



Short communication

Molecular aptamer beacon for myotonic dystrophy kinase-related Cdc42-binding kinase α

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ABSTRACT

A novel strategy for the development of molecular aptamer beacon for a signal transduction protein, myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) was proposed in this work. MRCK is an important downstream effector protein of Cdc42 that phosphorylates proteins involved in organizing actin structures responsible for forming stress fibres, lamellipodia or filopodia. The simple method for MAB design could potentially be applied to other aptamers for modification into a protein probe. The MRCK aptamer was modified into a MAB by adding nucleotides on the 5' end, which are complementary to the 3' end of the aptamer so as to destroy the existing structure and change it into a MB form. In the absence of MRCK, the MAB remained a hairpin structure. However, in the presence of MRCK, the equilibrium was shifted towards the formation of the MRCK-aptamer complex, resulting in the preference for the MRCK-binding conformer, where a fluorescence-quenching pair added to the 5' and 3' ends signaled any protein-dependent conformation change. The development of MABs for signal transduction proteins will have the potential to replace antibodies for diagnostic assays as well as protein studies in cellular imaging.

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1. Introduction

Myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) belongs to a family of serine/threonine kinases which are highly related to the myotonic dystrophy protein kinase (DMPK) [1,2] and Rho-associated kinases (ROKs/ROCKs/Rho-kinases) in term of protein sequences similarity and their downstream phosphorylation targets [3–5]. MRCK is a downstream effector of Cdc42 [6], an example of Rho GTPases that was reported to induce the formation of actin-rich membrane extension structures called filopodia [7,8]; and involved in the establishment of cell polarity [9]. Rho GTPase is one particular family of proteins that appears to play a pivotal role in regulating the biochemical pathways most relevant to cell migration. Cell migration is an essential event observed in wound healing, immune surveillance, and during the growth and development of multicellular organisms.

Multiple functional domains were discovered and characterized from MRCKs. All the isoforms of MRCKs, including MRCK α , MRCK β and MRCK γ , contain a highly conserved N-terminal ser-

ine/threonine kinase domain. MRCK α was found to bind more strongly to Cdc42-GTP than to Rac1-GTP and acts as an effector of Cdc42 that has the function of promoting cytoskeletal reorganization [6]. Previous studies show that MRCK α has implication in Cdc42-mediated peripheral actin formation and neurite outgrowth in HeLa and PC12 cells, respectively [10], and the mechanism in which MRCK α regulates actin cytoskeletal reorganization is via phosphorylation of a myosin binding subunit of protein phosphatase 1 (PP1) [11] and myosin light chain 2 (MLC2) [12]. MRCK α also forms a tripartite complex with LRAP3a (Leucine repeat adaptor protein) and myosin 18a, and is involved in modulating lamellar actomyosin retrograde flow that is crucial to cell protrusion and migration [13]. For this work, aptamers selected for MRCK α isoform was used for the design of molecular aptamer beacon (MAB). The development of aptamer probes for signal transduction proteins can facilitate the study of the effect of drugs on cells through perturbation of protein localization and aid in understanding biological pathways, which will be of great therapeutic importance.

To date, applications of aptamers as affinity probes in cellular imaging have been published but still in its infancy. Aptamers for protein targets such as thrombin and particularly cell-surface proteins, e.g. tenascin-C [14] and prostate-specific membrane antigen [15] were reported. Conversely, aptamers for imaging of protein localization within cells have yet been extensively exploited. Effective protein recognition mechanisms are necessary for monitoring proteins inside living cells for a better understanding of cellu-

Abbreviations: MAB, molecular aptamer beacon; MB, molecular beacon; MRCK, myotonic dystrophy kinase-related Cdc42-binding kinase; SELEX, systematic evolution of ligands by exponential enrichment.

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Table 1
Oligonucleotide abbreviations and their respective sequences.

Abbreviation	Sequence ^a
MRCK M6MAB	5'/FAM/CCTACTAATGATAAACCACTGGTGAATCGCTCAAGTCAGTAGTAGG/DABCYL/3'
MisM6MAB ^b	5'/FAM/CCTACTAATGATA AGG TCACTGGTGAATCGCTCAAGTCAGTAGTAGG/DABCYL/3'
Complementary M6	3'/TTACTATTTGGTGACCACTTAGCGAGTTCAGTCATCATCC/5'

^a Underlined bases are additional sequences added to form a hairpin structure from the aptamer.

^b Bases in bold are randomly changed bases from the original aptamer sequence.

lar behavior. Although fluorophore-labeled antibodies have been used for living cell protein detection, usually only large aggregation of proteins can provide enough signal contrast, as the unbound antibodies are also fluorescent. Tracking target proteins using fluorophore-labeled antibodies is difficult, because the fluorescence signal of the antibody remains the same irrespective of whether the antibody is bound to the target protein or not [16]. Antibodies are also not easily synthesized and may be immunogenic [17,18]. The green fluorescence protein (GFP) family can help to solve this problem by fusing GFP with host proteins. However, this requires using gene clone techniques and not all fusions are successful.

Similar to fluorophore-labeled antibodies, aptamers modified with a single fluorophore require large aggregation of proteins for sufficient signal contrast during live cell protein detection, as the unbound aptamers are also fluorescent, thus driving the direction towards aptamer probe designs adopting the molecular beacon (MB) mechanism that have the potential to overcome this problem. Li et al. claimed that while the quenching-type molecular aptamer beacon is suitable for homogeneous quantitation of target proteins in real time, it is less favourable for monitoring proteins in living specimen as the decrease in fluorescent signal upon binding makes it hard to detect minute amounts of protein and to trace a target protein in a living cell. Hence, they suggested that a protein probe needs to produce a signal increase when it recognizes target proteins [16]. This can be achieved by adopting the hairpin structure of MB for the design of MABs (Fig. 1), such as those reported for ssDNA-binding proteins, i.e. *E. coli* SSB protein [19] and lactate dehydrogenase (LDH) [20]. Hamaguchi et al. [21] constructed a thrombin-binding aptamer beacon by adding a half stem sequence to the thrombin aptamer to change the aptamer into a MB form. A fluorophore–quencher pair was used and upon protein binding, the MB form was changed back to an aptamer form (G-quartet conformer), restoring the quenched fluorescence.

In this work, the methodology as described by Hamaguchi et al. was implemented to design a MAB for a new MRCK α aptamer selected by our group using non-SELEX [22]. The G-quartet conformation of the thrombin aptamer has been well-established, leading to its use as proof-of-concept for many different beacon designs [23–27]. However, it is not known if such designs are readily applicable to other aptamers besides thrombin aptamer. By applying the reported beacon design by Hamaguchi to a new aptamer sequence with unknown conformation upon target-binding, preliminary studies of the MRCK α MAB gave insights to the feasibility of applying the hairpin structure for the development of MABs using aptamers selected for other proteins of interest.

2. Materials and methods

2.1. Materials

MRCK α kinase domain (MRCK α -KD) and its kinase-dead mutant (54.4 kDa; pI 4.954) were prepared at the Institute of Molecular and Cell Biology, Singapore as described earlier [28,29]. The molecular weight of MRCK α is around 197 kDa (pI 6.549), which is too big to be prepared by *E. coli* bacteria. The kinase domain that determines

Table 2
Binding constants of MRCK aptamer with various proteins.

Proteins	K_d /nM
Active MRCK α -KD	125 \pm 60
Inactive MRCK α -KD	nd ^a
Thrombin	nd ^a

Average of triplicates.

^a K_d is not determined as there was no observable complex peak in electropherogram.

the active or inactive form of MRCK α was prepared instead for the selection of aptamers. It was predicted that the high specificity of aptamers will have the capacity to distinguish various forms of MRCK despite a slight difference in structure. The sequences of the molecular aptamer beacons and complementary M6 aptamer used in this study were purchased from Sigma-Proligo (Singapore) as given in Table 1.

2.2. Design of molecular aptamer beacons

Secondary structures of DNA aptamers were predicted using the mFold program from the website (<http://eu.idtdna.com/Scitools/Applications/mFold/>) under the conditions (50 mM tris, 50 mM NaCl, 5 mM MgCl₂) at which the aptamers were selected for the design of molecular aptamer beacons.

2.3. Design and selectivity studies for MRCK α molecular aptamer beacons

The MAB was diluted to a concentration of 10 μ M in TE buffer, heated to 95 °C for 3 min and cooled to room temperature to ensure proper folding prior to the experiment. Fluorescence measurements were performed in the optimized aptamer selection buffer, i.e. 50 mM tris buffer pH 8.0, 50 mM NaCl and 5 mM MgCl₂. 0.5 μ M of M6MAB was incubated for 1 h with a range of MRCK α -KD concentrations (0–2.5 μ M) diluted to a final volume of 100 μ L. Further selectivity studies with the structurally similar kinase-inactive MRCK α -KD, another kinase p21-activated kinase (PAK1) and a mismatched M6MAB (MisM6MAB) were used. Fluorescence was read by BioTek Synergy™ 4 Multi-Mode Microplate Reader with Hybrid Technology™ in 96-well microtitre plates. The wavelengths for excitation and emission (Ex/Em) of carboxyfluorescein are 485 and 520 nm, respectively.

3. Results and discussion

3.1. Solution studies of MRCK aptamer and its corresponding beacon design

Many unique DNA aptamers for MRCK α protein were selected by our group using non-SELEX developed by Krylov et al. [22,30], which is a modified SELEX technique employing capillary electrophoresis (CE) as an instrumental platform. A representative MRCK α aptamer was used in this work for the design of MAB. Prior selectivity studies of the MRCK α aptamer were done using CE, with

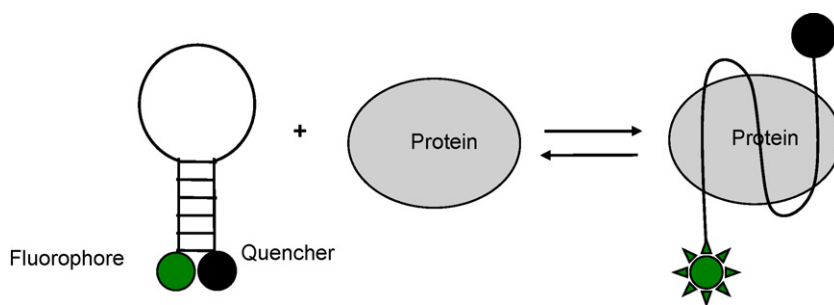


Fig. 1. Working principle of molecular aptamer beacon with hairpin structure.

K_d tabulated in Table 2, as determined by nonequilibrium capillary electrophoresis of equilibrium mixture (NECEEM) [31]. The secondary structure of the aptamer in Fig. 2a was predicted using the mFold program under the conditions (50 mM tris, 50 mM NaCl, 5 mM $MgCl_2$) at which it was selected.

We constructed a MRCK α -binding aptamer beacon by adding a half stem sequence (i.e. six additional bases CCTACT on the 5' end, which are complementary to the 3' end) to the MRCK α aptamer so as to destroy the existing structure and change it into an MB form. Prior CE-LIF studies found that the modification did not affect the binding affinity of the aptamer, with the dissociation constant determined to be within the same range. The 5' and 3' ends are then coupled to FAM fluorophore and a dark quencher DABCYL that quenches fluorescence through contact quenching. The secondary structure of the MRCK α M6MAB is as shown in Fig. 2b. To prove that MRCK α M6MAB indeed takes on a hairpin structure, a complementary M6 DNA sequence was used to verify the increase in fluorescence signal upon binding (Fig. 3). The quenching efficiency was calculated to be 93.5%.

The calculated change in Gibbs free energy (ΔG) and melting temperature (T_m) are -5.6 kcal/mole and 52.2 °C, respectively. Both ΔG and T_m were used to qualitatively assess the tendency for for-

mation of secondary structure and duplex formations under given experimental conditions. In comparison to the MRCK aptamer, where ΔG and T_m are -2.41 kcal/mole and 47.8 , respectively, the MRCK M6MAB forms the hairpin loop more favourably with the addition of a half stem sequence. In the presence of MRCK, however, the equilibrium is shifted towards the formation of the MRCK-aptamer complex, resulting in the preference for the MRCK-binding conformer, where a fluorescence-quenching pair added to the 5' and 3' ends would signal any protein-dependent conformation change.

Fig. 4 gives the binding curve of the MRCK M6MAB with varying concentrations of kinase-active MRCK α -KD. Lower concentrations of kinase-active MRCK α -KD in nanomolar range could not be measured as the background fluorescence of the M6MAB obscured any changes in fluorescence when the ratio of M6MAB to kinase-active MRCK α -KD was significantly higher. For example, when 0.5 μM of M6MAB was incubated with 5 nM of kinase-active MRCK α -KD, M6MAB binds to the protein to form an open structure that causes the fluorophore and quencher to be forced apart, giving rise to an increase in fluorescence. However, this increase in fluorescence was not sufficiently more than the background fluorescence resulting from inefficient quenching of the closed hairpin struc-

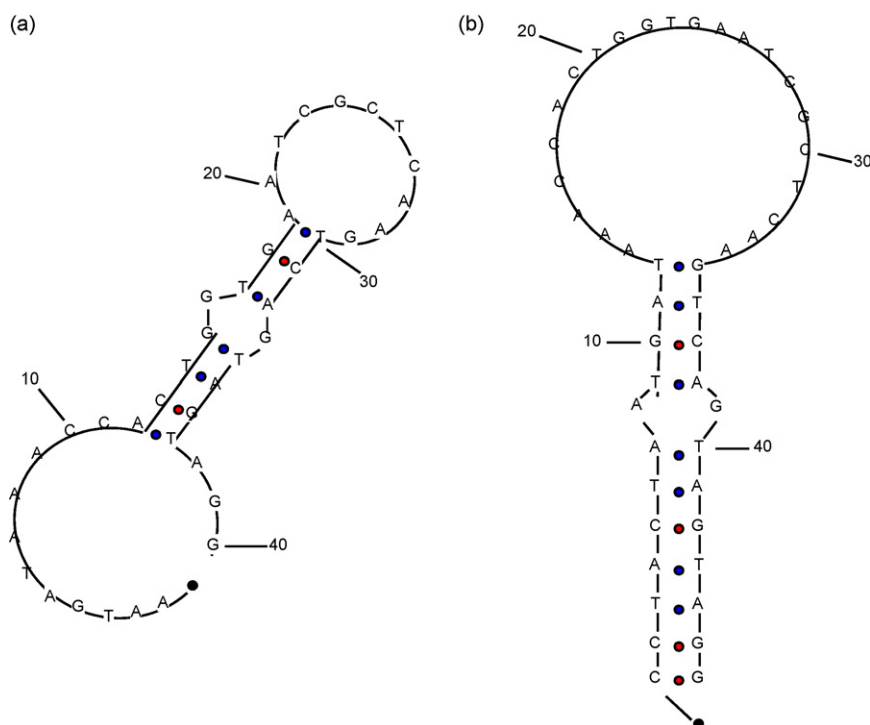


Fig. 2. Secondary structures of (a) MRCK α aptamer and (b) MRCK α molecular aptamer beacon predicted using mFold program under optimized selection conditions of 50 mM tris, 50 mM NaCl, 5 mM $MgCl_2$.

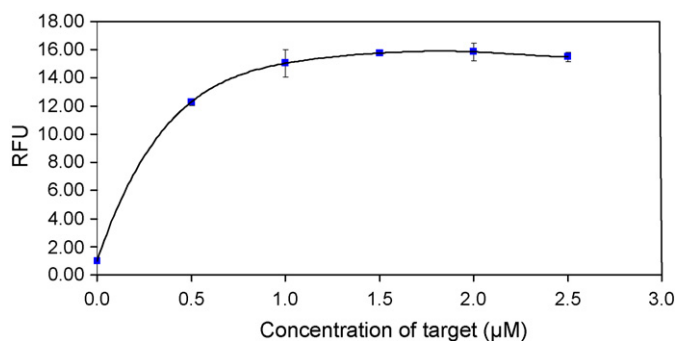


Fig. 3. Fluorescence emission of MRCK α M6MAB as a function of complementary sequence concentration, incubated under optimized selection conditions.

ture. Therefore, the detection limit of MRCK α -KD was found to be 0.5 μ M. When lower concentration such as 5 nM of M6MAB was used instead, the fluorescence signal could not be detected by the microplate reader. Hence, a concentration of 0.5 μ M of M6MAB, which was within the range of molecular beacons typically used in cellular studies as described in various publications [32–34], was used. Although other fluorophore–quencher pairs may be used to reduce the background signal, these investigations were considered beyond the scope of the present study since our main objective was to determine if the molecular beacon for MRCK α based on the hairpin structure design could exhibit fluorescence upon binding to the targeted protein *in vitro*.

A response of approximately 2.3-fold increase in fluorescence at saturating kinase-active MRCK α -KD concentrations was observed in Fig. 4. This is comparable to the reported work by Hamaguchi et al. [21] who obtained an approximate of 2.8-fold increase in fluorescence for thrombin aptamer beacon. The trend observed suggests that the hairpin structure can be used for MAB design, as the response with the concentration is quantifiable. However, the relative increase in fluorescence was significantly greater when a complementary M6 sequence was used. This suggested that upon binding of M6MAB to kinase-active MRCK α -KD, the change in distance between the quencher and fluorophore from the hairpin structure to the bound MAB form was not as pronounced as that when a complementary strand was used. Hence, more MRCK α aptamer sequences need to be tested as it is expected that different sequences would adopt different structures upon target binding, resulting in varying enhancement in fluorescence intensity. A larger magnitude change is necessary to achieve higher sensitivity for MRCK α for more practical purpose in cellular imaging as the concentration of MRCK α in the cell is in the low nanomolar range.

3.2. Selectivity studies of MRCK molecular aptamer beacon

The specificity of the MRCK aptamer was previously studied by our group using CE. However, there is a need to ensure that

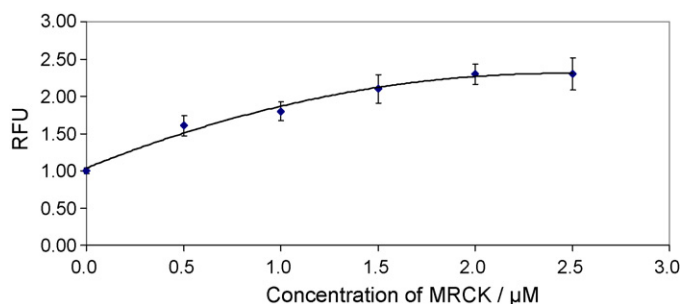


Fig. 4. Fluorescence emission of MRCK α M6MAB as a function of kinase-active MRCK α -KD concentration, incubated under optimized selection conditions.

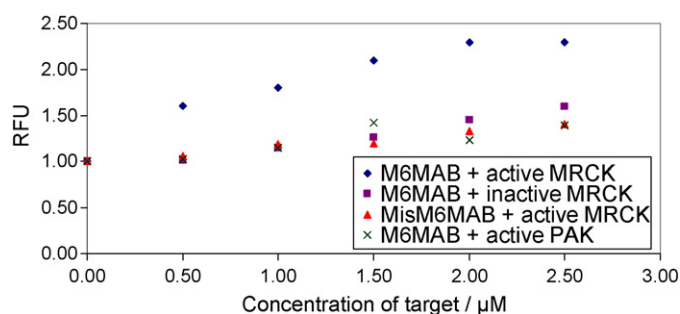


Fig. 5. Fluorescence emission of MRCK α M6MAB as a function of target concentration. Negative controls using kinase-inactive MRCK α -KD, active PAK1 and mismatched MAB (MisM6MAB) were plotted. Triplicates were done for each data point.

modification on the aptamer into a MAB does not affect its binding specificity for MRCK α -KD. Negative controls were carried out as shown in Fig. 5. When the structurally similar kinase-dead MRCK α -KD or another kinase-active PAK1 was incubated with the MRCK α M6MAB, fluorescence change was reduced. Structurally, kinase-active MRCK α -KD proteins adopt dimeric form in which the dimerization is mediated by their N termini. Following dimerization, transautophosphorylation takes place and the kinase is activated subsequently. Active MRCK α -KD acts as Cdc42 downstream effector and conveys its function in cytoskeleton reorganization upon binding to Cdc42. In contrast to active MRCK α -KD, though it is not defective in Cdc42 binding, kinase-dead mutant MRCK α -KD is unable to form dimer and thus does not undergo transautophosphorylation. The ability to differentiate the active form from the kinase-inactive MRCK α -KD indicates the selectivity of the MRCK M6MAB; unlike anti-MRCK α antibodies that have the capacity to recognize common epitope of active and inactive MRCK α but not the structural difference of active and inactive MRCK α kinase domain. Moreover, PAK1 was also used to test the cross-binding effect of M6MAB due to its similarity in kinase domain with MRCK α . Both PAK and MRCK belong to the same class of Rho-associated serine/threonine kinase, therefore there is a need to ensure that M6MAB does not interact with PAK1 kinase domain unspecifically. As for PAK1, inactive PAK1 is dimerized in head-to-tail fashion whereas active PAK1 is autophosphorylated and adopts monomeric structure.

A similar trend was observed when a mismatched MAB (MisM6MAB) was incubated with kinase-active MRCK α -KD. MisM6MAB was designed by randomly changing three consecutive bases within the aptamer sequence. This shows the high specificity of the aptamer sequence to the binding sites on the kinase-active MRCK α -KD, and also proves that addition of the half stem sequence did not alter the binding properties of the aptamer. Nonetheless, further confirmation on the conformational change based on NMR and X-ray crystallographic studies is necessary to facilitate probe design.

The MAB thus has the potential to be employed for the detection of the enzyme *in situ* in its active state but more work needs to be done for greater differentiation from its inactive form and further investigation on its inhibitory properties. The hairpin design for MAB can be easily applied for diagnostic assays that generate a change in fluorescence upon target binding. To reduce false positives as a result of nuclease degradation of nucleic acid probes, a FRET pair with fluorescent quencher can be used instead to give a decrease in fluorescence in the presence of the protein target. In addition, however, it is far more complicated in the cellular environment, which poses a greater challenge for the application of MABs in cellular imaging. Previous works on platelet-derived growth factor and thrombin by Vicens et al. [35] and Baldrich et

al. [26], respectively have established that MABs remain robust under various biological conditions in terms of pH, temperature and salt concentrations. However, these studies were done *in vitro* and thus the aptamer probe needs to be tested under the cellular environment. For existing anti-MRCK α antibodies used for cellular imaging, overnight staining is required to observe its localization due to the low expression of MRCK α within the cells. Prior CE studies found that the MRCK α aptamer was able to bind to the kinase-active MRCK α -KD within 15 min of incubation time, thus the proposed MAB design has the potential to replace traditional antibodies with greater efficiency as well as enhanced ability in the differentiation of active and inactive forms of the protein. Further work to optimize the application of aptamer probes for cellular studies is currently underway within our research group.

4. Conclusion

The hairpin structure for MAB design developed for thrombin aptamer was successfully adopted for our newly selected MRCK α aptamer, which suggests the possibility to apply such a design to other aptamers for probe development. The simplicity in design makes it suitable for diagnostic assays and it would be ideal if MABs can be used for cellular imaging, as the background fluorescence would be quenched by the quencher on the unbound MABs. Unfortunately, it is not so straightforward. More studies are required to ensure that the MABs maintain its configuration in the cellular environment. Further modifications are also necessary to improve the biostability for live cells applications as DNA/RNA MABs can be degraded by nucleases present in cells and sequestered by the nucleus. The potential for MABs to replace GFP and antibodies for cell imaging will simplify bench procedures for cellular studies as well as reduce the research costs. In addition, the successful development of MABs for signal transduction proteins for live cell applications can facilitate the study of biological pathways.

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