Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2

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A network of interacting proteins controls the activity of cyclin-dependent kinase 2 (Cdk2) (refs 1, 2) and governs the entry of higher eukaryotic cells into S phase. Analysis of this and other genetic regulatory networks would be facilitated by intracellular reagents that recognize specific targets and inhibit specific network connections. We report here the expression of a combinatorial library of constrained 20-residue peptides displayed by the active-site loop of *Escherichia coli* thioredoxin, and the use of a two-hybrid system to select those that bind human Cdk2. These peptide aptamers were designed to mimic the recognition function of the complementarity-determining regions of immunoglobulins. The aptamers recognized different epitopes on the Cdk2 surface with equilibrium dissociation constant in the nanomolar range; those tested inhibited Cdk2 activity. Our results show that peptide aptamers bear some analogies with monoclonal antibodies, with the advantages that they are isolated together with their coding genes, that their small size should allow their structures to be solved, and that they are designated to function inside cells.

We based our approach on the fact that peptide loops that are anchored at both their amino and carboxy termini, such as those found in antibody and T-cell receptor complementarity-determining regions, are capable of specific and high-affinity molecular recognition. The active-site loop of *E. coli* thioredoxin (TrxA) can be used as a scaffold to display such conformationally constrained peptides[3], which, when expressed as fusions to the flagellin gene product, can recognize distinct combinations of shape, charge and hydrophobicity[4]. Moreover, TrxA is small, soluble, rigid and readily expressed and purified from *E. coli*.

We constructed a library that directed the synthesis in yeast of constrained peptides of random sequence, displayed in the active site of *E. coli* TrxA and fused to a modified set of protein moieties from the original interaction trap[5], a yeast two-hybrid system (Fig. 1*a*). We chose 20 residues (20-mers) for the length of the displayed peptides, which is long enough to fold into many patterns of shape and charge, but sufficiently short that many of their encoding oligonucleotides lacked stop codons and to enable the structure of the encoded proteins to be easily solved. The library contained 2.9×10^9 members, of which $>10^9$ directed the synthesis of peptides. Because of the presence of fortuitous restriction sites in some coding oligonucleotides and because some library members contained double inserts, about one fifth of the constrained peptides were longer or shorter than unit length. We introduced this interaction library into a selection strain containing a LexA-Cdk2 bait. From 6.0×10^6 transformants, we isolated 14 plasmids that expressed peptide aptamers that interacted with Cdk2 but not with control proteins (Fig. 1*b*).

We used an interaction mating assay[6] to examine the strength and specificity with which the peptide aptamers bound Cdk2 (Fig. 2*a*). As judged by the blue colour on interaction matrix plates containing X-gal, all 14 aptamers interacted with the LexA-Cdk2 bait but not with unrelated proteins such as Max or Rb, or with certain CDK family members such as Cdk4, which shares 47% sequence identity with Cdk2. However, some aptamers interacted with other CDK family members. The fact that different peptide aptamers showed distinct patterns of crossreactivity with different CDKs indicated that these aptamers recognized different epitopes conserved among different subsets of CDKs. The sequence of the variable regions is shown in Fig. 2*b*. Sequences that were not of unit length occurred at the same frequency among the Cdk2-interacting aptamers as in the library as a whole. No variable region showed notable sequence similarity to known proteins, as would be expected if the 20-mer peptides

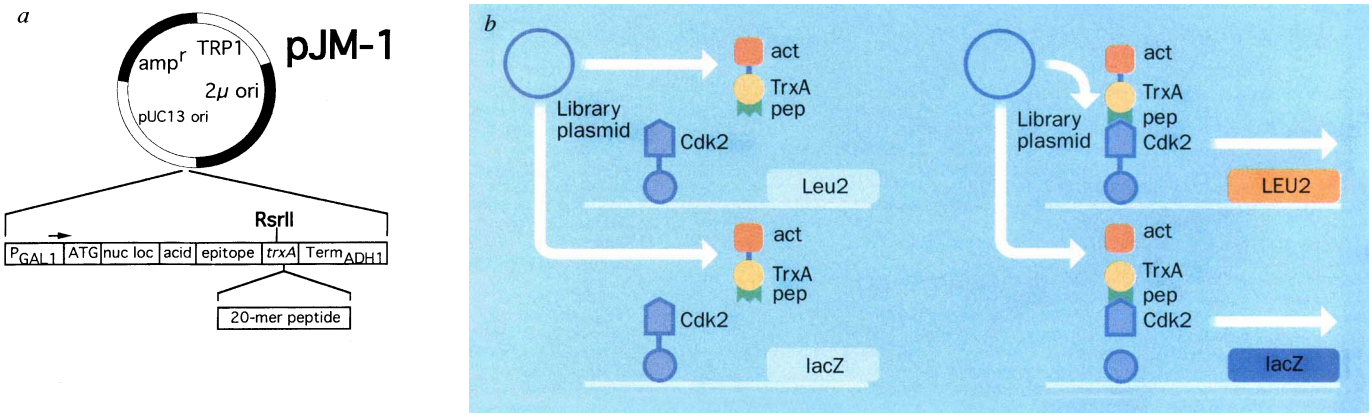
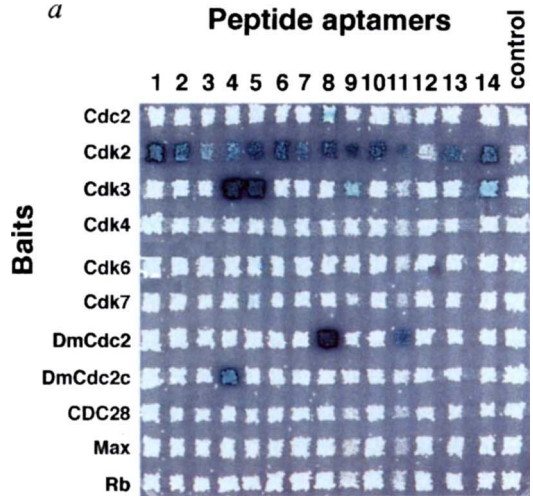


FIG. 1 *a*, Peptide aptamer expression vector. *b*, Selection of anti-Cdk2 aptamers.

METHODS. *a*, We used the oligonucleotides 5′-CAGTCAGTCAGTCAATTGAAGAAGGAGATATACATATGGGTGCTCCTCCAAAAAAGAAGAGAAAGGTAGCTGGTTCTGAGTTCCCGGGGATCACCTTGCGGATTCAGGA-3′ and 5′-ACTGACTGACTGCATATGGAATTCAGAGGCATAATCTGGCACATCATAAGGGTAGGACCCAAAACAAAGGTCTGTTCCGCCTGAGTGACGTTCAGCACGGAACTCACCGGATGACCGCCTTTCGCAACGG-3′ to generate a PCR fragment from pLexA-B112[21] encoding a fusion between the SV40 nuclear localization sequence, the B112 'acid blob', and the haemagglutinin epitope tag, flanked by *Mun*I and *Nde*I sites. We excised *trxA* from pALTRXA-781[3] with *Nde*I and *Sal*I. We introduced both fragments into the *Eco*RI–*Xho*I-cut pJG4-4[5] to create pJM-1. We made the library by annealing 1 nmol oligonucleotide 5′-GACTGACTGGTCCG(NNG/T)$_{20}$GGTCCTCAGTCAGTCAG-3′ to 1 nmol 5′-CTGACTGACTGAGGACC-3′, synthesizing the second strand with Klenow, cutting the product with *Ava*II, ligating it into *Rsr*II-cut pJM-1, and transforming *E. coli* GI724[3] to get 2.7×10^9 transformants. *b*, We transformed 100 μg of the library[22] into EGY48 containing the *LexAop-lacZ* reporter pSH18-34[23] and a LexA-Cdk2 bait[5]. We isolated interactors from 6×10^7 cells derived from 6×10^6 transformants[5]. Sixty-six colonies were galactose-dependent LEU$^+$ and blue on X-gal. Library plasmids from these colonies contained 14 different sequences.



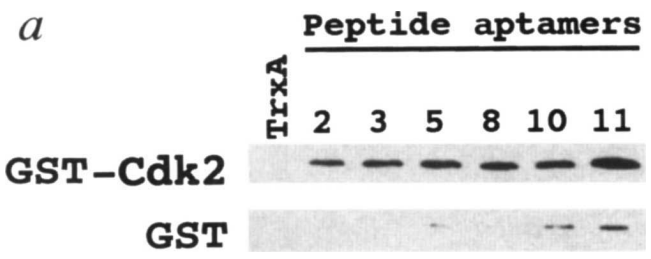
b

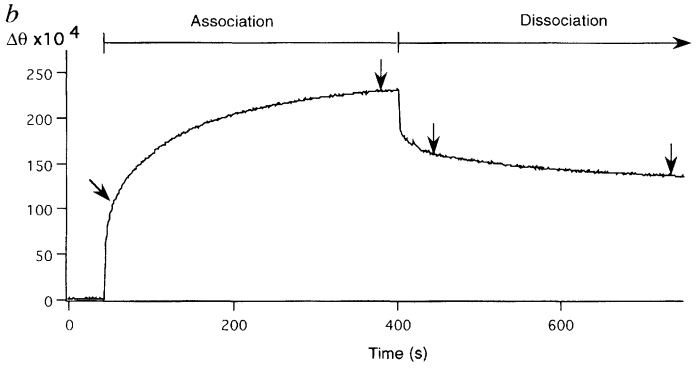
pep1	ELRHRLGRAL SEDMVRGLAW GPTSHCATVP GRSDLWRVIR FL
pep2	LVCKSYRLDW EAGALFRSLF
pep3	YRWQQGVVPS NMASCSFRCQ
pep4	SSFSLWLLMV KSIKRAAWEL GPSSAWNTSG WASLSDFY
pep5	SVRMRYGIDA FFDLGGLLHG
pep6	RVKLGYSFWA QSLLRCISVG
pep7	QLYAGCYLGV VIASSLSIRV
pep8	YSFVHHGFFN FRVSWREMLA
pep9	QQRFVFSPSW FTCAGTSDFW GPEPLFDWTR D
pep10	QVWSLWALGW RWLRRYGWNM
pep11	WRRMELDAEI RWVKPISPLE
pep12	RPLTGRWVVW GRRHEECGLT
pep13	PVCCMMYGHR TAPHSVFNVD
pep14	WSPELLRAMV AFRWLLERRP

FIG. 2 *a*, Specificity of peptide aptamer recognition. *b*, Sequence of Cdk2-interacting peptide loops.
METHODS. *a*, We transformed EGY48 with plasmids expressing the different anti-Cdk2 aptamers and one that contained a control 20-mer peptide loop, and then mated these transformants to different bait strains as described[6]. *b*, We sequenced the DNA encoding the peptide loops using a dideoxy kit (USB).

formed new recognition structures. All were charged, suggesting that some of their interactions with the Cdk2 target could be ionic. None of the peptides showed more than random similarity to any other, suggesting that we have not yet exhausted the peptide motifs capable of recognizing Cdk2.

We arbitrarily chose six aptamers for further study. The peptide aptamers interacted with a glutathione *S*-transferase(GST)–Cdk2 fusion protein *in vitro* (Fig. 3*a*), demonstrating that these interactions were independent of any bridge proteins native to yeast. On the basis of interpolation from interaction trap calibration experiments[7], the robust transcription that some aptamers directed from the pSH18-34 reporter suggested that the equilibrium dissociation constants (K_ds) of the interactions were $<10^{-6}$ M. To measure precisely the binding affinity of the aptamers to Cdk2, we used an evanescent wave instrument (BIAcore, Pharmacia). We coupled purified His_6-Ckd2 to CM-dextran chips, then flowed peptide





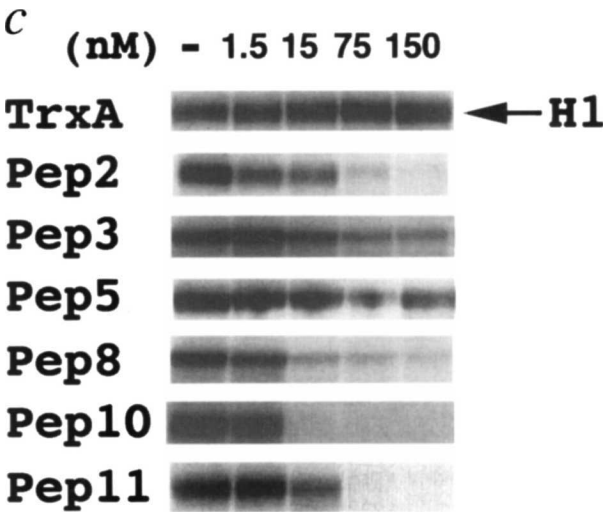


FIG. 3 *a*, *In vitro* interaction between peptide aptamers and GST–Cdk2. *b*, Representative affinity measurement. Portions of the curve used to calculate rate binding constants are shown. *c*, Inhibition of Cdk2 kinase activity *in vitro* by peptide aptamers.
METHODS. *a*, We purified GST and GST–Cdk2 as described[24]. We constructed pALHISTRX by annealing the oligonucleotides 5′-TAATGAGCTA-TAAACACCACCACCACCACCACGACGACGACGACAAAGG-3′ and 5′-TACCTTT-GTCGCTGTCGTCGTGGTGGTGGTGGTGGTGTTTATCGCTCATTA-3′ and ligating into *Nde*I-cut pALTRX-781[3]. We cloned *Ava*II fragments encoding peptide loops from the library plasmids into *Rsr*II-cut pALHISTRX. We expressed His_6-TrxA and His_6 aptamers in GI724 as described[25], purified them on a Ni^{2+}-NTA-agarose gel according to manufacturer's directions (Qiagen), and dialysed them against 10 mM HEPES, pH 7.4, 50 mM NaCl. We precipitated 1 μg His6-TrxA or His_6 aptamers with Gst or Gst-Cdk2 sepharose beads as described[24], and detected the aptamers by western blot with an anti-TrxA rabbit antiserum and ECL reagents (Amersham). *b*, We crosslinked His_6-Cdk2 in 10 mM MES, pH 6.1, 50 mM NaCl to CM5 chips with an amine-coupling kit (Pharmacia). We flowed purified aptamers in running buffer (HEPES 10 mM, pH 7.4, NaCl, 50 mM) onto the chips at 5 μl min^{-1}. We recorded association and dissociation of the His_6-Cdk2-aptamer complexes as variations in resonance angle with time. Association phase starts upon aptamer injection and dissociation phase upon running buffer injection. We fitted portions of association and dissociation curves that excluded the sudden variations in resonance angle caused by transitions between running buffer and aptamer-containing running buffer, which differed slightly in refractive index ('buffer fluxes'). *c*, We coinfected 2×10^7 Sf9 cells with recombinant bacculoviruses expressing haemagluttinin-tagged Cdk2 and His_6-cyclin E as described[9,26]. We lysed cells 40 h after infection in 500 μl 1× kinase buffer[9]. We used 5 μl 100-fold diluted extract in 30 μl reactions. We performed 20-min reactions at 25 °C by adding 2.5 μCi [γ^{32}P]ATP (3,000 Ci $mmol^{-1}$), 25 μM ATP, 100 ng histone H1 (Sigma) and indicated amounts of His_6-TrxA or His_6 aptamers. We ran samples on 15% SDS–PAGE gels and revealed them using autoradiography.

aptamers in running buffer (10 mM HEPES, pH 7.4, 50 mM NaCl) over the chip, allowed them to bind, and rinsed the chip with running buffer without aptamer (Fig. 3*b*). We determined association and dissociation rate constants by fitting the association and dissociation phases of at least two runs (typically four runs) for each aptamer to exponential functions using a nonlinear least-squares algorithm as described[8]. We calculated K_ds by dividing dissociation rate constants by association rate constants. Under these conditions all aptamers exhibited K_ds between 30 and 120 nM (Table 1).

Our results indicated that these peptide aptamers might inhibit the activity of Cdk2, perhaps by binding to a face of the molecule and by blocking its interaction with one of its partner proteins or substrates. Accordingly, we tested the ability of the aptamers to inhibit phosphorylation of histone H1 by Cdk2/cyclin E kinase (Fig. 3*c*). All tested aptamers did so; under standard conditions (10 mM HEPES, pH 7.5, ~2 mM KCl, 0–5 mM NaCl)[9], apparent half-inhibitory concentrations ranged from 1.5 to 100 nM. To rule out the possibility that a trace bacterial contaminant was responsible for the inhibition, we removed the the His_6 aptamer from the Pep2 preparation with a rabbit polyclonal anti-thioredoxin antiserum; this immunodepleted preparation no longer inhibited Cdk2 kinase activity (not shown). Half-inhibitory concentrations of aptamers were lower than the K_ds measured from evanescent wave experiments, consistent with the idea that some of the energy of each interaction is ionic and is reduced by the salt in the evanescent wave instrument running buffer.

In coprecipitation experiments[10], purified Pep2 did not compete with *in vitro* translated cyclin E for binding to *in vitro* translated Cdk2 (not shown). However, inhibition by Pep2 was reversed by addition of a 10-fold excess of histone H1 (not shown), suggesting that at least Pep2 inhibits kinase activity by competing with its H1 substrate.

Previous studies have established that libraries of unconstrained peptides contain sequences capable of recognizing targets *in vitro*[11–15] and in yeast[16]; unlike the sequences reported here, such isolated peptides often bear similarity to natural interactors. By contrast with unconstrained libraries, constrained peptide libraries are less conformationally diverse[17], but this lack of conformational diversity should lower the entropic cost if binding causes the loop to adopt a single conformation[18]; we imagine this reduction in entropic cost accounts for the fact that our Cdk2 peptide aptamers recognize their targets with higher affinity than is typically observed for unconstrained peptides[16,19,20]. This high affinity suggests that peptide aptamers could inhibit protein function *in vivo*, in the simplest case by binding to specific faces of the target molecule and disrupting its interaction with specific partners or effectors.

The ability to generate large numbers of aptamers from combinatorial libraries, taken together with the interaction trap, which offers a powerful selection for those that bind specific proteins, will aid the selection of peptide aptamers against a variety of intracellular targets. The use of such aptamers as inhibitors of protein contacts should aid the dissection of the networks of protein interactions that govern division of higher eukaryotic cells and greatly ease the analysis of gene function in those metazoan organisms for which isolation of specific missense alleles is now impractical. The analogy with antibodies suggests that peptide aptamers may also be useful in other applications in which immunological reagents are now used, such as ELISAs, immunofluorescence experiments, and sensors, and suggests routes by which their affinity might be increased—for example by increasing their valency, and using existing interaction technology to select mutants that bind more tightly.

Finally, the fact that the variable region is displayed by TrxA, a platform of known structure, should help in the solving of the loop structure by NMR and X-ray difference methods and help guide searches for peptide-mimetic compounds for use as drugs. This first generation of peptide aptamers may lead to recognition modules for intracellular nanotechnologies aimed at destroying, modifying and assembling macromolecules inside cells. □

TABLE 1 Rate and equilibrium binding constants of six aptamers

Aptamer	Dissociation rate constant $\times 10^{-6}$ (s^{-1})	Association rate constant ($M^{-1} s^{-1}$)	K_d (nM)
Pep 2	480 ± 109	7,474 ± 270	64 ± 16
Pep 3	246 ± 20	2,201 ± 160	112 ± 17
Pep 5	428 ± 16	8,263 ± 215	52 ± 3
Pep 8	120 ± 15	3,122 ± 23	38 ± 5
Pep 10	693 ± 64	6,555 ± 28	105 ± 10
Pep 11	484 ± 25	5,590 ± 168	87 ± 7

We fitted association and dissociation curves to exponential functions using a nonlinear least-squares method[8] and the data analysis program IGOR (Wavemetrics Inc., Lake Oswego, Oregon).

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Crystal structure of the kinesin motor domain reveals a structural similarity to myosin

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KINESIN is the founding member of a superfamily of microtubule-based motor proteins that perform force-generating tasks such as organelle transport and chromosome segregation[1,2]. It has two identical ~960-amino-acid chains containing an amino-terminal globular motor domain, a central α-helical region that enables dimer formation through a coiled-coil, and a carboxy-terminal tail domain that binds light chains and possibly an organelle