An Antiproliferative Genetic Screening Identifies a Peptide Aptamer that Targets Calcineurin and Upregulates its Activity

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Running title: screening of antiproliferative peptide aptamers
Summary

Peptide aptamers are combinatorial recognition molecules that consist of a constant scaffold protein displaying a doubly-constrained variable peptide loop. They bind specifically target proteins and interfere with their function. We have built a peptide aptamer library in a lentiviral expression system to isolate aptamers that inhibit cell proliferation in vitro. Using one of the isolated aptamers (R5G42) as a bait protein, we have performed yeast two-hybrid screening of cDNA libraries and identified calcineurin A (CNA) as a target protein candidate. R5G42 binds CNA in vitro and stimulates its phosphatase activity. When expressed transiently in human cells, R5G42 induces the dephosphorylation of Bad. We have identified an antiproliferative peptide aptamer that binds calcineurin and stimulates its activity. The use of this ligand may help elucidate the still elusive structural mechanisms of activation and inhibition of calcineurin. Our work illustrates the power of phenotypic screening of combinatorial protein libraries.
in order to interrogate the proteome and chart molecular regulatory networks.
Introduction

In absence of classical genetics, the deciphering of mammalian regulatory networks rests mostly on the reverse genetics methodology, and particularly on the use of transdominant negative agents such as dominant negative alleles (1), antibodies (2), nucleic acid aptamers (3), peptide aptamers (4), antisense or small interfering RNA (5), and small molecule inhibitors when available (6). In most applications, these agents are designed or selected to specifically target a protein and they are then introduced into cellular or animal models to assess the phenotypic consequences of the targeted perturbation they exert. Another approach consists of constructing large libraries of transdominant agents in retroviral vectors and performing genetic selections or screening to isolate library members that confer given phenotypes. Libraries of antisense cDNAs (7), random fragments of cDNAs (8), ribozymes (9), combinatorial peptides (10), shRNAs (11) have been used successfully to interrogate
proteomes and identify new members of mammalian regulatory pathways.

A straightforward selection of transdominant negative agents that inhibit cell proliferation is an oxymoron. Elaborated experimental schemes have thus been developed and used successfully to identify cytostatic random cDNA fragments (12) and random linear peptides terminally fused to GFP (13). In both cases, a counterselection against dividing cells has been devised and, in the latter case, coupled to a positive screening for cells that do not divide and thus maintain a fluorescent vital dye. Whereas different antiproliferative linear peptides have been isolated, their mechanism of action has not been elucidated so far (13).

We set out to isolate another kind of combinatorial protein reagents for their ability to inhibit tumor cell proliferation, with the hope of identifying proteins playing an unexpected role in this biological phenomenon.
Peptide aptamers are man-made combinatorial protein reagents that bind target proteins and can interfere with their function in living cells and organisms (14), reviewed in (4). They consist of conformationally-constrained random sequence peptide loops displayed by a scaffold protein. They bind their cognate targets with a strong affinity and, usually, a high specificity, which allows them to discriminate between closely related members within a protein family (14), or even between different allelic variants of a given protein (15). So far, peptide aptamers have been mostly selected through yeast two-hybrid screening experiments, for their ability to bind a given target protein. In fewer instances, peptide aptamers have been selected for their ability to confer selectable phenotypes to yeast (16,17) and bacteria (18). Peptide aptamers selected in yeast have been used successfully to identify their cognate target proteins by two-hybrid screening.

Here, we have constructed a peptide aptamer library in a simian immunodeficiency virus (SIV)-derived gene
expression system. We have performed an iterative genetic screening to isolate peptide aptamers that inhibit tumor cell proliferation. We have identified the catalytic subunit of the calcium-activated protein phosphatase calcineurin as a target of one of the isolated aptamers. We have shown that this aptamer upregulates the phosphatase activity of calcineurin in vitro and in cultured cells. Our work has identified an antiproliferative molecule that binds and stimulates calcineurin through a seemingly original mechanism.
Experimental Procedures

Cell culture
We maintained all mammalian cells in a 5% CO₂ atmosphere at 37°C in Dulbecco’s Modified Eagle’s Medium (Invitrogen-Gibco) supplemented with 10% v/v fetal calf serum and 100μg/ml penicillin-streptomycin.

Construction of lentiviral vectors
All the lentivectors were derived from pR4SA-EFS-GFP-W (19). We first digested this vector with Hind III, thus eliminating EGFP, WPRE and EcoRI sites, to create pVRV1. We blunted the remaining EcoRI site upstream of the CMV promoter and religated the vector to create pVRV2. We digested pVRV2 with BamHI and HindIII and we ligated the following hybridized oligodeoxynucleotides: 5’-
GATCGCTAAGCGAATTCCTCGAGGCGCGCGTCGACCAGGATCC-3’ and 5’-
AGCTTTGGATCCTGGTCGACGCGCGGCCTCGAGGAATTCCGTTCAGC-3’ to create pVRV3, that bears a multiple cloning sequence. We constructed pVRV4 by inserting an IRES-EGFPf
(farnesylated enhanced GFP) coding sequence in pVRV3. This was done by a multiplex ligation between SalI/BamHI-cut pVRV3, a SalI/NcoI-cut EMCV IRES cassette (from pIRES2-EGFP, Clontech) and a NcoI/BamHI-cut EGFP-f coding sequence (from pEGFP-F, Takara Bio). We then PCR amplified a HA-tagged HTRX fragment from pJMX-HTRX (Abed et al, in preparation) using the oligonucleotides 5’-GCGGCTAAGCCATGTACCCTTATGATGTGCCAG-3’ and 5’-GGAGACTTGACCAAACCTCTG-3’, and we ligated this fragment into BlpI/XhoI-cut pVRV4. The resulting plasmid, pVRV6, directs the bicistronic expression of an HA-tagged human TRX (with a modified active site) and of EGFP carrying a farnesylation sequence so as to anchor the marker protein to plasma membranes.

**Construction of the peptide aptamer expression library**

We constructed pBK1, a library of peptide aptamers bearing 10 amino acids within the active site of HA-tagged human TRX. We annealed the oligonucleotides 5’-TGGGCCGAGTGGAGCGGTCCG(NNS)9NNCGGACCGAGCAAGATGATCGCCCC-3’ and 5’-GGGGCGATCATCTTGCTCGGTCCG-3’ and we produced
duplexes using the Klenow DNA polymerase. We ligated the AvaII-cut duplexes into CpoI-cut pVRV6. We transformed the ligation product into ElectroTen Blue competent bacteria (Stratagene) and we obtained $8.5 \times 10^9$ transformants.

**Viral vector production**

We produced lentiviral particles by transfecting into 293T cells the following plasmids: i) pVRV6, pVRV12 (pVRV6 directing the expression of p21$^{\text{cip1}}$), pBK1 or any aptamer sub-library; ii) helper pSIV15, directing the expression of gag and pol (20); iii) FbmoSalf, directing the expression of a murine ecotropic envelope (19); iv) pRev (20). In some experiments, plasmids (iii) and (iv) were replaced by the G-rev plasmid (20), directing the expression of Rev and the VSV-G pantropic envelope. We collected and filtered lentivirus-containing supernatants 48h post-transfection through a 0.45μ filter. We determined viral titers by infecting XC or Hela cells and counting GFP-positive cells with a
cytometer (FACScan, Becton-Dickinson). We routinely infected from 40% to 100% cells.

**Screening of antiproliferative peptide aptamers**

We plated XC cells 24h before infection (2 x 10^5 cells/well, 6-well plates, 6 plates). To infect the cells, we added a medium containing a viral supernatant and 6µg/ml polybrene. Three days later, we collected the cells, washed with PBS, stained 5 x 10^5 cells/ml with 10µM CellTracker™ Orange CMTMR (Invitrogen) in PBS at 37°C for 30 min and incubated in culture medium for another 30 min at 37°C. We then plated the cells onto 10 cm dishes (10^6 cells/dish). After 72h, we collected the cells and we sorted the highest percentile of CMTMR fluorescent cells using a FACS Vantage flow cytometer (Becton-Dickinson). We pooled the sorted cells and we extracted their genomic DNA using a Wizard Genomic DNA purification kit (Promega). We PCR amplified aptamer coding genes using the oligonucleotides 5’-AACCGGTGCCTAGAGAAGGT-3’ and 5’-AGACCCCTAGGAATGCTCGT-3’. We cloned the EcoRI/XhoI-digested products into
EcoRI/XhoI-cut pVRV6, to create successive sub-libraries of peptide aptamers, named pCMTMR 1 to 7.

**Two-hybrid screening of R5G42-interacting proteins**

We digested pVRV6-R5G42 with EcoRI and XhoI and ligated the fragment into EcoRI/XhoI-cut pGILDA (Clontech) to create pGILDA-R5G42, a plasmid directing the galactose-inducible expression of a LexA-R5G42 fusion protein. We transformed MB226α pSH18-34 yeast (21) with pGILDA-R5G42 and MB210a yeast (21) with human foetal brain and human testes cDNA libraries, constructed in pJG4-5. We performed the yeast-two hybrid screening of both libraries essentially as described (21), using 4x10^8 cfu and 2.4x10^8 cfu from the brain and testes libraries, respectively. We estimated the mating efficiency at 50% and 58% and the number of diploid exconjugants at 0.2 x 10^8 and 1x10^8 for the brain and testis cDNA library transformed yeast, respectively. We induced the expression of the bait and the libraries at 30°C for 5h, from 10% of the diploids. We collected the yeast and plated them onto 10 Ura⁻His⁻Trp⁻Leu⁻ galactose/raffinose
plates for 5 days. We replica plated onto 10 Ura−His−Trp− Ade− X-gal galactose/raffinose plates. We picked 60 clones from the brain and 48 clones from the testes library that grew in absence of leucine and adenine, and that displayed a β-galactosidase activity. Library plasmids were recovered and re-transformed into EGY48α. The interaction phenotypes were confirmed by a mating assay with EGY42α transformed with pGILDA-R5G42. We then sequenced the library cDNAs from most reconfirmed clones.

Yeast two-hybrid mating assays

To build the different truncations of the CNAβ Cter interacting clone, we designed oligonucleotides that enabled cloning the PCR products into pJG4-5 by homologous recombination.

RH6: 5′-TTATGATGTGCGAAGACTGTATGCTCTCCCGAATTCAgtatgggttc tgatgatg-3′

RH4: 5′-AAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGCTActgtacagc atctttccg-3′
RH3: 5’- AAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGCTAggcactttgcaggtctgc-3’

RH7: 5’- ACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGTCAcctgagaacagagaagact-3’

The 5’ end of RH6 (upper case) matches part of the HA epitope tag and the 5’ ends of RH4, RH3 and RH7 (upper case) match the 5’ extremity of the ADH terminator. We performed the PCR reactions using pCMV-SPORT6-CnAβ (see below) as a template. We constructed CnAβCter Δ1, Δ2, CaM by combining oligonucleotides RH6/RH4, RH6/RH3, RH6/RH7, respectively. We co-transformed MB210α with the PCR products and EcoRI/XhoI-cut pJG4-5. We retrieved the prey plasmids from the transformants (21) and we checked the homologous recombination products by sequencing. We also transformed MB210α with positive and negative controls of interaction. We co-transformed TB50α with pSH18-34T (a plasmid bearing a high-sensitivity lacZ reporter gene) and pGILDA directing the expression of LexA, LexA-R5G42, LexA-R7G44 and LexA-R5G52. We performed the yeast two-hybrid mating assays as described (21).
In vitro binding assay

We first digested pVRV6-aptamer plasmids with EcoRI and XhoI and ligated the fragments into EcoRI/XhoI-cut pGEX4T1. We expressed GST-aptamer fusions in a BL-21(DE3) E.coli strain. We diluted 1/100 overnight cultures and let them grow at 37°C to reach an OD$_{600}$ of 0.6 to 0.8. We induced the expression of fusion proteins by adding 1mM IPTG and incubating overnight at 20°C with vigorous shaking. We collected the bacteria and resuspended them into a lysis buffer (50mM Tris pH8, 100mM NaCl, 1mM DTT) containing 1mg/ml lysozyme. We froze and thawed three times and sonicated on ice. We centrifuged the lysates at 13000g for 30 min and we collected the soluble fractions. We immobilized equal amounts of GST-aptamer fusion proteins on 100µl glutathion sepharose 4B beads (Amersham) at room temperature for 20 min. We washed three times the beads with lysis buffer. We incubated the beads with 1 or 3µg of bovine brain purified calcineurin (Upstate) for 1h at 4°C. We then washed five times the beads with lysis
buffer and we eluted the bound protein by boiling samples 10 min in presence of electrophoresis loading buffer. We loaded the samples onto a SDS-PAGE, transferred to nitrocellulose membrane, and detected calcineurin by western-blotting using an anti-calcineurin pan A antibody (1/1000, Chemicon International). We revealed the blot using a HRP-linked rabbit antiserum and an ECL kit (Perkin Elmer).

Cell proliferation assay

To stably express peptide aptamers in mammalian cells, we used the episomal eukaryotic expression vector pCEP4 that bears a CMV promoter and a hygromycin selection marker (Invitrogen). We PCR amplified aptamer coding sequences using the oligonucleotides 5’-GCAAGCTAGCATGTACCCTTATGATGTGCCA-3’ that hybridized to the HA coding sequence and 5’-CGTTGCAGCCGCTTAGACTAATTCAATATGGT-3’ that contained a stop codon. We digested the PCR products with NheI and NotI and ligated them into NheI/NotI- cut pCEP4 to create pEA-aptamer plasmids. We plated 3 x 10^5 cells/
well in 6-well plates and transfected 24h after using Jet PEI (Qbiogen), 3.7 µg pEA-aptamer plasmids and 0.3 µg pEGFP-C1 (Clontech) to monitor transfection. We added hygromycin (Invitrogen) at 200 µg/ml two days later and we cultured the cells for 2 weeks, renewing the medium twice a week. We then rinsed the cells in PBS and we fixed and stained them by incubating 30 min in crystal violet (0.05% crystal violet, 20% ethanol, 0.37% formaldehyde). We removed excess crystal violet by washing with water.

**In vitro phosphatase assay**

We first produced GST-aptamer fusion proteins as described above. For this experiment, we eluted GST-aptamer fusion proteins from glutathion sepharose beads using 20mM reduced L-glutathione (Sigma), and we dialyzed the eluates overnight against a phosphatase buffer (50mM Tris-HCl pH7.4, 0.1mM CaCl₂). We measured the phosphatase activity of calcineurin using pNPP (Sigma) as substrate, in a final volume of 100 µl. The sample solution contained 50mM Tris-HCl (pH7.4), 0.1mM
CaCl$_2$, 1mM NiSO$_4$, 0.15mg/ml BSA (Sigma), 0.1µM calcineurin (Upstate). We added purified calmodulin (Upstate) and GST-aptamer fusion proteins at different concentrations (see figure legend). After a 15 min pre-incubation at 37°C, we started the reactions by adding 4.1mM pNPP and we incubated the mixtures at 37°C for 20 min. We measured the nitrophenylate product at 405nm using an Envision plate reader (Perkin Elmer). We substracted the background level that we determined using a reaction mixture lacking calcineurin.

Monitoring of Bad phosphorylation

We cloned the peptide aptamer coding genes into pPEAt (a pCEP4-based vector that bears a tetracyclin-inducible promoter and a hygromycin resistance gene), as described above ("cell proliferation assay").

We seeded 4 x 10$^5$ Hela-tet cells/well in a 6-well plate 24h before transfection. We transfected with Jet PEI (Qbiogen) 1µg of pEBG-mBad (a plasmid directing the expression of the murine Bad protein; Cell Signaling Technology), 0.5µg of pCMV-SPORT6-CnAβ and pCMV-SPORT6-
CnB (plasmids directing the expression of human calcineurin Aβ and B; RZPD), and 1µg of pPEAt-R5G42, -R5G52 or –R7G44. After an overnight incubation of the transfection mix, we washed the cells once with culture medium and we added fresh medium, with or without 0.5µM FK506 (Calbiochem). We collected the cells 24h later, washed them twice in PBS, and lysed them 20min in ice-cold lysis buffer (20mM Tris, pH 7.4, 150mM NaCl, 2mM EDTA, 1% NP40, protease inhibitor cocktail complete EDTA free – Roche). We centrifuged the lysates to remove cellular debris and we quantified the protein content using the microBCA protein assay kit (Pierce). We loaded 50µg of the lysates on a 4–12% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-phospho-Bad (Ser112), anti-phospho-Bad (Ser136), and anti-Bad antibodies (Cell Signaling Technology). We revealed the blots using the enhanced chemiluminescence (ECL) system (Perkin Elmer).
Results

Peptide aptamer libraries and screening strategy

To construct our peptide aptamer libraries, we used a SIV-derived lentiviral expression vector directing the constitutive expression of bicistrons (transgenes and a GFP marker) under the control of an EF1α promoter (see Experimental Procedures). We first built 12 low-complexity peptide aptamer libraries, combining two scaffolds (human thioredoxin or a E.coli thioredoxin, whose coding sequence harbors codons optimized for expression in mammalian cells), two epitope tags (HA or 6His) and three variable region lengths (16, 10 or 7 amino acids). We performed pilot experiments to determine which library yielded the highest expression level of peptide aptamers upon transduction of XC cells with viral particles. We observed that the best combination was the HA-tagged, human thioredoxin displaying random peptide loops of 10 amino acids (data
not shown) and we constructed accordingly pBK1, a high-complexity peptide aptamer library (Figure 1A).

To isolate library members that inhibit tumor cell proliferation, we made use of the fluorescent vital dye CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine), which cells incorporate and dilute, as they proceed through division cycles. Those cells that do not divide maintain a high fluorescence level and can thus be sorted by flow cytometry. Because of a significant background of cells that do not grow or proliferate more slowly independent of the expression of peptide aptamers, multiple screening rounds were necessary to isolate peptide aptamers that exert an antiproliferative effect. We thus constructed a peptide aptamer sub-library from the highest percentile of CMTMR-positive cells obtained after each screening iteration and we submitted each sub-library to a subsequent screening round (Figure 1B).
Isolation of antiproliferative peptide aptamers

We used rat XC cells, derived from a RSV-induced sarcoma, which enabled us to use viral particles harboring a murine ecotropic envelope. We performed 7 screening iterations before isolating and characterizing individual peptide aptamers. We determined the antiproliferative activity of the sub-libraries both in XC cells and in human Hela cells. As shown in Figure 2A, the mean fluorescence intensity of both cell lines increases gradually with the number of screening iterations, thereby indicating a progressive enrichment of antiproliferative peptide aptamers within the sub-libraries.

We picked and sequenced 100 clones from the R5 and R7 sub-libraries, obtained from the fifth and seventh screening iteration, respectively. More than 40% of the peptide aptamers isolated after the seventh screening iteration corresponded to a single library member, named R5G42. The occurrence of this aptamer was already significant after the fifth iteration but was not detectable after the fourth iteration. Three other
peptide aptamers that showed a lower occurrence were also isolated (R7G11, R7G44 and R5G52) (Table 1).

We wished to establish the antiproliferative activity of these peptide aptamers using alternative cellular models and a non-retroviral vector to express individually each aptamer. We cloned the aptamer coding genes into a vector bearing a hygromycin selection marker. We also subcloned the pBK1 library into this vector, to create “AptaLib”. We continuously expressed the aptamers, the empty thioredoxin scaffold or AptaLib in Hela and MCF-7 cells for 2 weeks and we stained the cells that grew. Aptamers R5G42 and R5G52 significantly inhibited the proliferation of both cell lines, as compared to AptaLib and human thioredoxin. Aptamer R7G44, similarly to other aptamers (not shown), did not exert any antiproliferative effect (Figure 2B). These aptamers may originate from the remaining background of slowly proliferating cells during the seventh screening iteration, independently of the expressed aptamers. Surprisingly, aptamer R7G11 did not inhibit cell
proliferation in this assay (not shown), despite showing a high occurrence in the seventh sub-library (Table 1). This could be due to the fact that the CMTMR assay is more sensitive in detecting modest antiproliferative effects than the colony formation assay or that some peptide aptamers somehow enhance the CMTMR labeling of their host cells. From all these results, we decided to focus on peptide aptamer R5G42 and to identify its target protein.

Identification of calcineurin A as a target protein

We performed two yeast two-hybrid screening experiments against a LexA–R5G42 bait protein, using a human testis and a human fetal brain cDNA library. We obtained 29 and 42 reconfirmed clones, respectively. We disregarded those clones that either showed a barely detectable two-hybrid interaction phenotype, or that cross-interacted with control aptamers, or that corresponded to hypothetical proteins (Table 2). We thus retained two candidates. The highest occurring clone, from both libraries, corresponded to the NS5A-
TP2 protein, recently discovered through a systematic search for genes that are transactivated by the non-structural NS5A protein from hepatitis C virus (22). No biological knowledge is currently available for this protein. The other remaining target candidate was calcineurin A (CNA), for which two different isoforms (beta and gamma) were selected from the testes library (Figure 3A). We decided to focus our work on CNA.

We first set out to map the R5G42 binding site on CNA. The CNA interacting clones selected in the yeast two-hybrid experiments corresponded to the carboxy-terminal regions of the β and γ isoforms, encompassing the calmodulin-binding domain and the auto-inhibitory domain (Figure 3B). Among the 3 truncations constructed from the CNAβ selected clone (Figure 3B), only CNAβΔ1 retained its yeast two-hybrid interaction phenotype with R5G42 (Figure 3A and data not shown). These results indicate that the R5G42 binding site on CNA lies between the amino-terminus of the calmodulin-binding domain and the carboxy-terminus of the auto-inhibitory domain, and is not circumscribed to the CaM
binding domain. This yeast two-hybrid mating assay also supports the specificity of interaction between R5G42 and CNA, as R5G42 did not show an interaction phenotype with two unrelated bait proteins (RAS, FKBP12) and as R7G44 and R5G52 did not show an interaction phenotype with CNA. R5G52, however, did not show an interaction phenotype with peptide aptamer RG22, which interacts with LexA in the context of most (but not all) LexA fusion proteins. The LexA-R5G52 bait protein may thus not be properly expressed and/or folded in this yeast two-hybrid setting.

To confirm the interaction between R5G42 and CNA, we performed an in vitro binding assay between recombinant purified GST-aptamer fusion proteins, coupled to a glutathione-sepharose matrix, and purified CNA. The GST-R5G42 solid phase readily captured CNA, as opposed to a GST-R7G44 control (Figure 3C).

Modulation of calcineurin activity

We next explored the ability of R5G42 to modulate the enzymatic activity of its target protein. To this
end, we first performed an in vitro phosphatase assay using purified CNA and \textit{para}-nitrophenylphosphate (pNPP) as a substrate. As shown in Figure 4A, the addition of purified calmodulin (CaM) is required to activate CNA. The addition of recombinant purified GST-R5G42 did not result in an inhibition or an exacerbation of CaM-activated CNA phosphatase activity (not shown). However, the addition of high concentrations of GST-R5G42 activated CNA phosphatase activity in absence of CaM, to a level comparable to that observed using CaM. The addition of equal amounts of the control aptamer R7G44 did not produce a significant effect. This experiment indicates that R5G42, like CaM, binds and activates CNA phosphatase activity in vitro.

We set out to confirm this finding in human cells. Bad is a key pro-apoptotic protein whose activity is tightly regulated by its phosphorylation status, itself controlled by the balanced activity of several protein kinases and calcineurin. Therefore, the phosphatase activity of calcineurin in cells can be monitored by examining Bad phosphorylation. We transfected Hela
cells with plasmids directing the expression of Bad, CNAβ, CNB and either R5G42, R5G52 or R7G44. We observed that expression of R5G42 decreased the phosphorylation of Bad on serine 136, without affecting the phosphorylation on serine 112 (Figure 4B). To demonstrate that this effect was caused by an upregulation of calcineurin activity, we performed the same experiments in presence of FK506, a well-known inhibitor of calcineurin. The R5G42-induced dephosphorylation of Bad on serine 136 was no longer observed in presence of FK506 (Figure 4B).

**Discussion**

We have built and used a lentiviral peptide aptamer library to isolate aptamers that inhibit cell proliferation in vitro. We have determined the identity of the target proteins of one of the isolated peptide aptamers by performing yeast two-hybrid screening experiments. We have retained NS5A-TP2 and CNA as two strong target candidates. No biological information is currently available for NS5A-TP2, except that its
coding gene is transactivated by the non-structural NS5A protein from hepatitis C virus (22). In contrast, calcineurin (of which CNA is the catalytic subunit) is a well-studied protein phosphatase that plays a key role in coupling Ca^{2+} signaling to cellular responses (reviewed in (23)). The demonstration that calcineurin was the target of the immunosuppressants cyclosporin A and FK506 has sparked a considerable interest in this protein and has greatly facilitated the elucidation of its function, especially in T cell activation. Despite numerous studies, the role of calcineurin in cell proliferation remains less clear. Cyclosporin A has been shown to inhibit the proliferation of various cells, but at concentrations exceeding that required to observe an inhibition of T cell activation. FK506, although a more potent immunosuppressant than cyclosporin A, shows a weaker antiproliferative activity (reviewed in (24)). These observations suggest that the antiproliferative activity of these immunophilins may be caused by the modulation of other target protein(s). Contrary to the hypothesis that
calcineurin positively regulates cell proliferation, calcineurin has been shown to induce apoptosis through different mechanisms including the dephosphorylation of Bad, a pro-apoptotic Bcl-2 family member (25).

Here, we have shown that an antiproliferative peptide aptamer (R5G42) binds CNA and activates its phosphatase activity in vitro. Consistent with the in vitro results, the transient expression of R5G42 in human cells induces the dephosphorylation of Bad on Serine 136, which is totally reversed by FK506. The expression of R5G52, another antiproliferative peptide aptamer, does not affect Bad phosphorylation levels. Altogether, these results indicate that Bad dephosphorylation is specifically caused by the activation of CNA by R5G42, as opposed to being an indirect consequence of an antiproliferative activity. The antiproliferative effect of R5G42 could stem from a calcineurin-mediated induction of apoptosis, which would only occur upon prolonged expression of the peptide aptamer. Indeed, we have failed to show that transient expression of R5G42 caused conspicuous signs
of apoptosis or cell cycle arrest (not shown).
Importantly, we cannot rule out the possibility that
the targeting of NS5A-TP2 (or another unidentified
target protein) also contributes to the
antiproliferative effect of R5G42. NS5A-TP2 contains a
conserved HD domain, found in many phosphatases. The
use of the R5G42 peptide aptamer may help elucidate the
function of this protein, which may play a role in the
control of cell proliferation.

The structural mechanisms of the activation and the
inhibition of calcineurin by, respectively, calmodulin
and immunophilin-immunosuppressant complexes remain
poorly understood (26). Here, we describe a new CNA
ligand that activates CNA phosphatase activity through
a potentially original mechanism, since its binding
site is located between the CaM-binding domain and the
auto-inhibitory domain, but does not appear to be
circumscribed to the CaM-binding domain. Structural
studies of the CNA–R5G42 complex will be needed to
elucidate the activation mechanism, which may help
clarify the still elusive physiological activation and inhibition mechanisms of calcineurin.

This article describes the first phenotypic selection of peptide aptamers in mammalian cells. It also describes the first identification of a functional perturbation of a protein targeted by combinatorial protein molecules isolated from an antiproliferative screening. A number of arguments strongly support the choice of peptide aptamers to perform various phenotypic screening or selections, with the goal of interrogating proteomes to identify target proteins involved in the underlying regulatory networks. First, proof of concept has been obtained in yeast where peptide aptamers were selected for their ability to overcome the cell cycle arrest induced by a mating pheromone, and where target proteins were identified by yeast two-hybrid screening (16,17). Second, peptide aptamers can target many different kinds of intracellular proteins such as kinases, phosphatases, receptors, adaptor proteins, transcription factors, chaperones, etc., involved in many regulatory pathways.
(reviewed in (4) and unpublished results). Third, peptide aptamers have been shown to decorate their target proteins by binding to many different surfaces, involved in different functions (27). For this reason, peptide aptamers can induce a broader range of perturbations on protein function than other reverse genetics methods, such as gene knockout or the use of transdominant negative alleles (Abed et al. in preparation). Last, the double constraint imposed on the variable regions reduces the conformational freedom and yields typically high binding affinities for the target proteins, thereby facilitating their identification by different methods.

Our work illustrates the particularities of using combinatorial protein molecules for phenotypic screening of transdominant reagents. The main limitation lies in the tedious target identification step. When using nucleic acid molecules (cDNA fragments, antisense, shRNAs), the identity of the target proteins is immediately unveiled by sequencing the isolated library members. In contrast, selected
combinatorial protein molecules must be used as probes to determine the identity of their targets, by performing yeast two-hybrid cDNA screening \((10,16,17)\) or affinity capture experiments followed by mass spectrometry \((28)\). However, combinatorial protein molecules, and particularly peptide aptamers, present a considerable advantage above nucleic acid molecules. Whereas the latter can only inhibit the function of their target proteins \((by a dominant negative effect or by reducing expression levels)\), the former can cause more diverse perturbations on the function of their targets, including an activation as observed in this study. Therefore, the use of combinatorial protein molecules for phenotypic screening or selections allows a more extensive probing of proteomes, thus enhancing the chances to identify different target proteins whose perturbations cause a given phenotype. Another significant advantage of using peptide aptamers lies in their application for drug discovery. Once their target proteins are identified, peptide aptamers can guide the identification of small molecule mimicks that bind the
same molecular surfaces on the targets and induce the same biological effects (27). We anticipate that the use of retroviral libraries of peptide aptamers for phenotypic screening or selections will aid the unraveling of molecular regulatory networks that control major biological processes and will impact positively therapeutic research by facilitating the discovery of new targets and small molecule drugs.

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Author Contributions
The first two authors contributed equally to this work.
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Figure Legends

Figure 1. Design of the peptide aptamer library and of the antiproliferative screening

(A) Schematic representation of the pBK1 peptide aptamer library. This SIV-derived expression system directs the expression of two cistrons coding for a EGFPf transduction marker and HA-tagged peptide aptamers consisting of a 10 aminoacid variable region inserted within the active site of human thioredoxin.

(B) Workflow of the screening for antiproliferative peptide aptamers. Rat XC cells are transduced with pBK1 and labelled with CMTMR. The highest percentile of fluorescent cells is then isolated by flow cytometry, and the peptide aptamer coding sequences are amplified by PCR from genomic DNA to construct sub-libraries. The sub-libraries are used in successive iterations of this process (detailed in Experimental Procedures).
Figure 2. Antiproliferative effect of peptide aptamers

(A) Progressive enrichment of peptide aptamer sublibraries in antiproliferative peptide aptamers through screening iterations. XC or Hela cells were transduced with pBK1 or different R"n" sub-libraries obtained after n screening iterations, and were labelled with CMTMR. The mean fluorescent intensity increases with the number of screening iterations, indicating a progressive enrichment in peptide aptamers exerting an antiproliferative effect.

(B) Colony formation assays. Hela or MCF-7 cells were transfected with plasmids directing the stable expression of the Cdk inhibitor p21, a library of peptide aptamers from pBK1 (AptaLib), Human thioredoxin (HTRX), and peptide aptamers R7G44, R5G42, R5G52. The cells were cultured for two weeks and the colonies were stained with crystal violet.
Figure 3. Interaction between peptide aptamer R5G42 and calcineurin A

(A) Yeast two-hybrid mating assay. TB50α yeast were co-transformed with pSH18-34T (bearing a lacZ reporter gene) and plasmids directing the expression of LexA alone or in fusion with peptide aptamers R5G42, R7G44 or R5G52. MB210a yeast were transformed with the selected cDNA library plasmids directing the expression of CNAβ, CNAγ, and NS5ATP2 truncated proteins. To obtain negative controls, MB210a yeast were also transformed with the empty prey plasmid (pJG4-5) and with pJG4-5 directing the expression of Ras and FKBP12 prey proteins. To obtain a positive control, MB210a yeast were transformed with pJG4-5 directing the expression of RG22 peptide aptamer prey fusion protein that interacts with LexA in the context of most LexA fusion proteins.

(B) Schematic representation of the CNA clones selected through the yeast two-hybrid screening and of the truncations performed on CNAβ.
(C) Affinity capture assay. Comparable amounts of GST-R7G44 or GST-R5G42 recombinant fusion proteins were coupled to glutathione-sepharose beads. Purified calcineurin was added onto the beads and the captured molecules were revealed by a western blot experiment using an anti-calcineurin antibody.

**Figure 4. Stimulation of calcineurin activity by peptide aptamer R5G42**

(A) In vitro calcineurin phosphatase assay. Dephosphorylation of the model substrate pNPP by purified calcineurin was measured in presence of various amounts of purified calmodulin (CaM), GST-R5G42 or GST-R7G44 fusion proteins.

(B) Monitoring of BAD phosphorylation in cultured cells. Hela-Tet cells were transfected with plasmids directing the transient expression of BAD, CNAβ, CNB and peptide aptamers R5G42, R5G52 or R7G44. Transfected cells were treated or not with 500 nM FK506. The expression level of BAD and the phosphorylation of
serine 112 and 136 residues were monitored by western blot experiments using specific antibodies.

Table 1: Occurrence of antiproliferative peptide aptamers after the last screening iteration and variable region sequences
Amino acids in lower case correspond to the HTRX flanking residues.

Table 2: Results of the yeast two-hybrid screening against R5G42
The table lists the different clones selected from the brain and testes libraries and sequenced. Bold numbers correspond to strong, specific two-hybrid interaction phenotypes. Plain numbers correspond to weak, specific interaction phenotypes. Numbers in italics correspond to non-specific clones, which show two-hybrid interaction phenotypes with other peptide aptamers.
### Table 1

| Peptide Aptamer | Occurrence in 7th sub-library | Sequence of variable region       |
|-----------------|-------------------------------|-----------------------------------|
| R5G42           | 0.41                          | ...cgpSAVTFAVCALgpc...            |
| R7G11           | 0.09                          | ...cgpLHLAGRGWENgpc...            |
| R5G52           | 0.08                          | ...cgpIQSPPESPTGgpc...            |
| R7G44           | 0.014                         | ...cgpHQSTIGVAEFgpc...            |

### Table 2

|                      | Brain library | Testes library | Accession Number |
|----------------------|---------------|----------------|------------------|
| CNAβ                 | 1             |                | NP_066955        |
| CNAγ                 | 2             |                | NP_005596        |
| NS5ATP2              | 29            | 9              | NP_057147        |
| Proteasome β5 subunit| 1             |                | NP_002788        |
| Maspardin            | 3             |                | NP_057714        |
| K channel tetramer. domain | 5          |                | NP_076419        |
| Hypothetical protein | 1             |                | XP_943453.1      |
| Adaptor protein with PH and SH2 domains | 1 |                | NP_066189.1 |
| Sorting nexin 9      | 1             |                | NP_057308        |
| CDC42 (GEF9)         | 5             |                | NP_056000        |
| Promyelocytic leukemia Zn finger protein | 2          |                | NP_005997        |
| Fascin 3             | 1             |                | NP_065102.1      |
Figure 1

(a) Schematic representation of the experimental setup.

(b) Graph showing cell number against CMTMR fluorescence intensity. XC cells are transduced with CMTMR, followed by culture for 72h, sorting, and PCR on genomic DNA. The process is repeated n times, leading to sub-library construction and validation.
Figure 2

a

Overlay XC pBK1/R3/R5/R6/R7

Overlay HeLa pBK1/R3/R5/R6/R7

CMTMR fluorescence intensity

b

HeLa

MCF-7

p21 AptaLib HTRX R7G44 R5G42 R5G52
Figure 3

(a) Western blot analysis of Lex A and mutated forms (Lex A-R5G42, Lex A-R7G44, Lex A-R5G52) using anti-calcineurin and Coomassie GST-Aptamer.

(b) CNA γ/β and CNA Cαter and Cαter-Δ1, Cαter-Δ2, Cαter-CaM with different AAs.
Figure 4

(a) OD 405nm

(b) Anti-P-BAD (Ser 112)

Anti-P-BAD (Ser 136)

Anti-BAD

FK506 0.5 μM

- - + + +