Bi-functional, Substrate Mimicking RNA Inhibits MSK1-mediated cAMP-response Element-binding Protein Phosphorylation and Reveals Magnesium Ion-dependent Conformational Changes of the Kinase*

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The design of specific inhibitors for protein kinases is an important step toward elucidation of intracellular signal transduction pathways and to guide drug discovery programs. We devised a model approach to generate specific, competitive kinase inhibitors by isolating substrate mimics containing two independent binding sites with an anti-idiotype strategy from combinatorial RNA libraries. As a general test for the ability to generate highly specific kinase inhibitors, we selected the transcription factor cAMP-response element-binding protein (CREB) that is phosphorylated on the same serine residue by the protein kinase MSK1 as well as by RSK1. The sequences and structures of these kinases are very similar, about 60% of their amino acids are identical. Nevertheless, we can demonstrate that the selected RNA inhibitors inhibit specifically CREB phosphorylation by MSK1 but do not affect CREB phosphorylation by RSK1. The inhibitors interact preferentially with the inactive form of MSK1. Furthermore, we demonstrate that RNA ligands can be conformation-specific probes, and this feature allowed us to describe magnesium ion-dependent conformational changes of MSK1 upon activation.

Combinatorial RNA libraries can be used to isolated specific ligands, called aptamers, for virtually any target molecule by a procedure probably best known as SELEX (1, 2). Inhibitory aptamers can be isolated either directly by using the purified target molecule or indirectly by a variety of strategies like decoy selection, blended SELEX, and anti-idiotype approaches (3–7). Decoys are aptamers designed to compete with small, structured RNA sequences produced by viruses for binding to the target proteins that are essential for viral replication (8). Blended SELEX has been used to increase the affinity and specificity of an inhibitory chemical compound by attaching it non-covalently to an RNA library used subsequently to select for an additional, stabilizing contact for the target molecule (9). Anti-idiotype approaches use antibodies directed against interfaces of protein-protein interactions to isolate RNA mimics of one of the interaction domains (10, 11). A direct demonstration for the ability of RNA to mimic protein domains is supplied by the work of Nyborg and co-workers (12) who compared the crystal structures of the elongation factor (EF)1-G and a ternary complex of tRNA/GDP/EF-Tu. The structures appear to be very similar and provide evidence for the model that EF-G induces translocation of the ternary tRNA complex by functional mimicry.

Protein kinases form a large family of related enzymes that play important roles in intracellular signaling. Their key regulatory roles are subject to intensive investigation and targeted by pharmaceutical companies in drug discovery programs. Protein kinases are themselves very tightly regulated; however, information on the mechanisms of regulation or the nature of the conformational changes that take place are vastly lacking for the several hundreds of protein kinases present in the human genome.

Growth factor-induced transcription of target genes by the cyclic AMP-responsive element-binding protein (CREB) is dependent on the phosphorylation of a specific serine residue (Ser-133) (13, 14). MSK1 and RSK1, two structurally highly related kinases, have been shown to phosphorylate Ser-133 of CREB (15–18). We have combined the anti-idiotype approach and the blended SELEX strategy to select a bi-functional, substrate mimicking, RNA aptamer capable of specifically inhibiting CREB phosphorylation by MSK1. A similar approach had been suggested in the past and was termed “walking SELEX” but had not been performed experimentally so far, at least according to our knowledge.

We provide evidence that the aptamers can be indicators for magnesium ion-dependent conformational changes of kinases. The bi-functional CREB mimics were also used to precipitate specifically endogenous MSK1 from cellular extracts, further highlighting the strength and specificity of the interaction with the target protein. The aptamers do not bind to RSK1, a kinase structurally related to MSK1, nor do they inhibit RSK1 activity in vitro.

** Experimental Procedures

Antibodies—Rabbits were immunized with CREBtide coupled to a mixture of keyhole limpet hemocyanin/BSA, and the antisera were immunopurified on CH-Sepharose columns containing covalently linked, recombinant GST CREB protein expressed in bacteria. Briefly,
2–4 ng of GST-CREB dialyzed previously against coupling buffer (0.1 M NaHCO₃, pH 8.0, 0.5 mM NaCl) was coupled to 1 g of activated CH₄-B-Sepharose (Amersham Biosciences), and columns were washed with phosphate-buffered saline (PBS); 15–20 ml of antiserum was incubated overnight at 4 °C, and antibodies were purified. We recovered routinely 500–700 μg of purified anti-CREB antibody from 20 ml of antiserum.

For Western blots of co-precipitated proteins a sheep anti-MSK1 antibody and a rabbit anti-CREB antibody were used (16).

Purification of Recombinant Proteins—GST-MSK1, GST-RSK1, and GST-MAPK were expressed in 293 cells, and active or inactive GST proteins were purified as described (16). His₃-MSK1 was expressed and purified from baculovirus-infected cells as described for His₉-PDK1 (19).

Immobilization of Recombinant Proteins on Magnetic Beads—7.5 ng of GST-MSK1 (Dynal) was washed with water and resuspended in 500 μl of coupling solution (160 μg of purified, recombinant protein; 1× PBS; 1 mM NaSO₄). The beads were mixed in a rotating wheel overnight at 4 °C and washed 4 times with 1 ml of PBS and 4 times with 1 ml of PBS, 0.1 mg/ml BSA and resuspended in 1 ml of storage buffer (20 mM Hepes, pH 7.9, 110 mM KOAc, 6 mM MgOAc, 0.1% β-mercaptoethanol, 0.1 mg/ml BSA).

SELEX—Selection of CREB mimics with anti-CREB antibodies immobilized on M₂₈₀-anti-mouse IgG (Dynal) was performed in selection buffer NaK₁₅₀ (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 50 mM NaCl, 5 mM MgCl₂) as described (20). Selection of additional contact CREB mimics was done in an analogous way with the following modifications of the original protocol: (a) depletion of RNA bound to the magnetic beads was done with M₂₈₀-GST-CREB beads, selection with M₂₈₀-His₃-MSK1 beads; (b) new buffer for depletion, and washing steps (20 mM Hepes, pH 7.9, 110 mM KOac, 6 mM MgOAc, 0.1% β-mercaptoethanol, 0.1 mg/ml BSA, 0.1 mM ATP); (c) the temperature reaction for cycles 1–2 was 20 °C and 4 °C for cycles 3–5. (d) the primer 3′ RSP1 for the reverse transcription and PCRs with Llhex, was substituted by 3′ Ll-st5, 5′-CCC AAG CTT CCC AAG CCG GGG GGG GTT GCA CAC CGT CTC G-3′ with labeled 5′-ATP (final RNA concentration 10 μM) and RNA was eluted and radiactively labeled γ-ATP (final RNA concentration 10 μM) and incubated for 10 min at 30 °C to test for CREB phosphorylation by MSK1.

Real Time Interaction Analysis—Binding was analyzed directly by surface plasmon resonance in a BIAcore 3000 system. Monoclonal anti-GST antibody was coupled (amine coupling, 1000 response units) to specific and control cells of CM5 chips (BIAcore AB, Stevenage, UK). GST proteins (1000 response units) were coupled to the anti-GST antibodies. GST-MSK1 was bound to the specific channel and either GST-MAPK, or nothing was added to the control channel. No significant binding was recorded on the control cells. Interactions were studied in buffer HBS-P (0.01 M Hepes, pH 7.4, 0.15 M NaCl, 0.005% surfactant P20, supplemented with 1 mM dithiothreitol) at a flow rate of 30 μl/min. The sensor chip surface was regenerated with pulses of buffer containing 0.75 M NaCl, and injections were performed in HBS-P containing 10% RNA renaturation buffer and the indicated concentration of RNA.

RESULTS

Experimental Strategy—Substrates for individual protein kinases are frequently distinguishable by some kind of consensus sequence surrounding the amino acid phosphorylated by the kinase. We therefore considered that a substrate mimic of a specific kinase substrate might be a good starting point to generate a specific kinase inhibitor (Fig. 1A). A polyclonal antibody was raised against CREBtide, an 11-amino acid peptide corresponding to the CREB sequence surrounding Ser-133 (that is phosphorylated with high efficiency by MSK1) (16). A peptide sequence can usually adopt many different conformations when taken out of the context of the native protein. Because we were interested in an aptamer mimicking the native substrate, we decided to purify the anti-peptide antibodies on columns containing native CREB protein produced in bacteria (Fig. 1B).

Selection of RNA Mimics—Three different types of structurally constrained RNA libraries were utilized to select aptamers binding specifically to the antigen-binding site of the anti-CREB antibody (Fig. 2). The variable stretches of 22 or 30 nucleotides were placed in a structural backbone formed by the fixed sequences serving as the primer-binding sites for the reverse transcriptase-PCR steps of the selection cycle. The selection progress was followed; maximum enrichment for anti-CREB binding RNA was obtained after 4 cycles of selection. For each of the three libraries 24 clones were picked randomly, inserted into DNA vectors, and sequenced manually. The sequence data showed that we had selected a virtually identical RNA motif from all libraries despite the differences in the structural constraints and the length of the variable sequences (Fig. 3, A and B). A single sequence motif was isolated from the library LL22, whereas a number of highly similar sequences were obtained for the terminal loop-sequence from L30 and SSL30, although all clones showed an identical sequence and structure for the internal loop/bulge motif. This might be attributable to the lower structural complexity of LL22, which results in a higher enrichment of the prominent binder per

In Vitro CREB Phosphorylation—Unlabeled RNA (100 pmol/μl) was incubated for 10 min at 65 °C in renaturation buffer (10 mM Tris-HCl, pH 7.5, 7.0, 100 mM KCl, 1 mM MgCl₂, 10 μl of renatured RNA diluted in renaturation buffer to the desired concentration was incubated with 1 μl of active MSK1 (diluted in KB, 0.1 μg/μl BSA) and 6.5 μl of KB for 10 min at 30 °C; GST-CREB (eqinal = 0.5 μM) and γ-[32P]ATP (eqinal = 0.1 μM) were added, and the incubation was continued for 10 min at 30 °C. The reaction was stopped by the addition of protein loading buffer, and an aliquot of the reaction was analyzed as described.
Fig. 1. Experimental strategy. A, the transcription factor CREB is phosphorylated on serine 133 by the protein kinase MSK1. A peptide, also named CREBtide, corresponding to the CREB residues 126–136 is still phosphorylated by MSK1, indicating that this sequence might be sufficient to interact with the active site of MSK1. A polyclonal antibody was raised against the CREB peptide (step 1), purified on native CREB protein columns and used to select an RNA mimic of CREBtide (step 2). The RNA mimic of CREBtide was tested for its ability to compete with CREB protein for binding to the active site of MSK1. B, a peptide sequence taken out of the context of the folded, native protein might adopt "artificial" conformations that cannot be formed by this sequence in the native protein. When using an unconstrained peptide to elicit immune responses, it is therefore possible that distinct antibody populations could be generated, each of which recognizes specific peptide conformations. Only the antibodies against peptide conformations allowed in the native protein would be expected to bind to the properly folded protein (antibody C in this example).

Fig. 2. Structures of the RNA libraries. Three different, structurally constrained RNA libraries were used to select RNA mimics of the CREB peptide. In the LL-type libraries a constant, 18-bp stem was closed by a variable sequence of 22 or 30 nucleotides of random sequence. In the SSL-type, a variable sequence of 30 nucleotides was flanked by independent, stable stem-loop structures of the tetra-loop-type. The constant sequences corresponding to the primer-binding sites required for the selection cycle are shown. The variable part of the libraries is shown schematically (LL22, LL30, and SSL30).

selection cycle. Based on the common motif, we designed a set of seven RNA molecules to confirm the selected motif and to reduce the size of the minimal aptamer element (Fig. 3C).

Characterization of CREB Mimics—Radioactively labeled RNA corresponding to the motifs shown in Fig. 3C was analyzed by immunoprecipitation with anti-CREB antibodies (Fig. 4A). In the RNA analyzed in lanes 1–4 the selected core motif was left unaltered, but the length of the closing stem was reduced from 5 to 2 bp. In contrast, the RNA analyzed in lanes 5–7 contained point mutations in the internal and the terminal loop sequence, either separately or in combination. The experiment illustrated that a minimum sequence of 28 nucleotides
closed by a 3-bp stem structure is sufficient to bind to the anti-CREB antibody (Fig. 4A, IPP, lane 3; Fig. 5B, st5 secondary structure prediction) and that both the sequence of the internal loop as well as of the terminal loop were essential for binding (Fig. 4A, IPP, lanes 5–7). The original clone selected from LL22 containing the entire 18-bp stem and st5, both carrying the identical, selected internal and terminal loop sequences were binding with apparently identical efficiency to the anti-CREB antibody (data not shown). Furthermore, the anti-CREB aptamers competed with recombinant CREB for binding to the anti-CREB antibody (Fig. 4B, lane 1). To test whether the selected aptamers were true CREB mimics, we linked recombinant CREB or MSK1 covalently to magnetic beads and probed for binding. Because the RNA had been selected with an anti-CREB antibody from the libraries, it should bind to MSK1 only if mimicking structurally the CREB domain interacting with MSK1, whereas a CREB mimic should not bind to CREB itself. The experimental data showed that this was indeed the case because there was a clear correlation between the ability to bind to the anti-CREB antibody and the capacity to interact with the MSK1 protein. CREB mimic st5 (Fig. 4C, lane 3) but not the mutant st5 SA2TL (Fig. 4C, lane 4) was precipitated by MSK1, whereas neither bound to purified CREB (Fig. 4C, lanes 1 and 2).

Selection for an Additional, Stabilizing MSK1 Contact—The CREB mimics isolated by the anti-idiotypic approach and described in the previous section were binding specifically to MSK1 as anticipated. However, the interaction was stable only at low temperature due to the rapid off-rate of the MSK1–RNA complexes, a characteristic shared with genuine enzyme-substrate complexes. Formation of a long lived, stable complex would compromise the catalytic rate of the enzyme. But since the aim of our experiments was to generate a specific enzyme inhibitor, we had to increase the half-life of aptamer-MSK1 complexes. We designed a new library named LL22-st5 which contained the selected CREB mimic as part of the fixed sequence located at the 3′ end of the library (Fig. 5A) and selected for stable binding to recombinant MSK1. The underlying rationale was that the CREB mimic located at the 3′ end should direct all library molecules preferentially to the active site of MSK1, whereas the variable part of the library might be able to provide an additional MSK1 contact. The distance between the active site contact and the additional contact to be selected was obviously determined by the relative orientation and the distance between the aptamer domains present in the new library. To allow for a greater degree of rotational freedom between the CREB mimic domain and the putative additional contact domain, we created a variant library containing a stretch of 5 C residues between the two domains (LL22-C5-st5).

The additional contact libraries were used to select aptamers binding to His6–MSK1 produced in baculovirus-infected cells and immobilized on magnetic beads. MSK1 produced in baculovirus-infected cells was a mixture of active and inactive MSK1, with about 60% being in the active form (data not shown). The first two rounds of selection were performed at low temperature to favor the binding of the CREB mimic to the active site of MSK1. In fact, after two rounds of selection a large proportion of the RNA remained bound to MSK1 after the washing step, indicating enrichment for stably binding RNA. Subsequent rounds were therefore performed at room temperature to increase the stringency of the selection, and after an initial decrease of bound RNA in the first selection cycle at room temperature, a maximal enrichment was observed after 3 cycles executed at room temperature. Individual clones were sequenced both for the LL22-st5 as well as for the LL22-C5-st5 selection and analyzed. Curiously, no defined RNA structure was apparent, rather purine residues had been selected preferentially from the variable part of both libraries (Tables I and II). On average 16/22 residues of the initially variable part were purines, with many clones containing as much as 19/22, and the sequences selected from LL22-st5 and LL22-C5-st5 were similar.

![Fig. 3. Consensus structures for CREB mimics.](45796)
Two Distinct Contact Points Are Provided by Additional Contact-CREB Mimics—We devised a series of experiments to test whether the additional contact-CREB mimics were binding MSK1 independently in two sites. For all of the experiments presented in this work, we have used the additional contact sequence corresponding to clone 4.05 (Table II) with or without a stretch of 5 C residues interwoven between the two aptamer domains (named addcon-st5 and addcon-C5-st5). We have tested several other clones (among those also clone 3.03, Table I, which was the clone with the lowest number of purine residues), but no differences were observable in any of the assays performed (Fig. 6 and Fig. 10 and data not shown).

Additional contact-CREB mimics (addcon-st5 and addcon-C5-st5) or RNA corresponding only to the additional contact (addcon) or containing the additional contact in the context of a mutant CREB mimic (addcon-Cp-SA2TL) were incubated at room temperature with immobilized MSK1, and bound RNA was analyzed. Whereas the additional contact CREB mimic bound equally well with or without the stretch of 5 C residues (Fig. 6A, lanes 5 and 8), removal (Fig. 6A, lane 7) or mutation (Fig. 6A, lane 6) of the CREB mimicking aptamer sequence reduced binding to MSK1.

This could be also demonstrated by a protein sequestering assay that is more sensitive to changes in affinity than a direct precipitation assay, because the RNA to be analyzed for binding is present in molar excess and complexes have to be stable only during the preincubation step of the experiment but not during the subsequent washing steps (21). Immobilized MSK1 was incubated with unlabeled additional contact CREB mimic, additional contact RNA, control RNA (the same 18-bp stem present in LL22 but closed by a stable tetra-loop sequence instead of the selected sequences; Fig. 5B, TL18), or no unlabeled RNA before adding a small amount of labeled additional contact CREB mimic (addcon-Cp-st5). After a short incubation beads were washed extensively, and bound and labeled RNA was analyzed. Preincubation of MSK1 with excess of unlabeled addcon-Cp-st5 prevents subsequently added labeled addcon-Cp-st5 from binding to MSK1 (Fig. 6B, lane 2); no RNA (Fig. 6B, lane 1) or control RNA (Fig. 6B, lane 4) did interfere with binding of labeled addcon-Cp-st5, whereas preincubation with addcon alone resulted in partial reduction of labeled addcon-Cp-st5 binding (Fig. 6B, lane 3). This demonstrated that even an excess of additional contact RNA was not able to compete efficiently with additional contact CREB mimics for binding to MSK1.

Accessibility of the Additional Contact on Activated MSK1 Is Magnesium Ion-dependent—Conformational changes of kinases upon activation have been reported (22, 23). We therefore decided to compare the ability of the additional contact CREB mimics to interact with active and with inactive MSK1 in the presence or absence of Mg2+ ions. GST-MSK1 was expressed in 293 cells, which were untreated or treated with TPA to induce MSK1 activation. This allowed the purification of inactive, non-phosphorylated GST-MSK1 as well as of active GST-MSK1 (termed MSK1_TPA) and permitted us to analyze the effect of MSK1 activation on aptamer binding.

GST-MSK1 or GST-MSK1_TPA was attached to GT-Sepharose in the absence of magnesium ions and washed only subsequently with buffer with or without 5 mM MgCl2 depending on whether the following binding reaction was to be performed with or without magnesium ions. The coupling was performed uniformly in the absence of magnesium ions to exclude the possibility of different coupling efficiencies of GST-MSK1 to GT-Sepharose depending on the buffer composition.

When radioactively labeled addcon-st5 was incubated with immobilized MSK1 in the absence of magnesium ions, no difference in binding was observed for active (Fig. 7, lane 10) or inactive MSK1 (Fig. 7, lane 8). Addcon alone was showing identical behavior (Fig. 7, lanes 7 and 9); furthermore, the binding efficiencies of addcon and of addcon-st5 appeared to be similar although not identical (Fig. 7, compare lanes 7 and 8 or lanes 9 and 10). However, binding to active MSK1 was dramatically reduced in the presence of 5 mM MgCl2, both for addcon-st5 (Fig. 7, compare lanes 4 and 6) as well as for addcon (Fig. 7, compare lanes 3 and 5). In addition, addcon-st5 appeared now to bind much better than addcon both to inactive (Fig. 7, com-
pare lanes 3 and 4) as well as to active MSK1 (Fig. 7, compare lanes 5 and 6). Binding to inactive MSK1 was also reduced in the presence of magnesium ions, although the effect was less dramatic at least for addcon-st5 (Fig. 7, compare lanes 4 and 8).

These observations would be compatible with a model in which activation of MSK1 induced a conformational change in MSK1-dependent on the presence of magnesium ions (see below).

Surface Plasmon Resonance Analysis of Addcon-st5 Binding to MSK1—Real time interaction analysis was performed to characterize further and to quantify binding of addcon-st5 to MSK1. Inactive or active GST-MSK1 was coupled to a sensor chip containing covalently linked anti-GST antibodies, and binding of st5, addcon, and addcon-st5 was analyzed in real time under quasi-solution conditions at high flow rates. The control channel for nonspecific binding was coated with GST-MAPK. The measurements were performed with the buffer recommended for interaction analysis, which contained the surfactant P20 and did not contain magnesium ions. Note that due to the high flow rate and the presence of the detergent, binding conditions were more stringent than those of the precipitation experiments described in the previous sections. RNA concentrations ranging from 0.15 to 50 μM were analyzed in random order, and addcon-st5 at an intermediate concentration (1.25 μM) was inserted at the beginning and at the end of the measurements and also between any three samples during the measurement as a control for the integrity of the chip-bound MSK1.

Under these conditions a robust binding response was observed for addcon-st5 binding to inactive GST-MSK1, and a maximal response was obtained at 10 μM addcon-st5 (Fig. 8A); higher RNA concentrations did not result in a response increase (data not shown). The overall apparent KD for this interaction was about 0.65 μM (as estimated from the value of response units at equilibrium versus concentration, and fitting the data to a hyperbola; Fig. 8A and data not shown). The response obtained with addcon was too weak to be quantified; the signal observed at 50 μM was at best comparable with the response of addcon-st5 at 0.31 μM, whereas no response was

**Fig. 5. Structure of the “additional contact library.”** A, schematic presentation of the additional contact library. To select for RNA capable of contacting MSK1 in two distinct regions of the protein surface the additional contact library LL22-st5 was designed. The minimal structure of the CREB mimic (st5) was incorporated covalently into the 3′ end of the library LL22 and would be expected to direct the additional contact library to the active site of MSK1. The variable region of LL22-st5 should therefore be free to target surface residues at a distance from the active site limited or conditioned by the length of the constant 18-bp stem (~6 nm). To alter the relative geometry of LL22 and the CREB mimic a stretch of 5 C-residues was incorporated at the 5′ end of st5 in a second library LL22-C5-st5. The three structural domains present in LL22-C5-st5 are highlighted and indicated by arrows. B, secondary structures of st5 and TL18. The sequences and the proposed secondary structures of the CREB mimic st5 and the control RNA TL18 are shown. The stem sequence of TL18 is identical to the stem sequence of LL22/addcon.

**Table I**

| Selected sequences of library LL22-st5 |
|---------------------------------------|
| The sequences of the variable part of individual clones obtained from round 4 of selection with the library LL22-st5 are shown. Purine-residues are highlighted in gray. |
| 3.02 | U G B B B U C G B B G B G C G A U |
| 3.03 | C U G U C G U G G U A A C G U U C |
| 3.04 | U A U G G A G G S U G S G A R A G G C G A U |
| 3.05 | U A C G U G C A G U E U G U G U G U C A |
| 3.06 | U A C G U G C G C G C G A C G A C G A U |
| 3.07 | U A C G U G C G G C G G G A C G A C G A U |
| 3.08 | U A C G U G C G G C G G G A C G A C G A U |
| 3.09 | U A C G U G C G G C G G G A C G A C G A U |
| 3.10 | U A C G U G C G G C G G G A C G A C G A U |
| 3.11 | U A C G U G C G G C G G G A C G A C G A U |
| 3.12 | U A C G U G C G G C G G G A C G A C G A U |
| 3.13 | U A C G U G C G G C G G G A C G A C G A U |
| 3.14 | U A C G U G C G G C G G G A C G A C G A U |
| 3.15 | U A C G U G C G G C G G G A C G A C G A U |

**Table II**

| Selected sequences of library LL22-C5-st5 |
|---------------------------------------|
| The sequences of the variable part of individual clones obtained from round 4 of selection with the library LL22-C5-st5 are shown. Purine residues are highlighted in gray. |
| 4.01 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.02 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.03 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.04 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.05 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.06 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.07 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.08 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.09 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.10 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.11 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.12 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.13 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.14 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.15 | A U G B B U G B B G B B B B B A A U G G U A A A |
| 4.16 | A U G B B U G B B G B B B B B A A U G G U A A A |
C5st5 (1 pmol) was added, and the reaction was left for an additional room temperature for 20 min. Radioactively labeled RNA (1 pmol) containing either two potential MSK1-binding sites (addcon-st5; addcon-C5st5) or only the additional contact (addcon) were preincubated with beads-bound MSK1 (14 pmol) at room temperature. Beads were washed extensively, and RNA was extracted from the beads and analyzed on denaturing acrylamide gels. We observed with st5 at RNA concentrations up to 50 μM (data not shown). Binding of addcon-st5 to active MSK1 was also hardly detectable under these conditions, suggesting a much lower affinity for this interaction, in agreement with the IC50 of 10 μM estimated from the in vitro kinase assays (see below). Moreover, no binding of addcon-st5 to GST-RSK1 was detectable in control experiments (data not shown). The response curves obtained with addcon-st5 were also evaluated by fitting the data using the software of the BIAcore model 3000 used for the direct precipitation assay. Radioactively labeled RNA (1 pmol) containing either two potential MSK1-binding sites (addcon-st5; addcon-C5st5) or only the additional contact (addcon; addcon-C5-SA2TL) were incubated with beads-bound MSK1 (14 pmol) at room temperature. Beads were washed extensively, and RNA was extracted from the beads and analyzed on denaturing acrylamide gels.

During the measurements described above we had noticed changes in the binding profiles when including magnesium ions in the injection buffer. Increasing concentrations of MgCl2 changes in the binding profiles when including magnesium ions, which was subsequently visualized by probing Western blots of the precipitate obtained from 200 μg of whole cell extract and strongly suggest that the magnesium ion effects reported in the co-precipitation experiments were due to effects on the conformation of MSK1 and not to effects on RNA.

**Additional Contact CREB Mimics Interact with Endogenous MSK1 Present in 293 Cell Extracts**—The experiments presented above demonstrated that bi-functional CREB mimics can interact tightly and specifically with purified MSK1 under a variety of different assay conditions. We wanted to verify that this interaction could take place also in the context of a complex protein mixture and therefore used immobilized, biotinylated aptamers to precipitate endogenous MSK1 from 293 extracts which was subsequently visualized by probing Western blots of the precipitated 293 proteins with anti-MSK1 or control antibodies. We have reported the use of this experimental strategy previously (24) and have shown that this simple, one-step purification results in a large enrichment for the protein recognized by the aptamer. As controls for the specificity of the experiment, we also tested RNA corresponding to either of the independent contacts present in addcon-st5 or completely unrelated, biotinylated RNA. As documented in Fig. 9 only addcon-st5 precipitated endogenous inactive MSK1 from 293 extracts which was subsequently visualized by probing Western blots of the precipitated 293 proteins with anti-MSK1 or control antibodies. We have reported the use of this experimental strategy previously (24) and have shown that this simple, one-step purification results in a large enrichment for the protein recognized by the aptamer. As controls for the specificity of the experiment, we also tested RNA corresponding to either of the independent contacts present in addcon-st5 or completely unrelated, biotinylated RNA. As documented in Fig. 9 only addcon-st5 precipitated endogenous inactive MSK1 from 293 extracts (Fig. 9, lane 2, MSK1), whereas neither control RNA (Fig. 9, lane 5) nor addcon nor st5 alone precipitated MSK1 (Fig. 9, lanes 3 and 4, respectively). No precipitation of the structurally related kinase RSK1 was observed in control experiments (Fig. 9, RSK1) despite the fact that RSK1 appears to be far more abundant in 293 cell extracts (Fig. 9, compare lane 8, MSK1 + RSK1). According to our estimates reported previously (16) RSK1 appears to be 20–50-fold more abundant than MSK1 in 293 cells. Precipitation of MSK1 from 293 extracts by biotinylated addcon-st5 would appear to be rather efficient based on a comparison of the intensity of the signal observed in the precipitate obtained from 200 μg of whole cell extract and the amount of MSK1 present in 10 μg of 293 extract (Fig. 9,
depending on the kinase/substrate pair. The amount of nonspecific inhibition was identical for our control RNA TL18 and for tRNA and was not correlated with binding to the kinase or its substrate (Fig. 10A, TL, and Fig. 10B, tRNA, and data not shown). Because tRNA is a natural molecule present in high concentrations in cells, the observed effects would appear to be an intrinsic feature of our in vitro assays. However, increasing amounts of addcon-st5 inhibited specifically CREB phosphorylation by MSK1 with a half-maximal inhibition (IC50) at 10 μM (Fig. 10A, addcon-st5), whereas no specific inhibition of CREB phosphorylation by RSK1 was observed (Fig. 10B, addcon-st5). The additional contact alone (addcon) showed an intermediate behavior consistent with the reduced binding affinity for MSK1 (Fig. 10A, addcon, IC50 = 25 μM). The ability to inhibit MSK1 activity in vitro correlated therefore with the binding affinity of the RNA for MSK1.

As a further demonstration for the ability of substrate mimicking RNA to inhibit MSK1 activity, we activated inactive MSK1 in vitro with MAPK and analyzed CREB phosphorylation by the in vitro activated MSK1. For this experiment aptamers and control RNA were present already during the in vitro activation reaction. When in vitro activated GST-MSK1 was incubated with GST-CREB in the presence of radioactively labeled γ-ATP phosphorylation of CREB was observed (Fig. 10C, lane 2). The activity of MSK1 was strictly dependent on prior in vitro activation by MAPK because omission of MAPK during the activation reaction did not activate MSK1 (Fig. 10C, lane 1). When control RNA was included in the activation reaction no reduction of MSK1 activity was apparent in the subsequent CREB phosphorylation reaction (Fig. 10C, lane 7). In contrast, a dramatic reduction of CREB phosphorylation was observed when addcon-st5 was preincubated with inactive MSK1 and MAPK (Fig. 10C, lane 5). Preincubation of aptamers carrying only one of the two contact points present in addcon-st5 showed a differential behavior. The CREB mimic st5 did
correlated precisely with their ability to bind to MSK1. We experimentally investigated the effect of CREB mimics on MSK1 activation, using RSK1, MAPK, and aptamers (data not shown). Also, we performed an analogous experiment with addcon-st5, which serves as a control when we performed an analogous experiment with addcon-SA2TL, and addcon-SA2TL, no contact point (TLجل), or no unlabeled RNA (no RNA) and active MAPK was added to permit phosphorylation of MSK1 by MAPK. An aliquot was removed and added to a second reaction containing CREB protein (0.5 nM), and the reaction was continued for 10 min at 30 °C. Proteins were separated on denaturing acrylamide gels and visualized with an autoradiograph, and the relative amount of CREB phosphorylated by MSK1 was determined with the help of the image quantification program of the imager and plotted against the RNA concentration.

**DISCUSSION**

**Selection of Bi-functional Substrate Mimics**—Combinatorial RNA libraries have been used extensively to isolate specific RNA ligands (aptamers) that mimic protein domains. This is done experimentally by using antibodies raised against the peptide or protein domain to be mimicked for the selection of aptamers by the SELEX procedure. Here we have used the anti-idiotypic approach to isolate aptamers mimicking a peptide substrate (derived from the transcription factor CREB) of the protein kinase MSK1. Because the half-life of aptamer-MSK1 complexes is short, when incubated with the enzyme-substrate complexes, we selected for a second, stabilizing contact by incorporating the CREB mimicking aptamer into a secondary library. Based on the results of a number of different binding assays, we can conclude that the bi-functional CREB mimic, named addcon-st5, is indeed contacting MSK1 in two distinct sites. Furthermore, the contribution of the two contacts for binding to MSK1 would appear to be synergistic rather than additive in nature because additional contact CREB mimics bind better than additional contact RNA alone, although the CREB mimic alone does not bind detectably under these conditions. This hypothesis is also supported by the real time interaction analysis performed with the Biacore.

**Substrate Mimics Indicate Conformational Changes of MSK1**—Conformational changes of kinases upon activation would be suspected to be very common but are generally difficult to demonstrate unless the crystal structures of the inactive and active forms of the kinases can be compared. The properties of the aptamers generated for this study imply that they might be valuable tools to address these types of questions. The additional contact CREB mimics turned out to be specific probes for conformational changes of MSK1 because at least one of the contact points on MSK1 appears to be poorly accessible in the active conformation but very accessible in the inactive form. This behavior indicated conformational rearrangements of MSK1 upon activation and allowed us to show that Mg$^{2+}$ ions are required for this conformational change. The results suggest that the active form of MSK1 can switch from a form that does not bind addcon-st5 to one that readily interacts with addcon-st5 in an equilibrium that can be modulated by Mg$^{2+}$ ions and addcon-st5. Thus, in the presence of Mg$^{2+}$ ions the equilibrium is displaced toward the conformation that does not bind addcon-st5 and is active, whereas addcon-st5 displaces the equilibrium toward the inactive form of MSK1. Interestingly, the inactive form of MSK1 seems to bind addcon-st5 independently of the presence of Mg$^{2+}$ ions. This fact further strengthens the suggestion that the conformational change that is being studied is indeed the one that occurs physiologically for the regulatory inactive-active switch. As ATP was not present during the pull-down assays or in the Biacore analysis, the change being observed cannot be ascribed to the function of Mg$^{2+}$ ions bound to ATP and implies a
different site for this interaction. Mg$^{2+}$ ion-dependent conformational changes in protein kinases have not been thoroughly addressed previously since ATP-Mg is a requirement for the kinase activity. This is to our knowledge the first evidence that inactive-active conformational changes in protein kinases can be modulated by divergent cations independently of ATP. In other systems, divergent cations have been shown (26) to be involved in important conformational changes, for example in the case of integrin activation. Furthermore, insight into the molecular architecture of the inactive conformation of MSK1 or other kinases could be gained by co-crystallization of this type of inhibitor with the protein kinase of interest.

**Inhibition of MSK1 Activity**—The results of the *in vitro* kinase assays performed in the presence of additional contact CREB mimics suggest that the aptamers have the potential to inhibit MSK1 specifically. This could be achieved either by interfering with MSK1 activation or activity. Although most strategies to inhibit protein kinases have targeted the active kinase, inhibition of the kinase activation is in principle equally attractive. Indeed, inhibitors of ERK1/ERK2 activation have been shown to interfere with the activation of MEK1 rather than its activity (27).

The great impact that specific inhibitors of protein kinases have on the understanding of intracellular signaling pathways is well recognized. Nevertheless, most of the small molecule inhibitors developed to date and in use in academic laboratories for research have problems of specificity since they are mostly ATP competitive inhibitors. Furthermore, all these compounds have been generated by pharmaceutical companies and are extremely expensive to develop. As protein kinases are a major target for drug discovery (representing about 30% of the major targets approached (28)), the need for alternative strategies for validating specific protein kinases as good drug targets is of high importance to the industry too.

The present *in vitro* work suggests that the development of bi-functional substrate mimic inhibitors of protein kinases might be a promising strategy to inhibit specifically a protein kinase *in vivo*. However, we anticipate that further developments would be required before this type of inhibitors will routinely work for specific inhibition of signaling pathways in cells and in organisms. In support that this may be possible, it should be acknowledged that effective strategies have already been developed for the intracellular expression of ribozymes (29, 30). Furthermore, depending on the structural elements present in the expressed RNA, nuclear or cytoplasmic localization of the aptamers could be imposed (31, 32), a feature that would allow the incorporation of an additional level of complexity into RNA-based kinase inhibitors.

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