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Isozyme-specific Inhibition of Protein Kinase C by RNA Aptamers*

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***In vitro* selection technology has been used to purify RNA aptamers from a random sequence pool that can bind to, and specifically inhibit, protein kinase C βII. Two of the selected RNA aptamers bind to this isozyme of protein kinase C with nanomolar affinities and inhibit activation with unprecedented selectivity; the highly related, alternatively spliced βI isozyme, which differs by 23 residues, is inhibited with 1 order of magnitude lower potency; the next most similar isozyme, α, shows no detectable inhibition. The production of isozyme-specific inhibitors of protein kinase C opens the possibilities for dissecting the roles of specific protein kinase Cs in the myriad of intracellular signaling pathways.**

Protein kinase Cs are a family of enzymes that transduce the plethora of signals promoting phospholipid hydrolysis (Nishizuka, 1992). Although the regulation of protein kinase C isozymes by second messengers (diacylglycerol, Ca^{2+}) and membrane components (phosphatidylserine) has been well characterized (Nishizuka, 1992; Newton, 1993), the mechanisms by which protein kinase Cs can separately modulate different signals from disparate pathways remain unknown. Because much of the specificity may arise from differential regulation and substrate specificities of protein kinase C isozymes, one approach to addressing this problem is the development of isozyme-specific inhibitors of protein kinase C (Wilkinson and Hallam, 1994).

Specific inhibitors of protein kinases, in general, would be an invaluable asset to dissect the roles of protein kinases in signal transduction pathways. Unfortunately, the common chemistry of kinases has precluded the discovery of selective inhibitors, especially for isozymes within a particular protein kinase family. In the case of protein kinase C, selectivity of 3-fold between conventional protein kinase Cs has been achieved with bisindolylmaleimides (Wilkinson *et al.*, 1993). However, this degree of selectivity is not sufficient for identifying contributions of specific isozymes in signal transduction pathways (Wilkinson and Hallam, 1994).

In vitro selection has been used previously to isolate nucleic acid aptamers that can bind to extracellular proteins or protein domains not normally known for their interactions with nucleic acids, such as thrombin and basic fibroblast growth factor receptor (Ellington and Conrad, 1994). In order to develop reagents that could specifically bind to and disrupt intracellular protein-protein interactions that govern signal transduction pathways, *in vitro* selection technology has been used to purify a nucleic acid aptamer from a random sequence pool that can bind to and inhibit a specific protein kinase C isozyme.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain L-α-phosphatidylserine (PS)[1] and *sn*-1,2-diheptanoylphosphatidylcholine (PC_7) were obtained from Avanti Polar Lipids, Inc. HEPES buffer and *sn*-1,2-dioleoylglycerol (DG) were purchased from Sigma. Triton X-100 (10% w/v aqueous solution) was obtained from Pierce, and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was from DuPont NEN. A protein kinase C-selective peptide (FKKSFKL-NH_2 (Chakravarthy *et al.*, 1991)) was synthesized by the Indiana University Biochemistry Biotechnology Facility. Protein kinase C isozymes were purified from the baculovirus expression system, as described (Orr *et al.*, 1992), and stored at −20 °C in 10 mM Tris buffer, pH 7.5 (4 °C), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, and 50% glycerol. Oligonucleotides were a gift of Amgen, Boulder, Co. All other chemicals were reagent grade or molecular biology grade.

In Vitro Selection—The RNA pool used in these selections contained a 120-nucleotide randomized region and was initially described in Ellington and Szostak (1992). The primers used for the amplification of selected species were AGTAATACGACTCACTATAGGGAGAATTCCGACCAGAGGCTT and CATATGTGCGTCTACATGGATCCTCA. RNA for each round was quantified by spectrophotometry, diluted into binding buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 mM DTT, 0.05 mM ATP), heated to 80 °C, and cooled to ambient temperature to facilitate equilibration of conformers. The folded RNA (0.1–11 μM, depending on the cycle of selection) was then passed through a HAWP filter (Millipore, Bedford, MA) to remove filter binding species, mixed with PKC βII (10–160 nM, depending on the cycle of selection), and incubated at ambient temperature for 1 h. Samples were passed through a second HAWP filter at 5–10 p.s.i. RNA was eluted from filters with 400 μl of 4 M guanidine thiocyanate, precipitated, and amplified by reverse transcription, polymerase chain reaction, and T7 transcription as described previously (Giver *et al.*, 1993). For the 10th and 13th rounds, samples were mixed with protein immediately after folding ([PKC] = 160 and 580 nM), applied to a 6% acrylamide gel in 0.5 × TBE, and electrophoresed at 250 V (4 °C). Radiolabeled, shifted bands were excised, and the RNA was eluted into water for 18 h at 37 °C, ethanol precipitated, and amplified. After rounds 3, 6, 9, 12, and 14 of selection, radiolabeled RNA pools were assayed for their ability to be co-retained with PKC βII on filters.

Analysis of Aptamers—Polymerase chain reaction product from cycle 14 was ligated into pCRII (Invitrogen, San Diego, CA) and transformed into *Escherichia coli* DH5a. Double-stranded DNAs from individual colonies were sequenced using the Sequenase protocol (U. S. Biochemical Corp.).

Protein Kinase C Activity Assay—Activity of protein kinase C (2 nM) was measured in the presence of a protein kinase C-selective peptide (FKKSFKL-NH_2, 50 μg ml^{-1} in assay) in 20 mM HEPES buffer (pH 7.5 at 30 °C), 1 mM DTT, 50 μM [γ-^{32}P]ATP (150 Ci mol^{-1}), 10 mM $MgCl_2$, and either 0.4 mM $CaCl_2$ and Triton X-100 (0.1%, w/v) mixed micelles containing 15 mol % PS and 5 mol % DG or 0.5 mM EGTA. Incubations were without or with tRNA (10 μM) and in the presence or absence of the indicated RNA aptamers (64 nM). Samples were incubated at 30 °C for 5 min and quenched and analyzed as described previously (Orr *et al.*, 1992). Autophosphorylation was measured as described above except that peptide substrate was omitted from assays. In one experiment, Triton X-100/lipid mixed micelles were replaced with micelles composed of PC_7 (300 μM) and DG (16 μM).

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[1] The abbreviations used are: PS, L-α-phosphatidylserine; PC_7, *sn*-1,2-diheptanoylphosphatidylcholine; DG, *sn*-1,2-dioleoylglycerol; DTT, dithiothreitol; PKC, protein kinase C.

RESULTS AND DISCUSSION

A RNA pool that contained a core of 120 bases of random sequence was used as a starting point for selections (Ellington and Szostak, 1992) with the rat βII isozyme of protein kinase C. In the first cycle of selection, 10^{14} sequences were mixed with protein kinase C at a RNA:protein ratio of 69:1. In subsequent cycles, the protein:RNA ratio was manipulated, and a nonspecific RNA competitor (tRNA) was added to increase the stringency of selection. In most cycles, protein-nucleic acid complexes were captured on modified cellulose filters; however, to avoid the accumulation of filter binding sequences that some-

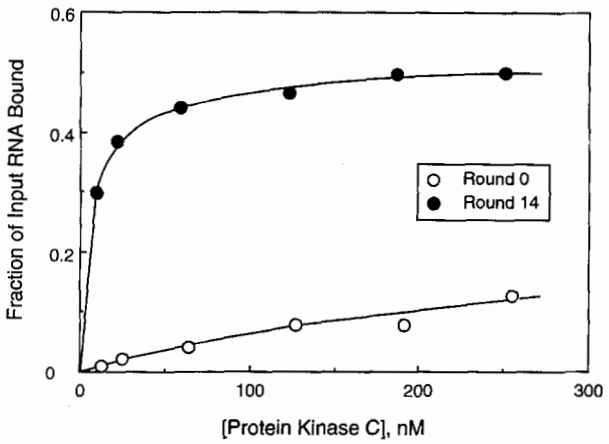

FIG. 1. **Binding curve for the cycle 14 RNA pool.** Following the 14th cycle, different concentrations of PKC βII were mixed with the selected RNA pool (final concentration, 11 nM), incubated as above, and then passed through a Schleicher and Schuell BA85 filter on a dot blot manifold. Radioactivity retained on the filters was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The fraction of counts co-retained with protein kinase C was determined by comparison with an unfiltered sample. Binding constants were determined via curve fitting of double-reciprocal and Scatchard plots of the data.

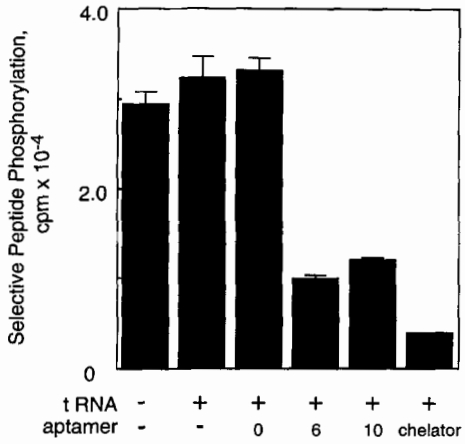

FIG. 3. **Inhibition of protein kinase C βII by aptamers PKC-6 and PKC-10.** Protein kinase C activity was measured in the presence or absence of tRNA (10 μM) and the presence or absence of 64 nM RNA aptamers 6, 10, or the unselected pool (aptamer 0). Reaction mixtures included the protein kinase C activators phosphatidylserine and diacylglycerol (15 and 5 mol %, respectively, in Triton X-100 mixed micelles) and Ca^{2+} (0.4 mM), as described under "Experimental Procedures," except for the column on the *far right*, which represents background activity in the absence of lipid and Ca^{2+} and the presence of 0.5 mM EGTA. Data represent the amount of ^{32}P incorporated into saturating amounts of the protein kinase C-selective peptide (see "Experimental Procedures") in 7 min (assay linear with time under the conditions of the assay).

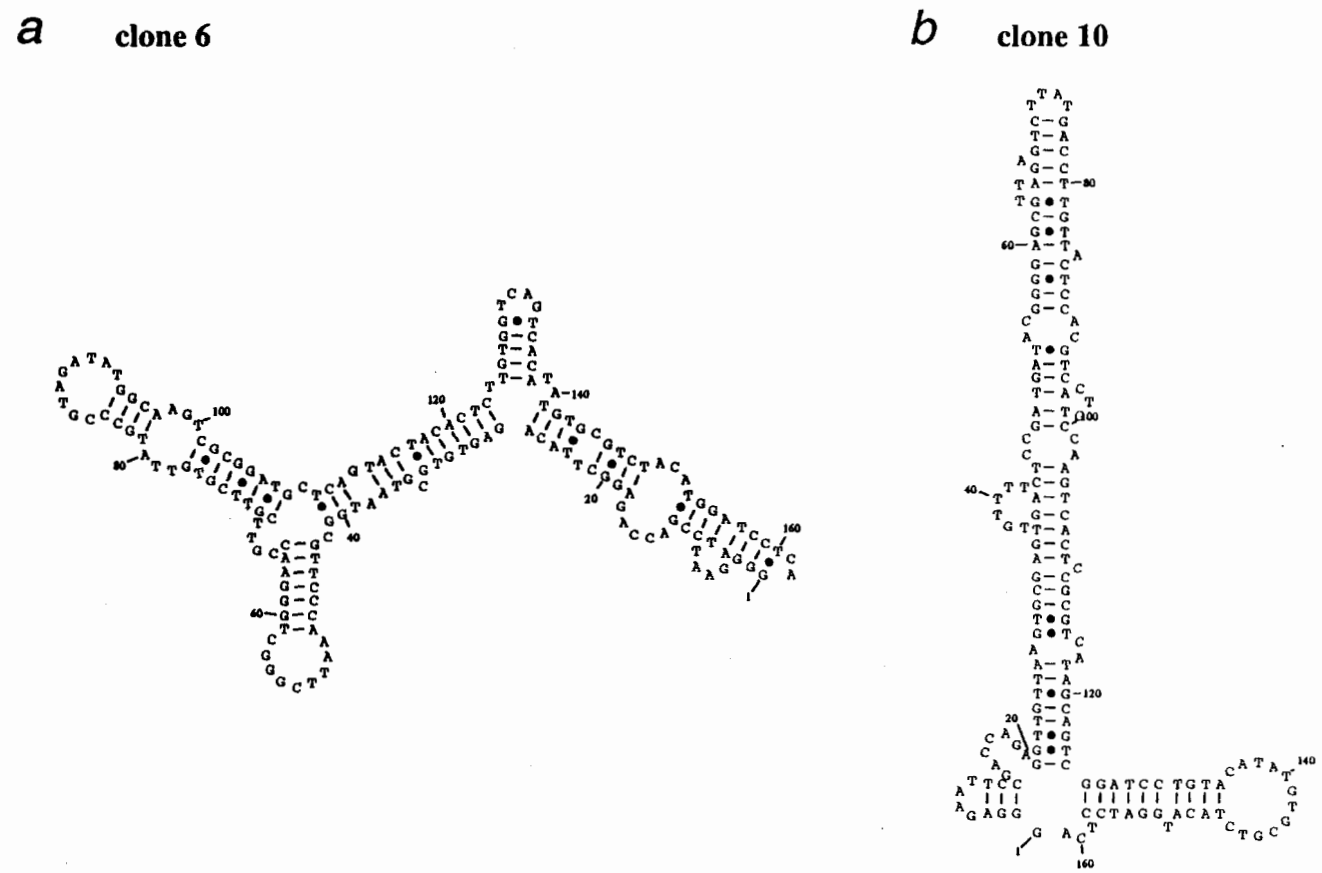

FIG. 2. **Secondary structural models for anti-PKC aptamers.** Secondary structure predictions were generated using the Mulfold program (Jaeger *et al.*, 1989) on a Macintosh Quadra 850. *a*, clone 6 (from Group I); *b*, clone 10 (from Group II).

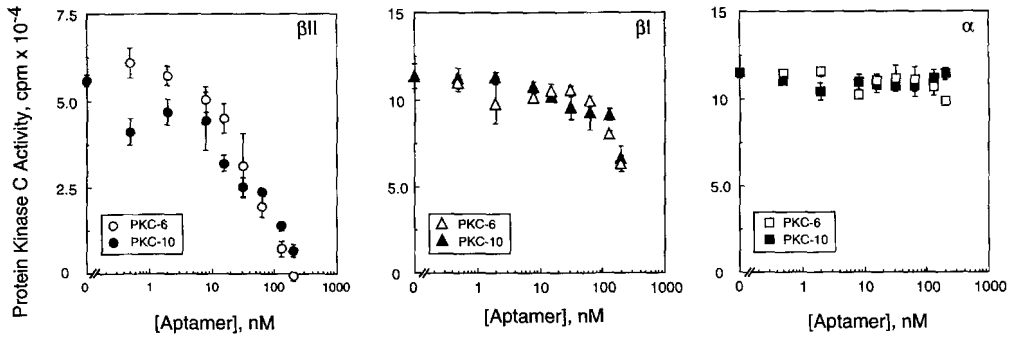

FIG. 4. **Specificity of PKC-6 and PKC-10 for protein kinase C β.** The activity of protein kinase Cs βII, βI, or α was assayed in the presence of increasing concentrations of PKC-6 or PKC-10. Activity represents the rate of Ca^{2+}/lipid-stimulated phosphorylation of the protein kinase C-selective peptide, presented as counts/min incorporated in 8 min (see Fig. 3). tRNA (10 μM) was included in the incubation medium.

times contaminate *in vitro* selections (Tuerk *et al.*, 1992), a gel shift selection was carried out in cycles 10 and 13. The RNA pool was assayed for its ability to bind protein kinase C at several points during the selection, and the affinity of the selected aptamers was found to increase steadily. After 14 cycles of selection and amplification, the aggregate equilibrium dissociation constant for protein kinase C-RNA complexes was found to be 7 nM (Fig. 1).

Individual aptamers from the round 14 population were cloned, sequenced, and aligned with one another. Two major classes of sequences predominated, and most clones were merely single or double substitution variants within these classes. Virtually no sequence or structural similarity was observed between these or other classes (Fig. 2).

Two clones from each of the two major classes and one from each of 14 unrelated minor classes were chosen for further analysis. Filter binding studies revealed that many of the isolated clones bound as well or better than the selected pool and had minimal interactions with the modified cellulose filter in the absence of the protein target (not shown). As expected, the major classes contained the tightest binding species. In addition, the individual aptamers from these classes, which differed from one another by only a few sequence substitutions, bound to protein kinase C βII with very similar affinities.

One sequence from Class I (PKC-6) and one from Class II (PKC-10) were tested for their ability to inhibit the enzymatic activity of protein kinase C βII. Fig. 3 shows that PKC-6 and PKC-10 (64 nM) strongly inhibited the activity of protein kinase C βII. In contrast, neither the unselected pool nor tRNA had any effect on the enzyme's activity. The autophosphorylation activity of βII was similarly inhibited by the selected aptamers, indicating that the aptamers were likely inhibiting activation or catalysis rather than merely interfering with substrate access. Consistent with disruption of activation (*i.e.* regulation), these aptamers did not inhibit the activity of the constitutively active, lipid-independent, carboxyl-terminal catalytic core of the enzyme (generated by mild trypsin treatment (Kishimoto *et al.*, 1983); data not shown). This result indicates that the aptamers likely bind to the regulatory domain and interfere with generation of the active conformation of the enzyme, presenting the intriguing possibility that the selected RNA molecules are acting as novel allosteric inhibitors of the enzyme. It is noteworthy that the inhibition of activity was not restricted to that mediated by PS; enzyme activated by the short-chained phosphatidylcholine, PC_7, which also exposes the enzyme's pseudosubstrate (Walker and Sando, 1988; Orr and Newton, 1994), was similarly inhibited by the RNA aptamers (data not shown).

To test the specificity of the observed inhibition, the effects of PKC-6 and PKC-10 on the activity of two related protein kinase Cs were tested (Fig. 4). Protein kinase C βI is an alternatively spliced variant of protein kinase C βII that is 96% homologous and differs only in the carboxyl-terminal 50 residues, where 50% of the residues are nonetheless still identical (Ono *et al.*, 1986). Remarkably, the IC_{50} for the βI isozyme was 1 order of magnitude higher than the IC_{50} for βII. Furthermore, neither aptamer had any significant effect on the activity of protein kinase C α, which is the next most closely related isozyme to protein kinase C βII (Dekker and Parker, 1994). These aptamers also had no effect on the activity of the more distantly related ε isozyme (data not shown). Thus, both PKC-6 and PKC-10 strongly inhibited only the isozyme they were originally selected to bind.

The unprecedented specificity of these aptamers suggests that they are binding surfaces unique to protein kinase C βII. The inability to alter the activity of just the catalytic core suggests that the binding surface is on the amino-terminal regulatory domain; furthermore, the slight inhibition of βI may indicate that the aptamer binding surface also interfaces with the carboxyl-terminal 5 kDa of the enzyme. Modeling studies suggest that these terminal residues form a separate domain near the active site of the catalytic core and near the basic pseudosubstrate (residues 19–31) of the regulatory domain (Orr and Newton, 1994).

While aptamers have previously been shown to discriminate between distantly related proteins, such as different reverse transcriptases (Tuerk *et al.*, 1992) or proteases (Bock *et al.*, 1992), this is the first demonstration that aptamers can be selected that recognize fine structure details between proteins. This work opens the possibility that the substrate recognition properties conferred on most proteins by natural selection can be mimicked by artificial evolution and that new allosteric inhibitors and activators of enzymes may be found using *in vitro* selection. As a practical example, expression of these aptamers in cells should now allow inhibition of one specific protein kinase C isozyme, thus opening the possibility for dissecting the roles of protein kinase C isozymes in signal transduction.

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