Aptamer Affinity Labeling

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Aptamer-Based Affinity Labeling of Proteins**

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Aptamers are versatile, single-stranded oligonucleotides that can be employed for the molecular recognition of different target structures by a strategy called systematic evolution of ligands via exponential enrichment (SELEX).[1] Traditionally, aptamers were evolved against small molecules[2] or proteins,[3] and subsequently modified to enable application in a variety of in vitro assays.[4] However, in recent years both the selection and application of aptamers has progressed towards considerably more complex targets, such as cells,[5] tissue slices,[6] or even live organisms.[7] On one hand, this attests to a huge advantage of the SELEX-approach as these target structures can be bound with high selectivity and affinity without knowledge of the actual molecule that is recognized by the aptamer. On the other hand, once an aptamer for targets of such complexity has been identified, this lack of knowledge immediately turns into a severe disadvantage, as it prevents further advancements, for example, as an analytical tool or as a biomarker for certain disease states. These restrictions require technologies that allow for the rational identification of unknown target molecules of an aptamer. Such methods would even be useful for complexes between an aptamer and a known target molecule, especially in cases in which the aptamer is employed (as a so-called intramer)[8] for target validation inside cells. Intramers are often used for affecting intracellular signaling pathways by inhibition of a particular protein to gain insight into its biological function. To be able to unambiguously assign a biological response to a particular protein of interest, it is essential to know whether there are other targets in the proteome that might also be inhibited by the same intramer ("off-target effects").

Similar demands are made on drug-like protein agonists and antagonists. Therefore, rational methods such as affinity-based proteomic profiling (ABPP), were developed for low-molecular-weight compounds, to identify off-target interaction partners within entire proteomes.[9] A central aspect of ABPP is the use of (photo)reactive derivatives of compounds that allow them to cross-link their binding partners. In this way, transient interactions can be captured and the stringent wash condition that are required for an efficient purification of the cross-linked species from the complex mixture of proteins can be applied. In contrast to ABPP, the proteome-wide profiling or target identification of aptamers is not well established.[6,10] A single example describes photo-cross-linking by photoreactive 5-iododeoxyuridines incorporated in an aptamer to identify its target on the cell surface.[10b] However, the method required tedious optimization of the position of the cross-linker moiety to prevent a loss in affinity. Because this approach probably requires similar optimizations for other aptamer/target pairs, its general applicability appears to be limited.

Herein we report a general strategy that we term aptamer-based affinity labeling (ABAL; see Figure 1 a,b), which allows for the rational identification of aptamer target structures within complex samples. In ABAL, the loss in affinity commonly observed when introducing photoreactive nucleotides into an aptamer is prevented by attaching the cross-

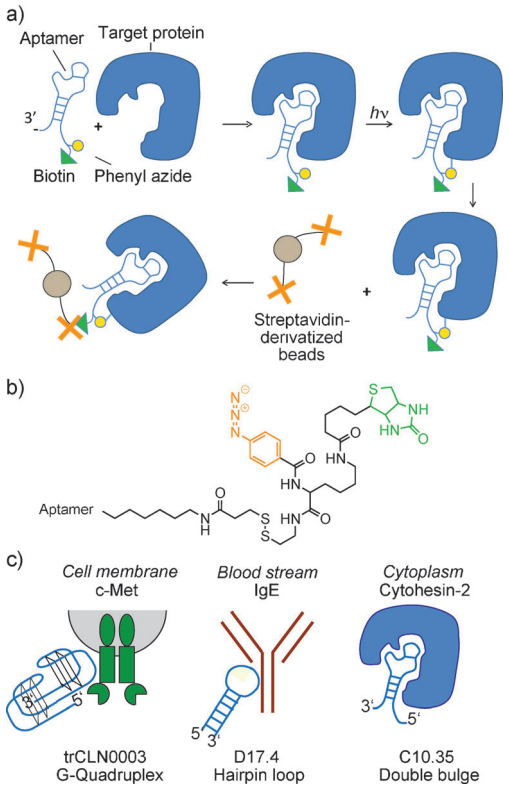

Figure 1. principle of aptamer-based affinity labeling. a) After incubation with its target protein, UV light is used to cross-link the aptamer to its target. Subsequent incubation with streptavidin-coated magnetic beads allows purification of the aptamer–protein complex. b) Structural formula of the ABAL moiety. c) The aptamer–protein pairs used.

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linking moiety to the 5-end of the aptamer (Figure 1b). This probe was originally developed for performing the so-called "label transfer reactions" to identify protein–protein interactions.[11] In addition to a phenyl azide moiety required for cross-linking, which could in principle be changed for other cross-linkers, such as diazirine derivatives, the probe also contains a biotin residue that, after incubation with avidin or streptavidin beads, can be employed for enriching the aptamer–protein conjugate. To demonstrate a broad applicability of the ABAL strategy, we chose three different aptamer–protein pairs as test systems that fulfill certain criteria. Firstly, we sought to employ aptamers that differ in their respective secondary structures, thereby representing a diversity of motifs of secondary structures, such as G-quadruplexes, hairpin-loops, or internal bulges (Figure 1c and Supporting Information Figure S1). Secondly, we chose three aptamer–protein pairs that differ in the cellular environment in which their respective target primarily resides, namely at the cell-surface, in the blood stream, and in the cytoplasm (Figure 1c).

ABAL was applied to the recently reported DNA aptamer CLN0003 (hereafter referred to as CLN3), which recognizes the ectodomain of the membrane protein hepatocyte growth factor receptor (HGFR, also known as c-Met).[12] According to our analysis, this aptamer consists of two G-quadruplex structures (Supporting Information Figure S1). A variant of CLN3 that was truncated to 40 nucleotides (trCLN3) and binds c-Met with nanomolar affinity[12] (Supporting Information Figure S1) was functionalized at its 5′-end with the ABAL probe. The resulting functionalized aptamer was separated from the excess ABAL moiety and residual trCLN3 by reverse-phase HPLC. To test the influence of the ABAL moiety on aptamer binding we performed filter retention assays in which the 5′-ABAL-functionalized aptamer competed with non-derivatized aptamer for binding to c-Met (Figure 2a). The IC_{50} value of ABAL-trCLN3 was (50 ± 8) nM, which is similar to the (76 ± 17) nM obtained for the unlabeled trCLN3. This result demonstrates that functionalization of the 5′ site of the aptamer did not affect its binding affinity.

We also designed a non-binding G25A point-mutant of trCLN3 (Supporting Information Figure S2) and showed that this ABAL–trCLN3 G25A variant displayed no binding in the tested concentration range (Figure 2a). In this way, we not only confirmed the G-quadruplex structure of the aptamer, but also established a meaningful negative-control to estimate the degree of non-specific cross-linking.

We next investigated whether ABAL–trCLN3 could be cross-linked to purified c-Met. To do so, we incubated c-Met in a concentration range between 0.5 μM and 19 nM with 250 nM of the ABAL-functionalized aptamers, and then treated the sample with UV irradiation at 365 nm. No cross-linking occurred in absence of UV irradiation (Figure 2b). But after exposure to 365 nm light, a high level of cross-linking was observed between ABAL–trCLN3 and 0.5 μM c-Met. Even at concentrations as low as 19 nM of c-Met cross-linking was still clearly detectable (Figure 2b). In contrast, even the highest concentration of c-Met resulted in very low cross-linking when ABAL–trCLN3 G25A was used; quantification of the band intensities at 500 nM c-Met revealed a 24-fold lower signal than for ABAL-trCLN3. This difference clearly shows that the reaction strictly depends on binding combined with UV irradiation.

Although functionalization of the 5′-end makes the ABAL approach easily applicable to the multitude of existing aptamers it could be argued that the phenyl azide is too distant from the bound protein, thereby preventing efficient cross-linking. However, comparison of the total amount of c-Met and the cross-linked c-Met fraction after UV irradiation revealed a cross-linking efficiency of approximately 30% (Supporting Information Figure S3). Although a photo-cross-linking efficiency of over 80% has been reported in individual cases,[13] the ABAL efficiency is higher than the 5–20% efficiencies typically observed when using aptamers function-

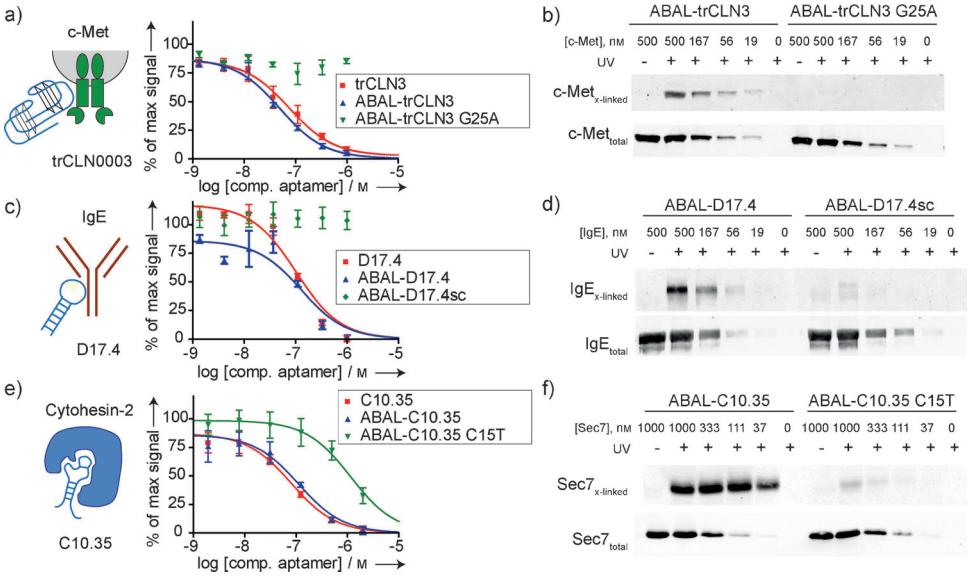

Figure 2. Characterization of ABAL-functionalized aptamers. a) Filter retention assay in which increasing concentrations of ABAL-functionalized variants of trCLN3 competed with radiolabeled trCLN3 for binding to the target protein c-Met. The curve fit assumed competition for a single binding site. b) Western blot analysis of cross-linking between ABAL–trCLN3 and a fusion protein of the ectodomain of c-Met and an IgG Fc domain (c-Met-Fc). c) and d) correspond to (a) and (b), but using ABAL–D17.4 and IgE. e) and f) correspond to (a) and (b) but employing ABAL–C10.35 and the Sec7 domain of cytohesin-2. In (b), (d), and (f), fluorescence-labeled neutravidin was used to determine the cross-linked protein amount. The total amount of protein was determined using an antibody specific for c-Met in (b), for IgE in (d), and for a histidine tag in (f).

alized with 5-bromo- or 5-iododeoxyuridine residues,[14] and similar to the efficiency observed with mRNA containing 2,4-dinitro-5-fluoro phenyl azide.[15] Thus, the observed high levels of functionalization demonstrate that positioning of the ABAL-probe at the 5′-end of the aptamer places the phenyl azide sufficiently close to the protein target to enable efficient cross-linking, yet at the same time distant enough from the binding site to prevent a loss in affinity.

Having established the ABAL principle for trCLN3, we next expanded ABAL to two other aptamer/protein pairs to estimate the generality of the approach. We chose the immunoglobulin E (IgE) binding DNA aptamer D17.4,[16] whose target proteins primarily reside in the blood stream. In addition, we employed the aptamer C10.35,[17] which binds the Sec7 domain of the guanine nucleotide exchange factor cytohesin-2, a cytoplasmic protein required for activating small GTPases and receptor tyrosin kinases.[18] In terms of their secondary structure, D17.4 and C10.35 not only differ from one another but also from CLN3. D17.4 forms a simple hairpin-loop structure, whereas C10.35 comprises a stem with a double bulge (Supporting Information Figure S1). As before, non-binding variants for both aptamers were used as negative controls. In the case of D17.4, a scrambled sequence variant (D17.4 sc) was created, whereas the non-binding point mutant C10.35 C15T was already available for C10.35.[17] For neither C10.35 nor D17.4 were the binding affinities for their respective targets altered after 5′-modification with the ABAL moiety (Figure 2c,e). Again, high levels of cross-linking were observed for both aptamers, whereas for the non-binding aptamer variants, marginal cross-linking was detectable at only the highest protein concentrations (Figure 2d,f). Quantification of the cross-linking bands at the highest protein concentrations yielded differences in cross-linking of 17-fold between D17.4 and its negative control for IgE, and sevenfold between C10.35 and its point mutant for Sec7, respectively. These results are similar to those observed for ABAL–trCLN3, indicating that ABAL can potentially be applied to a broad variety of aptamer/protein complexes.

To work in as many different biological assays and contexts as possible, an important prerequisite for ABAL is the ability to specifically and reliably cross-link targets in complex media. Therefore, the ABAL–C10.35/Sec7 complex was irradiated with UV light in the presence of lysate from human H460 cells. We indeed detected a band corresponding to the molecular weight of Sec7 in the lysate, whereas this band was absent when the negative control ABAL–C10.35 C15T was used (Figure 3, arrow 1). This result demonstrates that the cross-linking of ABAL-C10.35 to its target protein is possible in complex mixtures of different proteins. However, the blot also showed a number of additional bands. The intense bands indicated by arrow 2 are probably endogenous biotinylated proteins, as they were also observed when using a low-molecular-weight ABPP-probe for Sec7.[19] This notion is further supported by the fact that these bands disappeared when the lysate was pretreated with streptavidin-coated beads (data not shown). Arrow 3 in Figure 3 indicates bands that appear both for the binding and non-binding aptamers, but not in the absence of any ABAL–oligodeoxynucleotide. These cross-links therefore result either from non-

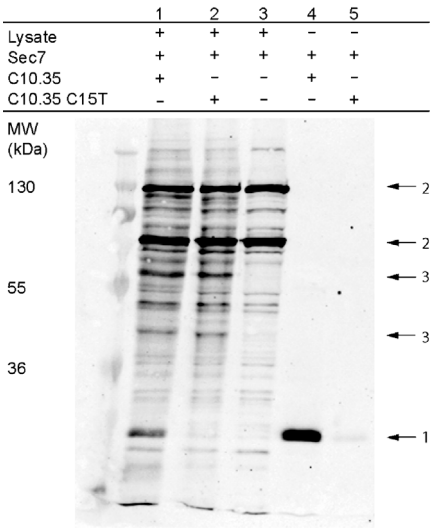

Figure 3. Western blot analysis of cross-linking between Sec7 and ABAL-functionalized variants of C10.35 in the presence of H460 cell lysate. 250 nM of aptamer was added to a solution containing 2 mg mL^{-1} lysate spiked with 1 μM of Sec7 (lanes 1–2). Lane 3: lysate/Sec7 without aptamer. Lanes 4–5: no lysate. For visualization of cross-linked products, fluorescence-labeled neutravidin was used.

specific binding of the ABAL moiety to certain proteins, or from nucleotide-binding proteins. In any case, these bands demonstrate the importance of the non-binding aptamer as a negative control. The remaining bands in lanes 1 and 2 are found even if no aptamer is added at all (lane 3). They result from the unspecific binding of the fluorescence labeled neutravidin to proteins present in the lysate.

In principle, ABAL could be directly applied to the cell-surface proteins of living cells. This would not only enable the identification of the aptamer target proteins evolved by cell-SELEX, but would also augment the repertoire of methods for specific cell-surface labeling. To test this possibility, we incubated the non-small-cell lung carcinoma (NSCLC) cell line H1838, known to overexpress c-Met,[20] with 250 nM of ABAL–trCLN3 and subsequently irradiated with UV light. After addition of streptavidin-coated magnetic beads, the cells were inspected by light microscopy. As evident from Figure 4a, left panel, cells incubated with ABAL–trCLN3 showed enhanced bead binding to their surface. Virtually no bead binding was observed when using ABAL–trCLN3 G25A (Figure 4a, middle panel), or no aptamer (Figure 4a, right panel). This result not only demonstrates that ABAL can be employed to target proteins on living cells, but also shows that the specificity of ABAL observed in vitro is maintained. Furthermore, the efficient labeling of cells with magnetic beads also expands the repertoire of methods for magnetic cell sorting, which to date has been restricted to the introduction of magnetism by antibodies.[21]

To test whether the bound c-Met could be enriched, we lysed H1838 cells after cross-linking and then added streptavidin-coated magnetic beads. Western blot analysis revealed enrichment of c-Met when using ABAL–trCLN3 (Figure 4b) whereas no band corresponding to c-Met was observed in the bead fraction when using the G25A mutant or in the absence

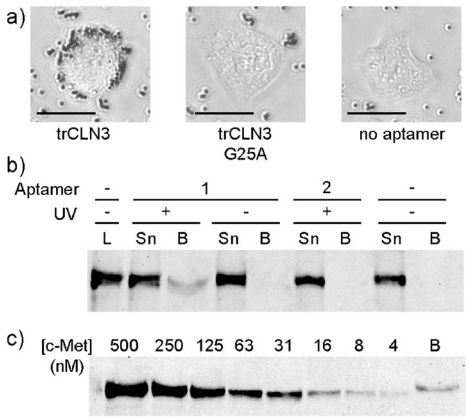

Figure 4. Photo-affinity labeling of c-Met on the surface of H1838 cells. a) Light microscopy images of magnetic-bead binding to H1838 cells incubated with ABAL–trCLN3, ABAL–trCLN3 G25A, or no aptamer (scale bars 25 μm). b) Western blot analysis of the supernatant (SN) or bead (B) fractions of samples treated with ABAL–trCLN3 (1), ABAL-trCLN3 G24A (2), or no aptamer (−) under or without UV irradiation. Sample L contains H1838 cell lysate without aptamer. c) Western blot analysis displaying decreasing concentrations of c-Met-Fc (R&D systems, MW = 123 kDa) together with the bead fraction of the cross-link from (b) involving aptamer 1 under UV-light. In (b) and (c), cross-linked products were visualized using rabbit-anti-c-Met in combination with fluorescence-labeled goat-anti-rabbit IgG.

of aptamers. This result further underlines the importance of specific binding in combination the UV-induced cross-linking for the enrichment of the target proteins from complex mixtures. Quantification of the enriched c-Met band revealed a total amount of 45–90 fmol (Figure 4c), which is within the detection range of nano-LC-MS.

In conclusion, our study demonstrates that aptamer-based affinity labeling (ABAL) is a powerful cross-linking strategy that exploits the affinity and specificity of aptamers together with their straightforward chemical modification. By using the 5′-end to introduce the photoreactive ABAL probe, highly efficient and specific cross-linking of three aptamer–protein pairs with large differences in secondary structure was achieved without loss in affinity. In this respect, our approach is different from previous studies in which aptamers containing 5-bromo-2′-deoxyuridine residues were evolved by the photo-SELEX method.[22] The photoreactivity of the resulting aptamers was elegantly used for either binding site identification[23] or to increase the sensitivity of multiplex microarrays.[10a,24] However, this approach requires an entirely new photo-SELEX for each individual target protein. ABAL was not limited to purified protein samples, but could be extended to highly complex biological samples, such as cellular lysate or even directly at the membranes of living cells. Thereby, this approach paves the way for target identification of current and future aptamers, thus enabling detailed studies of aptamer interactions in the complex environment of cells or biomedical samples. In principle, the ABAL-method should also be applicable to other terminal bioconjugation modules that have been established for nucleic acids.[25]

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