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From selection to caged aptamers: Identification of light-dependent ssDNA aptamers targeting cytohesin

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ABSTRACT

Caged aptamers represent valuable tools for the spatiotemporal control of protein function by light. Here we describe a general route starting with the de novo selection process targeting cytohesin-1 and aiming at the synthesis of caged aptamers without the prior knowledge of detailed structural determinants of aptamer-target binding.

The light-control of biological processes via artificial ligands is a promising and highly valuable route to analyzing the spatiotemporal function of biomolecules with high resolution.[1–4] In this regard protein inhibitors are required that can be subjected to chemical derivatisation allowing the introduction of light-sensitive moieties, such as photolabile groups or light-dependent *cis-trans* switches, thereby gaining light-control of the inhibitors. We recently started to develop aptamers whose activity can be controlled by light by incorporating photolabile protecting groups at strategic positions.[5–8] Aptamers are short, single stranded nucleic acids that fold into a well-defined 3D shape upon which a strong and specific interaction with a cognate target molecule is obtained.[9,10] Within our first studies we made use of a well-known ssDNA aptamer with 15 nucleotides that targets the human blood clotting factor α-thrombin.[11] Based on the co-crystal structure, we were able to locate those nucleotides that were either directly in contact with thrombin or those that are necessary for proper formation of the aptamer's active G-quadruplex structure.[12] Here we report on the selection, characterization, and subsequent generation of light-dependent aptamer variants without either a priori knowledge of the structure or of specific contact points of the aptamer with the target protein.

As a target molecule for the in vitro selection process we chose the cytoplasmic regulatory protein cytohesin-1.[13,14] Cytohesins are involved in signaling events during the immune response and they play a critical role during insulin receptor signaling. In this regard, the spatiotemporal control of cytohesin activity would provide a further level of precision in analyzing these events. The target decision was further nurtured by the fact that cytohesins represent a suitable class of target molecules for a SELEX experiment and therefore a functional RNA aptamer targeting cytohesin-1 has been already described.[15,16] Thus, we would be able to compare our results with those reported in the literature. The selection was performed on an automated liquid handling station, as described previously, and we employed biotinylated cytohesin-1 coupled to streptavidin-coated magnetic beads as target molecule.[17,18] After 12 selection cycles the resultant ssDNA library was cloned and sequenced. The enriched library contained merely the sequence C10 indicating a significant depletion of unbound sequences during the selection process (Fig. 1).

The aptamer C10 was analyzed for cytohesin-1 binding using filter retention analysis and a dissociation constant (K_D) of 67.8 nM regarding cytohesin-1 binding could be determined (Table 1). No binding of the aptamer to control proteins, such as lysozyme and BSA was detectable (data not shown). However, C10 interacts also with other members of the cytohesin protein family, such as ARNO (cytohesin-2) and cytohesin-3 (data not shown). In this regard the ssDNA aptamer C10 behaves similar to the RNA aptamer M69, which was described previously and also reported to interact with at least three members of the cytohesin protein family.[15,19] Indeed the aptamers display homology regarding the primary

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5′- AGCATAGAGACATCTGCTAT

5′- AGCATAGAGACATCTGCTAT N40 TAGACTCCAGACTTCAGGTA

ATCTGAGGTCTGAAGTCCAT-biotin-5′

C10 ACTCGGGAGGACTGCTTAGGATTGCGAACCCGGGTGTGGT

Figure 1. Top: sequence of the ssDNA library used for the automated SELEX procedure (primer binding sites in red and green and the 40 nucleotide random region is shown as N40) and the primer pair used for amplification. *Note*: the reverse primer bears a 5′-biotin moiety to facilitate strand displacement after PCR to obtain the non-biotinylated coding sequence. Bottom: the sequence of the aptamer C10 (only initial random region given) identified after 12 selection cycles.

Table 1
Dissociation constants of the ssDNA aptamer C10 and its truncated and mutated variants

Aptamer	Cytohesin-1[a]
C10	67.8 (±0.8)
C10.35	69.0 (±0.9)
C10.35 G7A	59.2 (±16.1)
C10.35 GG6/7AA	>500
C10.35 GGG5/6/7AAA	>500
C10.29	>500
C10.35 G14A	n.d.
C10.35 C15T	n.d.
C10.35 G14C/C15G	n.d.

[a] Values given in nM and are means of two experiments, standard deviation is given in parentheses, for all experiments the Sec7 domain of cytohesin-1 was used. (n.d. = not detectable).

sequence and both aptamers maintain cytohesin binding via the Sec7 sub-domain of cytohesin (Table 1). Having established C10 as cytohesin binding ssDNA aptamer we next began to determine the minimal binding motif of the aptamer. Based on the in silico calculated secondary structure of C10 we started to synthesize truncated variants either lacking the primer binding sites (C10.40) or variants that maintain the formation of the central stem structure bearing 35 (C10.35) and 29 (C10.29) nucleotides in length, respectively (Fig. 2).[20,21]

As shown in Table 1 the truncated variant C10.35 still interacts with cytohesin (K_D = 69.0 nM) without loss of affinity whereas further truncation of the central stem (C10.29) results in a strong decrease of affinity (K_D > 500 nM) (Table 1).

After having identified the 35 nucleotides minimal binding motif we next investigated whether C10 competes with the RNA apt-

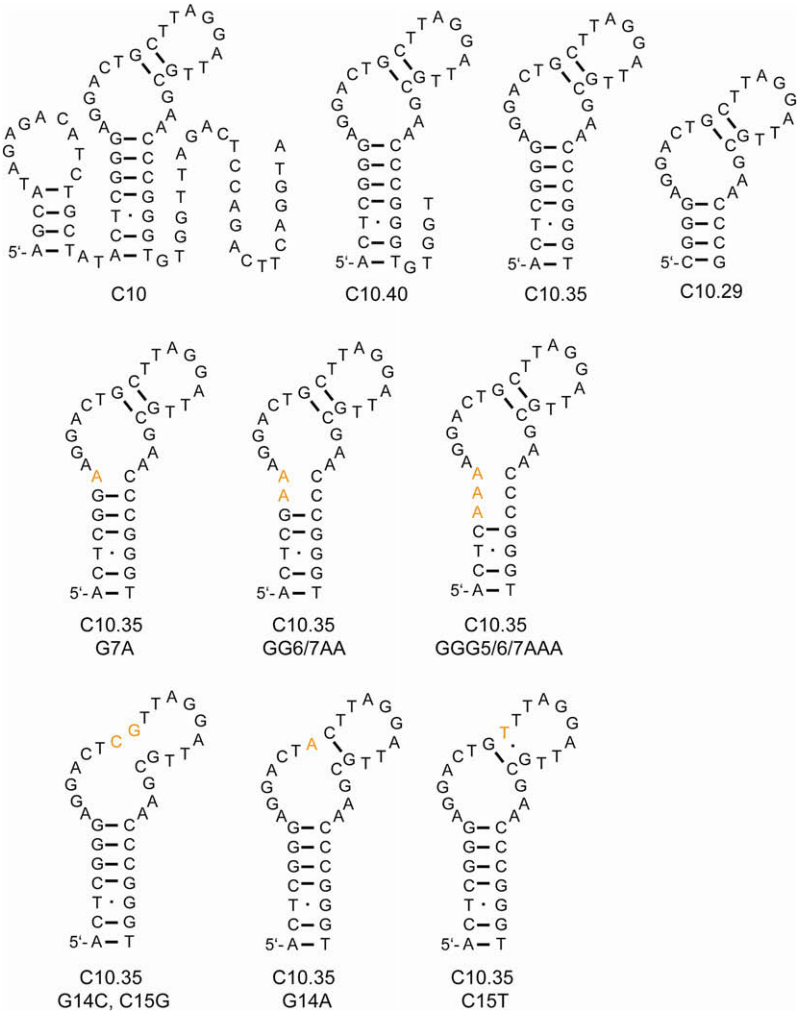

Figure 2. Putative in silico calculated secondary structures of the C10 aptamer and its truncated and mutated (highlighted in orange) variants.

amer M69 for cytohesin-1 binding. As shown in Figure 3A the aptamers C10.35 and M69 compete for cytohesin binding whereas a control RNA had no effect. These data indicate that either the aptamers target the same epitope of cytohesin-1 or the binding of one aptamer influences the binding of the other through steric constraints. To further validate the activity of the aptamer C10 we investigated the influence of the aptamer on the guanine nucleotide exchange factor function (GEF) of cytohesin. As shown in Figure 3B, the aptamer inhibits the GEF activity of cytohesin-1 in a concentration dependent manner whereas a scrambled control oligonucleotide (bearing the same nucleotide composition as C10.35 but in a different arrangement) has no effect. Likewise, the inhibition of the mutant aptamer C10.35 GG6/7AA that has a strongly reduced affinity regarding cytohesin is less pronounced.

Having the functional and truncated progeny of the ssDNA aptamer C10 in hand, we next sought to generate photo-dependent variants. Therefore we started to synthesize mutants of the aptamer and analyzed their binding activities employing filter retention analysis (Table 1). At first, we began to introduce mutants that were thought to destabilize the central stem structure. To this end we synthesized the mutants G7A, GG6/7AA, and GGG5/6/7AAA (Fig. 2). As shown in Table 1 the binding capacity of the mutants decreases with an increase of G to A substitutions, albeit cytohesin binding was not completely abrogated. Especially the mutant G7A still interacts with cytohesin with high affinity (K_D = 59.2 nM). This indicates that the stability of the stem is important but not crucial to maintain cytohesin binding. Next we introduced point mutations that were thought to interfere with the formation of the putative two nucleotide stem structure located in the loop region connecting the strands of the central stem structure (Fig. 2). Therefore we synthesized the mutants G14A, C15T, and G14C/C15G (Fig. 2). All three mutants revealed a total loss of cytohesin binding indicating that either the formation of the short stem is necessary for target binding or the nucleotides in question interact directly with cytohesin.

Based on these findings we synthesized the caged variants of the aptamer C10.35 with the modified nucleotides G^{NPP} (NPP: *o*-nitrophenylpropyl) or C^{NPE} (NPE: *o*-nitrophenylethyl) at the positions G14 and/or C15, respectively (Fig. 4A and B).

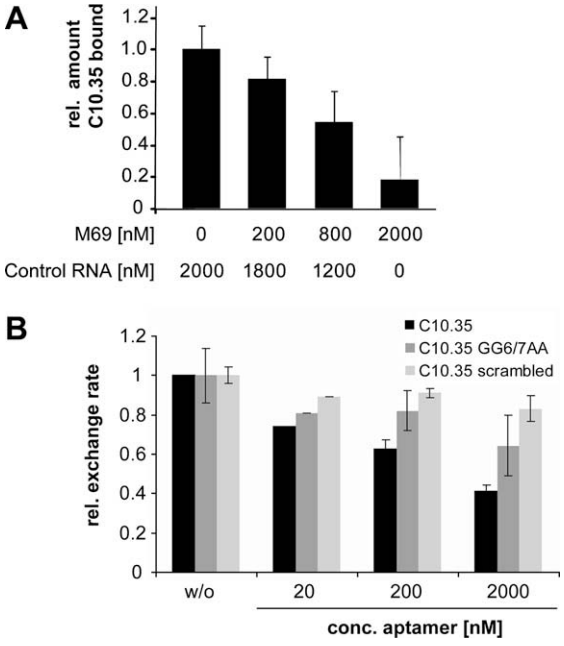

Figure 3. The aptamer C10.35 competes with the RNA aptamer M69 for cytohesin-1 binding (A) and inhibits the guanine nucleotide exchange factor (GEF) activity of cytohesin-1 (B).

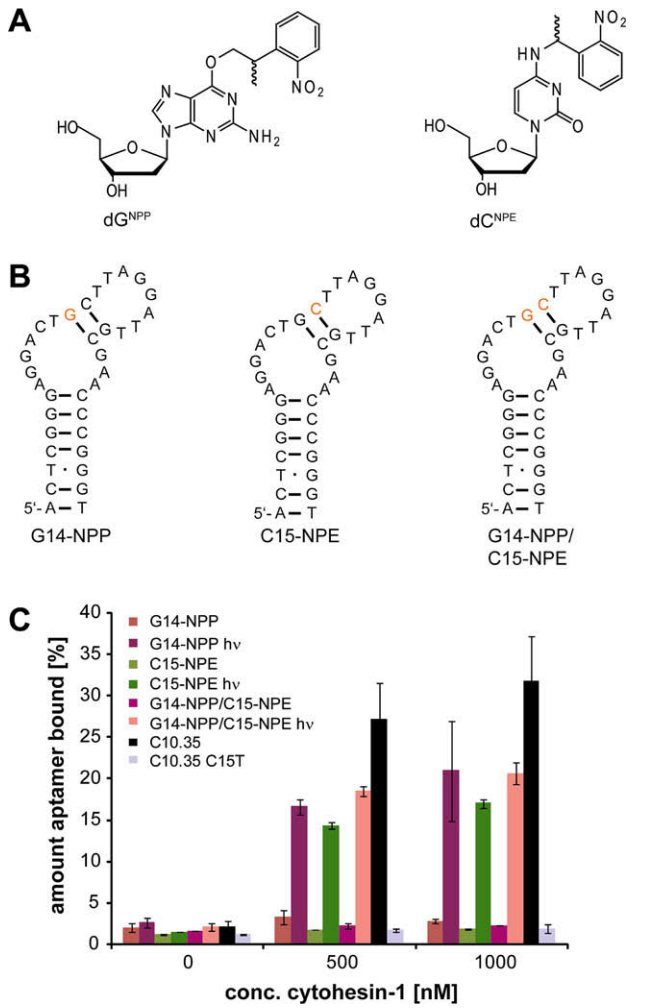

Figure 4. Caged variants of the aptamer C10.35. (A) Chemical structures of the phosphoramidites dG^{NPP} (*o*-nitrophenylpropyl) and dC^{NPE} (*o*-nitrophenylethyl) used to synthesize the caged aptamers described in (B). Positions highlighted in orange (G14 and C15) were modified with caged nucleosides. (C) Filter retention analysis of the caged aptamers regarding cytohesin binding prior and after irradiation with light (λ = 365 nm).

As shown in Figure 4C the caged variants do not interact with cytohesin-1 and binding can be reconstituted by irradiation with UV-A light (λ = 365 nm).

In conclusion we demonstrated a route that describes the de novo selection and characterization of ssDNA aptamers and the subsequent construction of caged variants thereof. Noteworthy, this procedure does not rely on the knowledge of the 3D structure of the aptamer a priori nor is the detailed mechanism of aptamer-target binding mandatory. We introduced a short 35 nucleotide ssDNA aptamer that targets cytohesin-1 and inhibits its GEF activity. The introduction of solely one caged nucleotide enables the quantitative regulation of cytohesin binding by light. The caged aptamers described here represent valuable tools for the characterization of cytohesin-1, in vitro and in cell culture experiments with spatiotemporal control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.032.

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