

# An artificial cell-cycle inhibitor isolated from a combinatorial library

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Edited by Sydney Brenner, The Molecular Sciences Institute, Berkeley, CA, and approved October 1, 1998 (received for review July 17, 1998)

**ABSTRACT** Understanding the genetic networks that operate inside cells will require the dissection of interactions among network members. Here we describe a peptide aptamer isolated from a combinatorial library that distinguishes among such interactions. This aptamer binds to cyclin-dependent kinase 2 (Cdk2) and inhibits its kinase activity. In contrast to naturally occurring inhibitors, such as p21<sup>Cip1</sup>, which inhibit the activity of Cdk2 on all its substrates, inhibition by pep8 has distinct substrate specificity. We show that the aptamer binds to Cdk2 at or near its active site and that its mode of inhibition is competitive. Expression of pep8 in human cells retards their progression through the G<sub>1</sub> phase of the cell cycle. Our results suggest that the aptamer inhibits cell-cycle progression by blocking the activity of Cdk2 on substrates needed for the G<sub>1</sub>-to-S transition. This work demonstrates the feasibility of selection of artificial proteins to perform functions not developed during evolution. The ability to select proteins that block interactions between a gene product and some partners but not others should make sophisticated genetic manipulations possible in human cells and other currently intractable systems.

Most cellular processes are governed by genetic regulatory networks whose members participate in complex patterns of interactions (1–3). Whereas genetic tools such as “knock-outs” (4, 5) illustrate the phenotypic consequences of disrupting all of the interactions in which a given protein is involved, reagents that could block, *in vivo*, specific interactions between proteins would help elucidate the functional significance of individual network connections.

Peptide aptamers are a new class of molecules that were designed to interfere with protein interactions inside cells (6). We have described peptide aptamers that consist of a random-sequence amino acid-variable region displayed in the active site of *Escherichia coli* thioredoxin (TrxA). We and others have used a yeast two-hybrid system to isolate from combinatorial libraries aptamers directed against cyclin-dependent kinase 2 (Cdk2; ref. 6), Ras (7), the HIV type 1 (HIV-1) Rev protein (B.C. and R.B., unpublished data), E2F (E. Fabbriozzi, L. Le Cam, J. Polanowska, M. Kazzorek, N. Lamb, R.B., and C. Sardet, unpublished data), and cyclins (50). These aptamers are capable of highly specific recognition; for example, some aptamers can discriminate between wild-type and oncogenic alleles of Ras (7). Peptide aptamers bind tightly to their targets with dissociation constants ( $K_d$ s) as low as 38 nM (6), presumably because of the conformational constraint and stability provided to the variable region by the TrxA scaffold (6, 8, 9). Peptide aptamers can inhibit protein function *in vitro*: several Cdk2-interacting peptide aptamers inhibit kinase activity in an

*in vitro* assay (6), raising the possibility that peptide aptamers may also inhibit protein function inside cells.

In mammalian cells, Cdk2 activity is required for transition between the G<sub>1</sub> and the S phase of the cell cycle. Two model substrates are routinely used to monitor Cdk2 function, histone H1 and the retinoblastoma protein (Rb). Although the functional significance of H1 phosphorylation is unclear, the H1 kinase activity of Cdk2 is highest during late G<sub>1</sub> (10, 11), when Cdk2 is promoting the G<sub>1</sub>-to-S transition (12–14). By contrast, at least one functional consequence of phosphorylation of Rb by cyclin-dependent kinases, including Cdk2, is clear; phosphorylation releases E2F and allows E2F to activate transcription of cell-cycle-specific genes (15).

Here we show that an anti-Cdk2 peptide aptamer, pep8, interferes specifically with the interaction between Cdk2 and one of its substrates but not another. We also demonstrate that pep8 inhibits cell-cycle progression in human cells. These results show that peptide aptamers will be useful for identifying and dissecting specific protein interactions in intracellular genetic regulatory networks.

## MATERIALS AND METHODS

**Plasmid Constructions.** All Cdk2 mutants except Cdk2-145 were created by using the QuickChange kit (Stratagene) according to the manufacturer's directions, starting from the original LexA-Cdk2 plasmid described by Gyuris *et al.* (16). The Cdk2-145 allele was amplified from CMV-HACdk2DN (a gift from Ed Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA) by using PCR and cloned into *EcoRI/XhoI* sites of pEG202 (16). For cytomegalovirus (CMV)-PepC and CMV-Pep8, peptide aptamers were amplified from their original library vectors (6) by using PCR with the primers 5'-CGGGATCCACCATGAGCGATAAAATT-3' and 5'-CGGAATTCCTATTACGCCAGGTTAGC-3' and cloned into the *BamHI/EcoRI* sites of pCDNA3.1 (Invitrogen).

**Kinase Assays.** *In vitro* kinase assays were performed as described (6), except that all of the reactions were performed with 100 mM NaCl present in the reaction mix.

**Protein and Peptide Purification.** His-pep8 and His-pepC were expressed and purified as described (6). Glutathione *S*-transferase (GST)-p21 (a gift of Anindya Dutta, Massachusetts General Hospital Cancer Center, Charlestown, MA) and GST-Rb (a gift of David Livingston, Harvard Medical School, Boston) were grown in *E. coli* BL21(DE3) (17) to OD<sub>600</sub> = 0.5, induced with 1 mM isopropyl  $\beta$ -D-thiogalactoside, harvested after a 3-hr incubation at 37°C, resuspended in 1× PBS (pH 7.2), 5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: TrxA, *Escherichia coli* thioredoxin; Cdk2, cyclin-dependent kinase 2; Rb, retinoblastoma protein; CMV, cytomegalovirus; GST, glutathione *S*-transferase.

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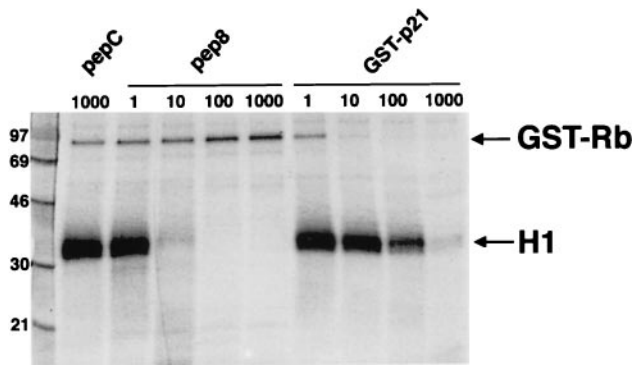


FIG. 1. Inhibition of Cdk2/cyclinE kinase activity by pep8 and p21 *in vitro*. Histone H1 and GST-Rb ( $5 \mu\text{M}$  each) were used as substrates for Cdk2/cyclin E kinase. Kinase assays were performed with extracts from sf9 cells that had been infected with baculoviruses expressing human Cdk2 and cyclin E.

fluoride,  $2 \mu\text{g/ml}$  aprotinin, and  $2 \text{ mg/ml}$  lysozyme, and incubated for 15 min on ice. The suspension was then sonicated, cleared by centrifugation, and bound to 1 ml of glutathione-Sepharose resin (Pharmacia). The column was washed with 8 ml of  $1\times$  PBS (pH 7.2), 5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride,  $2 \mu\text{g/ml}$  aprotinin, 1% Triton X-100, and 250 mM KCl, washed again with 3 ml of 50 mM Tris-HCl (pH 8.0)/25% glycerol, and eluted with 50 mM Tris-HCl (pH 8.0)/25% glycerol/10 mM glutathione. For kinetic experiments, we made a new preparation of GST-Rb immediately before the series of experiments; this preparation had a higher specific activity than the one we used for our initial characterizations. Free peptides (a gift from Hemchand Sook-Deo, Genetics Institute, Cambridge, MA) were synthesized on an Applied Biosciences 430A Peptide Synthesizer and were purified by reverse-phase HPLC on a  $\text{C}_{18}$  column by using a trifluoroacetic acid/acetonitrile-gradient elution. The purity ( $>90\%$ ) was estimated by HPLC and Coomassie blue staining on 20% Tris-Tricine SDS/PAGE gels. Each peptide was synthesized with a protein kinase A site (RRASV) at its amino

terminal followed by CGP-20-mer-GPC (for peptide variable region sequences see ref. 6).

**Two-Hybrid Mating Assay.** Interaction mating assays were performed as described (18), except that EGY42 (*MATa, ura3, his3, trp1, leu2*) was used in place of RFY206 as the bait-containing strain.

**Cell Culture and Flow Cytometry.** Saos-2 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Calcium phosphate transfections were carried out with  $2 \mu\text{g}$  of CMV-CD20 (gift of Brian Seed, Massachusetts General Hospital, Boston, MA) and  $20 \mu\text{g}$  of either CMV-PepC, CMV-Pep8, or CMV-p21 (gift of Wade Harper, Baylor University, Houston, TX). Forty-eight hours after transfection, cells were processed for flow cytometry as described (12). Transfected cells were washed in phosphate-buffered saline, stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD20 mAb (Becton Dickinson), and fixed overnight in 80% ethanol. Fixed cells were then treated with RNase and propidium iodide and analyzed for DNA content on a Coulter Epics XL flow cytometer. FITC-positive cells (3,000) were analyzed for each sample. MACCYCLE software (Phoenix Flow Systems, San Diego) was used to determine the number of cells in each phase of the cell cycle. The number of cells in the  $G_1$  phase was determined as the number of cells within the first peak of the bimodal distribution.

## RESULTS

We originally isolated pep8 in a two-hybrid hunt for peptide aptamers that interacted with human Cdk2 (6). *In vitro*, pep8 inhibited Cdk2/cyclin E-dependent phosphorylation of histone H1 with an  $\text{IC}_{50} = 5 \text{ nM}$  (6). Surprisingly, however, pep8 did not inhibit the phosphorylation of the Rb by Cdk2/cyclin E *in vitro*. (Fig. 1). When both substrates (H1 and GST-Rb) were present in the same reaction, pep8 inhibited only phosphorylation of H1, whereas p21 inhibited the phosphorylation of both H1 and GST-Rb (Fig. 1; refs. 13 and 14). A control aptamer (pepC) that does not interact with Cdk2 did not inhibit Cdk2/cyclin E kinase activity (Fig. 1). The distinct substrate specificity of pep8 has not been observed for naturally occurring Cdk inhibitors (14, 15, 19).

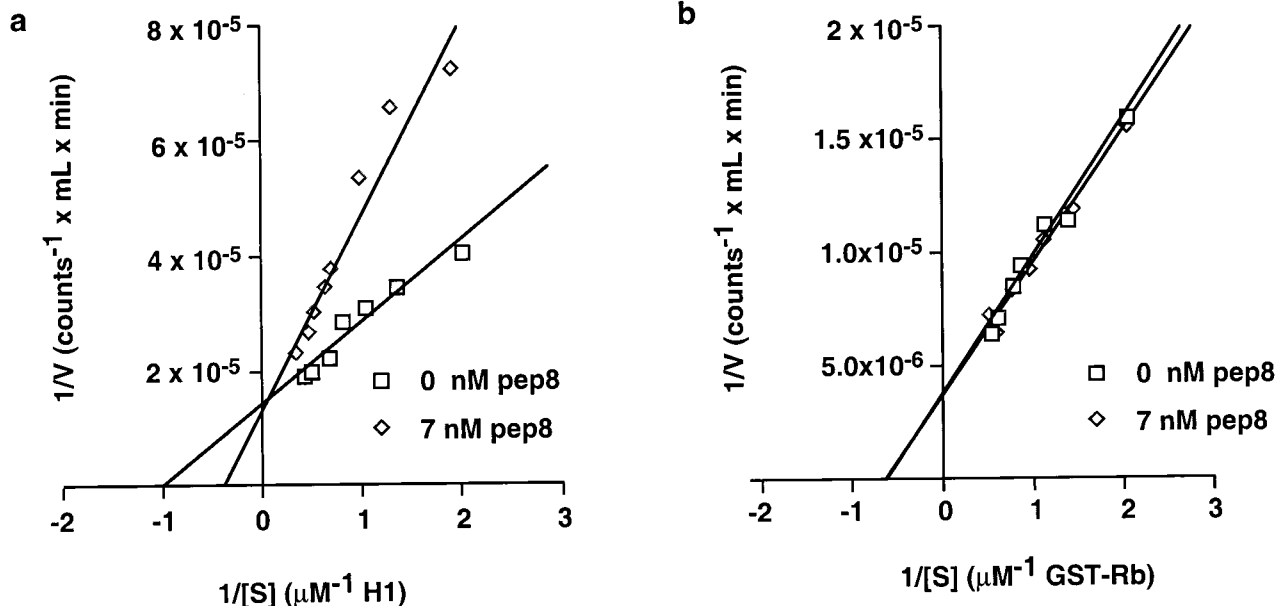
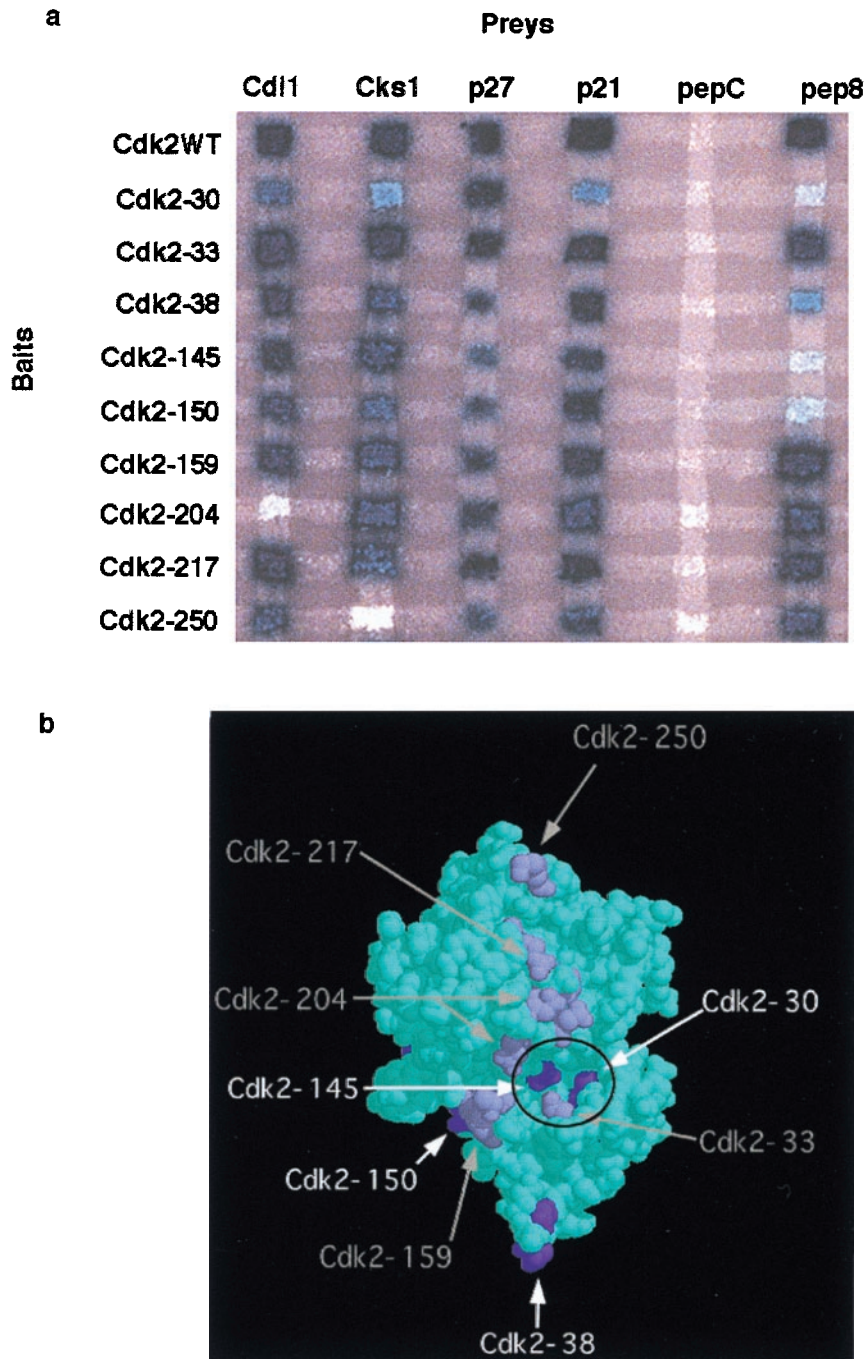


FIG. 2. Lineweaver-Burk double-reciprocal plots of substrate concentration versus kinase activity. Increasing concentrations (from 0.48 to  $1.6 \mu\text{M}$ ) of histone H1 (*a*) or GST-Rb (*b*) were added to kinase reactions in the presence of either 0 or 7 nM His-pep8. Reactions were performed at  $30^\circ\text{C}$  for 5 min before being stopped by the addition of sample buffer and electrophoresed on 12% SDS/PAGE gels. The signal from each reaction was quantitated on a PhosphorImager (Molecular Dynamics).

We speculated that pep8 may be a competitive inhibitor of Cdk2/cyclin E that blocked Cdk2's interaction with histone H1 but not with Rb. Because the Cdk2-H1 interaction cannot be detected by coimmunoprecipitation or in our two-hybrid system (data not shown), we could not directly observe a competition between pep8 and H1 by using these methods. Accordingly, we determined whether the kinetics of inhibition of H1 phosphorylation by pep8 was consistent with competitive inhibition. To do so, we measured Cdk2/cyclin E phosphorylation of increasing amounts of H1 in the presence or absence

of pep8 and graphed the results on a double-reciprocal Lineweaver-Burk plot (Fig. 2*a*). These results are consistent with competitive inhibition by pep8. In the presence of the inhibitor,  $V_{\max}$  is constant at 75,000 counts·ml<sup>-1</sup>·min<sup>-1</sup>, whereas the  $K_m$  rises from 1  $\mu$ M to approximately 3  $\mu$ M at 7 nM pep8. As before, pep8 has no effect on phosphorylation of Rb; the  $K_m$  for this reaction remains 1.5  $\mu$ M (Fig. 2*b*).

If pep8 is a competitive inhibitor of Cdk2, it should bind to the kinase at or near its active site to interfere with substrate binding. To test this hypothesis, we constructed a set of nine



**FIG. 3.** Interaction of pep8 with mutant alleles of Cdk2. (*a*) LexA fusions to the indicated Cdk2 mutants were tested for interaction with activation-domain fusions of Cdi1, Cks1, p27, p21, pepC, and pep8. Cdk2 mutant alleles are *Cdk2-30*: V30A, A31F, L32A; *Cdk2-33*: K33A; *Cdk2-38*: D38A, E40A; *Cdk2-145*: D145N; *Cdk2-150*: R150A, A151F, F152A; *Cdk2-159*: Y159A, T160D; *Cdk2-204*: P204A, D206A, E208A, D210A; *Cdk2-217*: R217A; and *Cdk2-250*: P250L. (*b*) Mutations that affect pep8 binding to Cdk2. We made this representation by using RASMOL (23) to display the structure of the Cdk2/Cyclin A complex (21) and to delete the cyclinA structure and display the indicated residues. In this figure, Cdk2's C-terminal lobe is on the bottom, with the active site facing the viewer. Residues that decrease binding to pep8 when they are mutated are shown in purple; those residues that do not affect binding are shown in light gray. The circle denotes the vicinity of the active site; only one mutation that does not affect pep8 binding, Cdk2-K33A, lies within this circle.



Cdk2 point mutants and used these in interaction-mating experiments (18) to map residues important for pep8 binding. From Western analysis with anti-LexA antiserum (20), all of the mutant LexA-Cdk2 fusions were expressed to equivalent levels (data not shown). These mutations do not affect the general structure of Cdk2; binding of other Cdk2-interacting proteins such as Cdi1, Cks1, p27, and p21 was only diminished by mutations previously shown to be required for binding of these partners (Fig. 3*a*). Four Cdk2 mutants showed reduced affinity for pep8 (Fig. 3*a*). We projected the locations of these mutations onto the structure of the Cdk2 moiety of a Cdk2/cyclin A complex (ref. 21; Fig. 3*b*). Two of the mutations that showed reduced binding to pep8, Cdk2-V30A, A31F, L32A and Cdk2-D145N lie within the Cdk2 active site; the Asp residue at position 145 actually coordinates ATP (21, 22).

These data are consistent with the idea that some of the contacts pep8 makes with Cdk2 are at or near its active site. By contrast, only one mutation that did not affect pep8 binding, Cdk2-K33A, lay in the active site, whereas the rest of the mutations that did not affect binding lay outside this region, mainly in the N-terminal lobe of the molecule. By contrast, the other two mutations that showed decreased binding to pep8 (Cdk2-D38A, E40A and Cdk2-R150A, A151F, F512A) lie along the cyclin-binding surface of Cdk2 (21, 22), relatively near the active site (below the circle in Fig. 3*b*). Three of the mutants that were deficient for binding to pep8 were normal in their interaction with p21, whereas the fourth, Cdk2-V30A, A31F, L32A, showed decreased binding to p21. These results argue that some of the contacts that Cdk2 makes with p21 are different from those that Cdk2 makes with pep8.

These results also suggest that the binding of cyclin to Cdk2 could enhance the interaction of pep8 with Cdk2. This hypothesis is supported by the observation that the  $IC_{50}$  of pep8 (5 nM), which was measured by using Cdk2/cyclin E complexes (6), is much lower than the  $K_d$  of the interaction between pep8 and Cdk2 (38 nM), which was measured by using monomeric His-tagged Cdk2 (6). Cyclin binding induces a conformational change in Cdk2 that exposes its active site (21, 22) and could facilitate pep8 binding.

The mutational and kinetic studies suggested that pep8 is a competitive inhibitor of Cdk2-dependent H1 phosphorylation that functions by binding the kinase near its active site. Given that mechanism, there are two explanations as to why pep8 inhibits Cdk2 phosphorylation of one substrate but not the other. Cdk2 may have a higher affinity for Rb than for H1, and pep8's affinity for Cdk2 may not be strong enough to compete with Rb for binding. Alternatively, pep8 may bind a region of Cdk2 needed for a rate-limiting interaction with H1 but not Rb. Two lines of evidence argue for the second idea. First, if Cdk2 had an affinity for Rb high enough to prevent pep8 from competing with Rb for interaction, then high concentrations of pep8 should overcome this effect. Fig. 1 shows that they do not; concentrations of pep8 nearly 1,000-fold higher than the  $IC_{50}$  for H1 phosphorylation do not affect Rb phosphorylation. Second, the  $K_m$  for phosphorylation (1.5  $\mu$ M) of GST-Rb by Cdk2 (Fig. 2*b*) is almost identical to the  $K_m$  for phosphorylation of H1 by Cdk2 (1  $\mu$ M). The fact that  $K_m$ s for these reactions are so similar argues that there are not likely to be large differences in Cdk2's affinity for these two substrates. Thus, these observations favor the idea that the binding sites for Rb and H1 are at least partially nonoverlapping and that substrate-specific inhibition by pep8 results from occluding sites on Cdk2 needed for a rate-limiting interaction with H1 but not Rb.

In pep8, the aptamer's variable region is sufficient for activity. *In vitro*, a 20-mer peptide containing the pep8 variable region inhibited the H1 kinase activity of Cdk2/cyclin E (Fig. 4) but had no effect on phosphorylation of GST-Rb (data not shown). Inhibition of H1 kinase activity had an  $IC_{50}$  of 7  $\mu$ M, a  $\approx$ 1,000-fold higher concentration than when the variable

## free peptides (7 $\mu$ M)

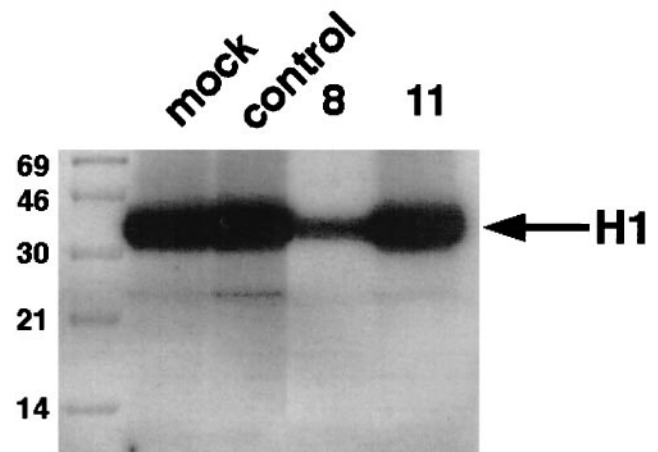


FIG. 4. Inhibition of Cdk2/cyclin E kinase activity by free peptides *in vitro*. The variable regions of two Cdk2-interacting aptamers (6), pep8 and pep11, and a control noninteracting aptamer were synthesized as free peptides and added to *in vitro* kinase reactions using histone H1 as substrate.

region is displayed on TrxA ( $IC_{50} = 5$  nM; ref. 6). These results are consistent with the idea that the key contacts pep8 makes with Cdk2 are with the pep8 variable region and that the conformational constraint imposed on the variable region by the TrxA scaffold allows for a high-affinity interaction by lowering the entropic cost of binding (6, 8, 9).

Although its substrates are not well characterized, it is clear that Cdk2 kinase activity is required for mammalian cells to move from  $G_1$  to S phase of the cell cycle (24). Inhibition of Cdk2 kinase activity in cell culture by overexpression of p21<sup>Cip1</sup> (13, 14), p27<sup>Kip1</sup> (19), or dominant-negative Cdk2 mutants (12) causes an accumulation of cells in  $G_1$ . To test the function of pep8, we used flow cytometry to observe Saos-2 cells that had been transiently transfected with a CMV-pep8 expression plasmid. Expression of pep8 in Saos-2 cells caused a significant and reproducible increase in the number of cells we observed in the  $G_1$  peak (Fig. 5). The magnitude of this retardation in the  $G_1$ -to-S transition by pep8 was less than what we and others observe for directed expression of p21 (ref. 14; Fig. 5). These results suggest that pep8 prevents Cdk2 from phosphorylating physiologically relevant substrates required for cells to pass through  $G_1$ . In light of our results *in vitro* demonstrating substrate specificity of pep8 inhibition, these results raise the possibility that pep8 inhibits cell-cycle progression *in vivo* by inhibiting the phosphorylation of a subset of Cdk2 substrates.

## DISCUSSION

We have described a peptide aptamer that inhibits the action of Cdk2 kinase on one substrate, histone H1, but not another, Rb. Our results with pep8 operationally define the existence of two classes of Cdk2 substrates that were not distinguished by the naturally occurring inhibitor, p21<sup>Cip1</sup>. Our results suggest that, *in vivo*, Cdk2 kinase acts on substrates other than Rb to effect the  $G_1$ -to-S transition, and that the aptamer blocks the action of Cdk2 on one or more of those substrates. These results illustrate the utility of selecting artificial proteins that perform functions that have not arisen during evolution and using these proteins to develop and test functional inferences. The ability to select proteins that block interactions between a gene product and some partners but not others should make possible sophisticated genetic manipulations in human cells and other currently intractable systems.

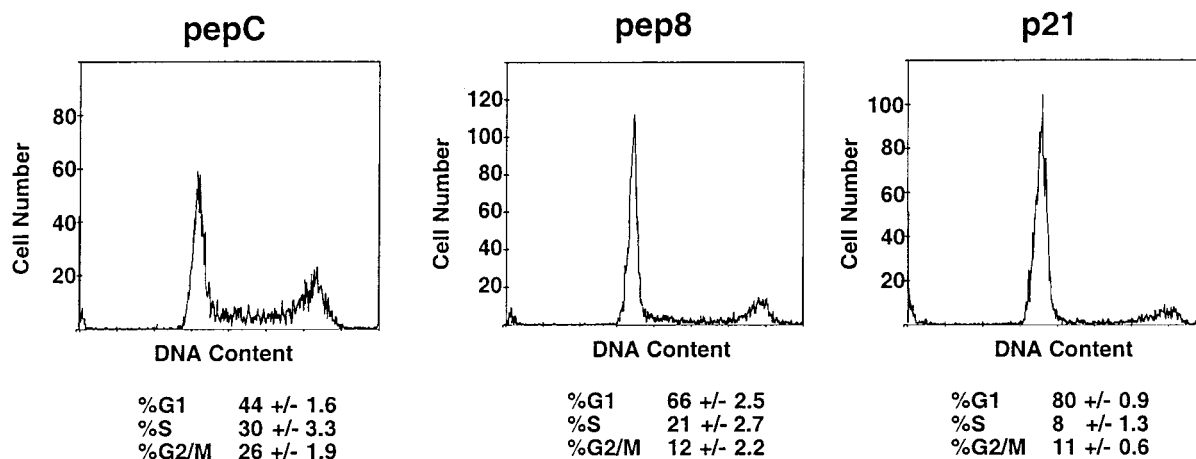


FIG. 5. Cell-cycle retardation by CMV-pep8. Saos-2 cells were transiently transfected with either 20  $\mu$ g of CMV-pepC, CMV-pep8, or CMV-p21 and 2  $\mu$ g of CMV-CD20 and analyzed for DNA content by flow cytometry as described in *Materials and Methods*.

The idea that there are substrates for Cdk2 other than Rb needed for the G<sub>1</sub>-to-S transition is consistent with earlier observations. Although Rb is likely to be a relevant substrate for the Cdk4/cyclinD complex (25–27), its role as a substrate for Cdk2/cyclin E is not clear (28). Cdk2/cyclin E kinase activity is required in Rb-deficient cells, including the Saos-2 cells used in this study (12, 29). Cdk2/cyclin E activity can also overcome cell-cycle arrest by phosphorylation-deficient Rb mutants (30, 31). Pep8 and other aptamers may help dissect the functions of different Cdk2 substrates and also may aid in their isolation.

The two-hybrid selection used to isolate peptide aptamers has the advantage that it preselects library members that are stable inside cells. Most ligand-binding peptides selected by phage-display methods (32–34) have been against extracellular targets such as surface antigens (35–37), receptors (38–40), or adhesion molecules (8, 41). Many of the peptides that bind tightly to these targets are conformationally constrained by disulfide-bridged cysteines (36, 42–45) that form under oxidizing conditions. Our results in yeast and in Saos-2 cells indicate that fusion to TrxA or other platforms [potentially including EglinC (A. Saunders and R.B., unpublished data), Tendamistat (46), and SP1 (47)] provides stability and conformational constraint to the variable region even in intracellular milieu where cysteine bridges are normally reduced.

Recent advances in two-hybrid technology should facilitate the direct selection of peptide aptamers that break specific protein–protein interactions (48, 49) and aptamers that interact with specific alleles of gene products (7). Such peptide aptamers will find use as dominant genetic agents in complex genetic backgrounds such as human cells and in other biological systems in which conventional manipulative genetics is unlikely to be developed.

We thank Hemchand Sook-Deo for synthesizing and purifying free variable regions. We thank Brian Seed, David Livingston, Anindya Dutta, Wade Harper, and Ed Harlow for plasmids used in this work. We thank Josh LaBaer and Adrian Ting for advice concerning flow cytometry, Andrew Mendelsohn for help with the figures, and Andrew Mendelsohn and other members of the Brent lab for useful advice and discussions. This work was supported by a grant to R.B. from the National Institute of General Medical Sciences.

1. Miklos, G. L. & Rubin, G. M. (1996) *Cell* **86**, 521–529.
2. Molkenin, J. D. & Olson, E. N. (1996) *Curr. Opin. Genet. Dev.* **6**, 445–453.
3. Struhl, K. (1996) *Cell* **84**, 179–182.

4. Brandon, E. P., Idzerda, R. L. & McKnight, G. S. (1995) *Curr. Biol.* **5**, 625–634.
5. Brandon, E. P., Idzerda, R. L. & McKnight, G. S. (1995) *Curr. Biol.* **5**, 758–765.
6. Colas, P., Cohen, B., Jessen, T., Grishina, I., McCoy, J. & Brent, R. (1996) *Nature (London)* **380**, 548–550.
7. Xu, C. W., Mendelsohn, A. R. & Brent, R. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 12473–12478.
8. Koivunen, E., Gay, D. A. & Ruoslahti, E. (1993) *J. Cell Biol.* **124**, 373–380.
9. McConnell, S. J., Kendall, M. L., Reilly, T. M. & Hoess, R. H. (1994) *Gene* **151**, 115–118.
10. Dulic, V., Lees, E. & Reed, S. I. (1992) *Science* **257**, 1958–1961.
11. Koff, A., Giordan, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D., Frauzza, B. R., Roberts, J. M. (1992) *Science* **257**, 1689–1694.
12. van den Heuvel, S. & Harlow, E. (1993) *Science* **262**, 2050–2054.
13. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993) *Cell* **75**, 805–816.
14. Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., *et al.* (1995) *Mol. Biol. Cell* **6**, 387–400.
15. Dynlacht, B. D., Flores, O., Lees, J. A. and Harlow, E. (1994) *Genes Dev.* **8**, 1772–1786.
16. Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. (1993) *Cell* **75**, 791–803.
17. Studier, F. W. & Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113–130.
18. Finley, R. L., Jr., & Brent, R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12980–12984.
19. Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M. & Koff, A. (1994) *Genes Dev.* **8**, 9–22.
20. Brent, R. & Ptashne, M. (1984) *Nature (London)* **312**, 612–615.
21. Jeffery, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. & Pavletich, N. P. (1995) *Nature (London)* **376**, 313–320.
22. DeBondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. & Kim, S. (1993) *Nature (London)* **363**, 595–602.
23. Sayle, R. A. & Milner-White, E. J. (1995) *Trends Biochem. Sci.* **20**, 333–379.
24. Morgan, D. O. (1995) *Nature (London)* **374**, 131–134.
25. Dowdy, S. F., Hinds, P. W., Louie, K., Reed, S. I., Arnold, A. & Weinberg, R. A. (1993) *Cell* **73**, 499–511.
26. Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J. & Livingston, D. M. (1993) *Cell* **73**, 487–497.
27. Lukas, J., Bartkova, J., Rohde, M., Strauss, M. & Bartek, J. (1995) *Mol. Cell. Biol.* **15**, 2600–2611.
28. Weinberg, R. A. (1995) *Cell* **81**, 323–330.
29. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M. & Pagano, M. (1995) *Mol. Cell. Biol.* **15**, 2612–2624.
30. Lukas, J., Herzinger, T., Hansen, K., Moroni, M. C., Resnitsky, D., Helin, K., Reed, S. I. & Bartek, J. (1997) *Genes Dev.* **11**, 1479–1492.
31. Leng, X., Connell-Crowley, L., Goodrich, D. & Harper, J. W. (1997) *Curr. Biol.* **7**, 709–712.

32. Burritt, J. B., Bond, C. W., Doss, K. W. & Jesaitis, A. J. (1996) *Anal. Biochem.* **238**, 1–13.
33. Cortese, R., Monaci, P., Luzzago, A., Santini, C., Bartoli, F., Cortese, I., Fortugno, P., Galfre, G., Nicosia, A. & Felici, F. (1996) *Curr. Opin. Biotechnol.* **7**, 616–621.
34. McGregor, D. (1996) *Mol. Biotechnol.* **6**, 155–162.
35. Heiskanen, T., Lundkvist, A., Vaheri, A. & Lankinen, H. (1997) *J. Virol.* **71**, 3879–3885.
36. Lundin, K., Samuelson, A., Jansson, M., Hinkula, J., Wahren, B. & Persson, M. A. A. (1996) *Immunology* **89**, 579–586.
37. Peletskaya, E. N., Glinsky, V. V., Glinsky, G. V. & Deutscher, S. L. (1997) *J. Mol. Biol.* **270**, 374–384.
38. Barry, M. A., Dower, W. J. & Johnston, S. A. (1996) *Nat. Med.* **2**, 299–305.
39. Lorimer, I. A., Keppler-Hafemeyer, A., Beers, R. A., Pegram, C. N., Bigner, D. D. & Pastan, I. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14815–14820.
40. Wrighton, N. C., Farrell, F. X., Chang, R., Kashyap, A. K., Barbone, F. P., Mulcahy, L. S., Johnson, D. L., Barrett, R. W., Joliffe, L. K. & Dower, W. J. (1996) *Science* **273**, 458–463.
41. Terskikh, A., Le Doussal, J., Crameri, R., Fisch, I., Mach, J. & Kajava, A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 1663–1668.
42. Koivunen, E., Gay, D. A. & Ruoslahti, E. (1993) *J. Biol. Chem.* **268**, 20205–20210.
43. McLafferty, M. A., Kent, K. A., Ladner, R. C. & Markland, W. (1993) *Gene* **128**, 29–36.
44. O’Neil, K. T., Hoess, R. H., Jackson, S. A., Ramachandran, N. S., Mousa, S. A. & DeGrado, W. F. (1992) *Proteins* **14**, 509–515.
45. Wely, J. K., Steininger, C. N., Caparon, M., Michener, M. L., Howard, S. C., Pegg, L. E., Meyer, D. M., Deciechi, P. A., Devine, C. S. & Casperson, G. F. (1996) *Proteins* **26**, 262–270.
46. McConnell, S. J. & Hoess, R. H. (1995) *J. Mol. Biol.* **250**, 460–470.
47. Cheng, X., Boyer, J. L. & Juliano, R. L. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 14120–14125.
48. Leanna, C. A. & Hannink, M. (1996) *Nucleic Acids Res.* **24**, 3341–3347.
49. Vidal, M., Brachmann, R., Fattaey, A., Harlow, E. & Boeke, J. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 10315–10320.
50. Kolonin, M. G. & Finley, R. L., Jr. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14266–14271.