the full block elicited by two individual clones evidencing that the reswelling is not due to proteoliposomes leakage. Note also the inhibitory effect of the COX epitope (blue curve; numbers refer to the amino acids in the protein sequence). d, the enzymatic function of COX is enhanced in vitro by increasing concentrations of VDAC (0 mM, black; 60 nM, red; 250 nM, green; 1 μM, blue). The inset shows the apparent dissociation constant of the interaction.
this context, novel adaptations of the yeast two-hybrid (Y2H) systems and phage display would be of value. The former is usually not membrane protein-friendly because among the false negative results from the Y2H systems 60% are membrane protein. Recently developed membrane-based Y2H methods have not been thoroughly tested yet, but obtaining in such systems a huge variation of epitopes to screen from is quite labor-intensive. Alternatively the phage display, although easy to perform and cost-effective, remains a prokaryotic system that is likely to miss interactions that are carried by the post-translational modifications of the protein epitopes. Although some eukaryotic cell display methods have been set up, the epitopes selectively targeted against membrane proteins remain once again mostly out of reach (35). The phage display, because it is an in vitro system, thus remains a powerful method especially when dealing with reconstituted membrane proteins and smaller epitopes (36).

In addition to being biased toward putatively better ligands and designed for epitopes against membrane proteins, our own adaptation of this method offers several other advantages. Because of the fractionation possibilities, the same set of epitopes can undergo various selection strategies depending on the needs of the experimentation. We did not “elute” the very strongly attached viral particles by breaking their binding with an acid or a detergent washout; we let them elute under running buffer conditions. We found that, based on the stability of the association, we could obtain phage fractions with different bulk affinities and different enrichment in terms of cellular epitopes (like the enrichment in mitochondrial proteins seen in fraction 3). Performing the quality control steps within the machine using an identical setup is also advantageous. In some phage display setups, panning and testing happen in different conditions; this is not experimentally optimal. The use of machines allows the method to be scaled up and automated with the appropriate design modifications of the equipment.

In our case, about 375 insert sequences allowed us to identify highly similar (>90%) portions of genes from 580 different proteins. Although identifying related domains in different proteins yields interesting functional clues, it does not solve the relevance of all obtained epitopes. It trims down the genome to a subset of possible candidates. To further sort out the most relevant clones from a putative subset, one has to use another independent method to control each selected clone. The most obvious strategy is direct binding (in the form of ELISA plates, of SPR binding measurement, etc.). But binding in vitro does not demonstrate cellular function. Functional assays are thus better as long as the function of the target protein is known. In our case this was realized under the form of a VDAC proteoliposome permeability test. This yielded a putatively more relevant subset of epitopes on which to focus. This subset consists of the clones displaying a relative permeability range at least double or less than half the value obtained with the naïve library. According to these criteria, we narrowed the dataset to 55 functional epitopes of 375 clones, likely trimming down the occurrences of false positives. To further validate these functional epitopes, more independent assays now ought be performed. Depending on the function of each of these putative VDAC binders, enzymatic, colorimetric, and/or other function-specific assays could be developed in addition to direct binding verification and permeability.

Our system also has its own built-in biases. Obviously hydrophobic epitopes could have been favored by our setup. The fact that we cleared out such clones on the first three flow cells decreased this possibility. A possible misfolding of VDAC into proteoliposomes could allow the selection of irrelevant phages. However, the careful VDAC reconstruction protocol minimizes this possibility. Another (uncontrollable) bias of our system is that the phage needs to replicate itself in between panning rounds. It is possible that an effective selective pressure geared toward binding may render the bearer of such epitope less able to compete during our reamplification rounds. This would lead to the loss of potentially relevant epitopes.

Another way to gauge our methodology would be to critically review its outputs with what is already known for VDAC-interacting proteins. We found at least nine of 24 reported protein-protein interactions, and in five more cases we hit on related proteins. Although the chances of this resulting by sheer chance are trivial and support our approaches, we still missed some expected and well documented interactions like the hexokinase or the TOM20 protein. According to our method (including a 5-min washout), we are likely to lose rapidly reversible ligands. Such would be the case of the TOM20 protein whose interaction is reported as being highly labile (37). Furthermore the structure of our target protein functionally embedded into phospholipid membranes is totally different from the looser structure recognized by the import machinery (37, 38). Although also readily reversible and hence labile, the interactions between VDAC and hexokinases or glycerol kinases may also have been missed due to our choice of protein isofrom; the correct ligand remains the human VDAC1 (39). Moreover VDAC-bound hexokinases are not expressed in the liver. As for the mitochondrial creatine kinase, its association with VDAC is calcium-dependent (40); as our selection buffer contained no Ca2+, it is an expected miss. Of all the apoptosis-related proteins presumably interacting with our target (41–44), we found Bcl-2 and three other proteins not yet reported but not Bax, Bak, Bid, Bim, or Bcl-xL. We did not expect Bak. Bak is reported to bind HVDAC2 not HVDAC1, our target ortholog (19). Recent data suggest that Bcl-xL needs to be inserted into the phospholipid membrane to bind and affect VDAC (42); so we could not have found this protein either. Thus the situation is likely very complex, and it is also worth mentioning that there still is a

2 Myriad Genetics Corp., I. Fraser, personal communication.
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debate about the precise nature of the apoptotic proteins interacting with our target. Among other possible false negative results, we should mention that some relevant binders are likely lost due to the experimental processing; there could be sequencing errors/problems or epitope sequences for which there are no matches in the database or that match still unidentified DNA. More importantly, we should not forget to include epitopes that will be underrepresented or absent from the initial library. Having used a liver cell library from normal human could explain the poor representation of some proteins and the absence of the mitochondrial benzodiazepine receptor (mPBR) and of the hexokinase that are not expressed in normal hepatocytes (45). Starting with another cell type, a cancer cell line, or other disease-related libraries may have yielded other results. The fact that we used a cDNA instead of a genomic library may also have biased our results toward heavily translated genes with more stable RNA. Thus, we could convincingly argue that we found most proteins that we could reasonably expect to find. This amounts to at least 38% of the known interactions and compares more than favorably with other reports of 13% of known interactions found (46) or even less than 5% (47–49). This useful approach is generally applicable for any protein and could be automated and scaled up even without the use of a SPR device.

Other mitochondrial proteins from our potential VDAC binders list demand a further analysis, but time limited the development and/or adaptation of additional assays. As for all high throughput methods, an ensuing validation of the ligands must be performed by biochemical methods. It is evident that an ensuing step is required to measure the accuracy of each interaction. Although some of the identified potential VDAC partners could readily tempt us to functional speculations, independent biochemical verification is very much required.

Another way to select a subset of the putative binders for further validation is to focus on the most functionally relevant clones. We performed a rapid functional screening assay based on the channel function of VDAC. This yielded a first subset of 75 functional clones, from which, 32 could be reliably identified with a protein in the database. Some of these clones were VDAC openers and some inhibited the VDAC permeability. A more accurate assay should now be performed to validate these potential VDAC regulating protein-epitopes.

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