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COMMUNICATION

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Apt-clean: Aptamer-mediated cleavage of extracellular antigen for the inhibition of membrane protein functions

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Recently, targeted protein degradation (TPD) has attracted much attention as a powerful strategy for effective inhibition of disease-related proteins. However, development of ligands with high affinity and specificity for a target protein is still a demanding task and poses a particular challenge for designing TPD therapeutics. In this work, we report a novel TPD strategy called aptamer-mediated cleavage of extracellular antigen (Apt-clean), where oligonucleotide-based affinity agents are used for selective recruitment of proteases to target membrane proteins. Our data demonstrate that Apt-clean induces selective degradation of the target protein both in vitro and in cellulo. In addition, potential of Apt-clean was demonstrated through the inhibition of a tumor-related growth factor signaling. This novel TPD modality may serve as an efficient and flexible strategy for targeting membrane proteins.

Introduction

In the last few decades, remarkable progress has been witnessed in targeted protein degradation (TPD) technology.[1,2] One of the most successful examples is a proteolysis-targeting chimera (PROTAC),[3] by which a target protein is recruited to an E3 ligase to induce its ubiquitination and proteasomal degradation. This can be achieved using bispecific molecules that recognize both target proteins and E3 ligases. TPD offers the opportunity to target various therapeutically relevant proteins, including those that have not been addressed by conventional small-molecule inhibitors and antagonists. Additionally, TPD often demonstrates better efficacy than conventional inhibitors owing to its catalytic nature.

Membrane proteins represent a major drug target in the human proteome.[4] Recently, non-small-molecule TPD strategies specialized in degrading membrane proteins have been extensively investigated.[5–11] In most of these strategies, E3 ligase or lysosome-shuttling receptors expressed on the cell surface are hijacked for the selective degradation of target proteins. Affinity agents, such as antibodies, nanobodies, synthetic glycopeptide ligands, and aptamers, have been used as building blocks for bispecific binders that transport the target membrane protein to the degradation machinery in the cell. However, these approaches are also not without their limitations. First, the expression patterns of cell surface E3 ligase and lysosome-shuttling receptors vary depending on the cellular context; thereby, limiting the cell type to be targeted specifically. Second, occupancy of the E3 ligases or lysosome-shuttling receptors with bispecific ligands potentially risks unexpected side effects, as these proteins are involved in the degradation and downregulation of other endogenous proteins.

To establish a new modality for TPD of membrane proteins, we revisited the concept of "catalytic antagonists," originally proposed by Davis *et al*.[12], in which a protease with broad substrate specificity is modified with a small-molecule ligand binding to the target protein. Protease recruitment in proximity to the target protein substantially increases the efficiency and selectivity of target degradation.[12–14] Theoretically, the target protein can be customized by the choice of ligand to be modified on the protease. Although a proof-of-concept study demonstrated the targeted degradation of proteins in solution;[12] the approach so far has seen limited success. This is presumably due to the technical difficulty and cumbersomeness of the biochemical conjugation of ligands to the protease, which should occur at an optimal modification site and stoichiometry. Additionally, the development of small-molecule ligands with high affinity and specificity for a target protein is still a

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demanding task. Antibody can be used as an alternative targeting agent to recruit the enzyme to the target.[15,16] However, antibody–protease chimera may be subjected to autoproteolysis, thereby making their design a challenging task.

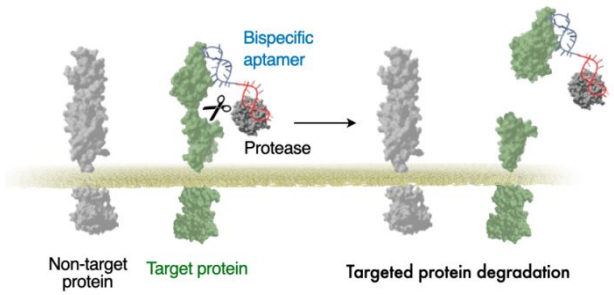

Figure 1. Schematic diagram of Apt-clean strategy. A bispecific aptamer binding to target protein and a protease with broad substrate specificity is used to recruit the protease to the proximity of target membrane protein.

Here, we propose an advanced concept called aptamer-mediated cleavage of extracellular antigen (Apt-clean), where oligonucleotide-based affinity agents (aptamers) are employed for the selective recruitment of proteases to the target protein without the need for bioconjugation (Figure 1). The aptamer, a single-strand oligonucleotide that recognizes a target molecule, is an ideal building block for the design of bispecific ligands because of these characteristics. (1) Aptamers are inert to proteolytic degradation; thus, they can be used as robust affinity agents for proteases. (2) One can generate an aptamer to a given membrane receptor and protease by a method called systematic evolution of ligands by exponential enrichment (SELEX).[17,18] (3) Given the structural programmability of oligonucleotides, bispecific aptamers can be designed rationally from two distinct aptamer sequences without losing their functionality. In this study, we targeted receptor tyrosine kinases (RTKs),[19] growth factor receptors whose aberrant activity is involved in various human diseases, including cancer. Our data demonstrate that the Apt-clean strategy substantially facilitates protease-mediated degradation of target receptors both in solution and on the living cell surface, and leads to downregulation of oncogenic RTK signaling in living cells.

Results and discussion

Thrombin, a serine protease that regulates the coagulation process,[20] was selected as a protease for our initial Apt-clean setting. Thrombin-mediated cleavage occurs at the carboxyl group of arginine residues (P1 position) in substrate proteins (Figure 2a, top). According to recent comprehensive studies,[21,22] thrombin shows a degree of promiscuity in the recognition of substrate sequences around the arginine residue, while the strictest substrate specificity was observed in P2 and P1′ positions of substrate peptides. Thrombin-binding aptamers (TBAs) have been extensively studied to regulate the coagulation process. Among several existing thrombin-binding aptamers, we focused on HD1[23] and HD22,[24] which bind to exosite 1 and 2 of thrombin, respectively (Figure 2a, bottom). Exosite 1 is a binding site for various thrombin substrates including fibrinogen, factor V, and factor VIII.[25] Exosite 2 is a binding site for heparin which facilitates the interactions with the antithrombin III and heparin cofactor II.[26] Notably, these exosites are on the opposite sides of the active pocket; thus, binding TBAs does not necessarily hinder the access of substrate peptides to the catalytic center. As demonstrated in previous studies, thrombin catalyzes the cleavage of peptide substrates even in the presence of TBAs, further supporting this contention. [27,28]

We first examined whether thrombin recruitment to a target membrane receptor could facilitate proteolytic cleavage. As a target receptor, we selected fibroblast growth factor receptor 1 (FGFR1).[29,30] Along with other growth factor receptors, aberrant FGFR1 activity has been associated with cancer development and malignancy.[29] Although several FGFR1 inhibitors and pan-FGFR inhibitors have been developed, their administration risks severe side effects due to the low kinase specificity.[31] We used a 38-mer DNA aptamer (SL38.2) that binds to the extracellular domain of FGFR1 with nanomolar affinity (K_d = 13 nM)[32] for the design of bispecific aptamers recruiting thrombin to FGFR1, which have different aptamer configurations (Figure 2b). The designed bispecific aptamers (500 nM) were incubated with thrombin (500 nM) and an Fc-fusion chimera of FGFR1 extracellular domain (0.5 μg) for 60 min. The cleavage of FGFR1 was monitored by SDS-PAGE (Figure 2c). Without

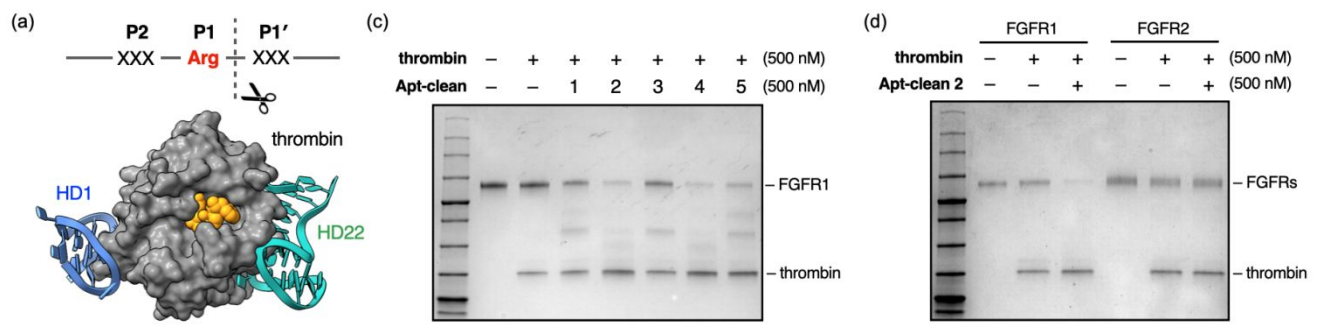

Figure 2. (a) Cocrystal structure of thrombin complexed with HD1-ΔT3 (blue) and HD22 (Cyan) (PDB ID: 5EW1). The catalytic site is occupied by a covalent inhibitor (orange). (b) Sequence of designed bispecific aptamers binding to FGFR1 and thrombin. Sequence of SL38.2 (FGFR1-binder), HD1 (exosite 1-binder), and HD22 (exosite 2-binder) are indicated in black, blue, and green, respectively. In Apt-clean 5, a flexible $d(T)_{10}$ linker (black, underlined) is introduced between the aptamers. (c) Cleavage of FGFR1-Fc mediated by bispecific aptamers. FGFR1-Fc (0.5 μg) was incubated in the presence or absence of bispecific aptamers (500 nM) and thrombin (500 nM) in DPBS for 60 min at 37 °C. (d) Cleavage of FGFRs mediated by bispecific aptamers. FGFR1-Fc or FGFR2-Fc (0.5 μg) was incubated in the presence or absence of Apt-clean 2 (500 nM) and thrombin (500 nM) in DPBS for 60 min at 37 °C.

bispecific aptamers, thrombin treatment did not induce FGFR1 cleavage. In contrast, a substantial decrease in FGFR1 band intensity was observed in samples treated with both bispecific aptamers and thrombin, suggesting that the aptamers facilitated thrombin-mediated proteolysis of FGFR1. The combination and configuration of aptamers affected proteolysis efficiency. The data indicate that Apt-clean 2 and 4, in which HD22 is tethered to the 3′ or 5′ terminal of SL38.2, demonstrated the highest target. We also verified that a control HD22 sequence without FGFR1-binding ability did not facilitate FGFR1 degradation (Figure S2), thereby eliminating the possibility of allosteric activation of thrombin.

Having identified a potent FGFR1 degrader in our hands, we tested its ability to degrade FGFR1 expressed on the living cell surface and inhibit FGFR1 signaling. FGFR1 is activated by the binding of FGF ligands such as FGF1, which induce FGFR1

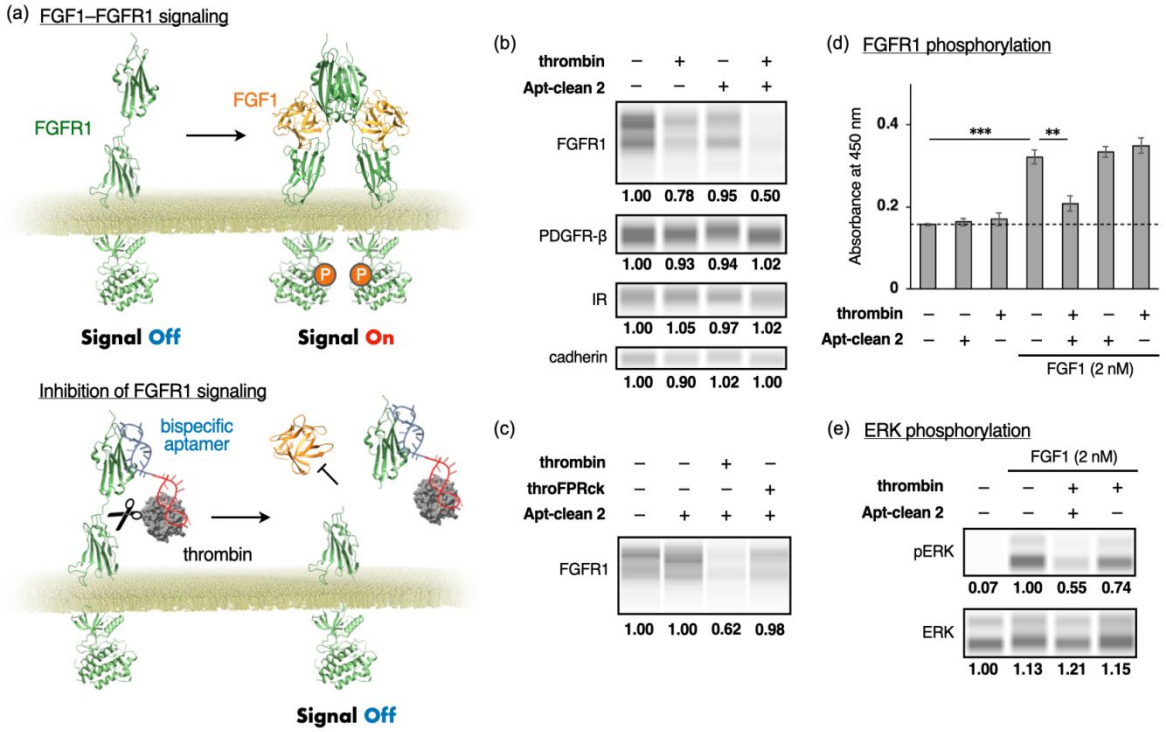

Figure 3. (a) Top; Schematic diagram of FGF1-induced FGFR1 signaling. FGF1 binds to FGFR1 and induces dimerization and activation of the receptor. Bottom; Schematic diagram of FGFR1 signal inhibition with Apt-clean strategy. Thrombin-induced cleavage of the extracellular domain renders FGFR1 inactive to FGF1. (b) Cleavage of FGFR1 and non-target membrane proteins expressed in 3T3-L1 cells with Apt-clean 2. The cells were incubated in the presence or absence of Apt-clean 2 (100 nM) and thrombin (100 nM) for 2 h. (c) Proteolytic activity-dependent cleavage of FGFR1. 3T3-L1 cells were treated with Apt-clean 2 and thrombin or throFPRck (100 nM) for 2 h. (d) ELISA of FGFR1 phosphorylation level induced by FGF1 (2 nM) for 15 min after the incubation in the presence or absence of Apt-clean 2 (300 nM) and thrombin (300 nM) for 2 h. The absorbance at 450 nm was measured and the mean values are indicated (N = 3). Error bars indicate SD. The statistical significance was tested using the t-test; $^{**}p < 0.01$, $^{***}p < 0.001$. (e) Phosphorylation of ERK, downstream molecules in FGFR1 signaling, induced by FGF1 (2 nM) for 15 min after the treatment with Apt-clean 2 (300 nM) and thrombin (300 nM) for 2 h.

degradation efficiency among the tested samples. Apt-clean 2 induced more efficient FGFR1 degradation than Apt-clean 5, suggesting that the flexible $d(T)_{10}$ linker adversely affects the degradation.

It is worth noting that the current system showed excellent specificity for the target proteins owing to the high specificity of the aptamer. FGFR2, an FGFR family member, has high sequence homology with FGFR1 in the extracellular domain (*ca.* 70%, Figure S1). Nevertheless, SL38.2 binds selectively to FGFR1 and does not bind to other FGFR members.[33] Our assay showed that a detectable degradation was observed only in FGFR1 when these proteins are incubated with Apt-clean 2 in the presence of thrombin (Figure 2d). Although the number of Arg residues in the FGFR2 extracellular domain (11 sites) was greater than that of FGFR1 (8 sites, Figure S1), such excellent specificity was achieved by selective thrombin recruitment to the dimerization and phosphorylation (Figure 3a). FGFR1 expressing 3T3-L1 cells were incubated with Apt-clean 2 and thrombin, and protein expression level after the treatment was evaluated using capillary electrophoresis immunoassay (Figure 3b). In the assay, the detected luminescence was normalized to the total protein amount of each sample, and relative intensity of normalized luminescence is indicated below the band images. While a slight decrease was also observed in the thrombin-treated group, a substantial decrease in FGFR1 expression was observed in cells treated with both Apt-clean 2 and thrombin (Figure 3b). To test the specificity of thrombin-mediated proteolysis, we also examined the expression levels of non-target membrane proteins, platelet-derived growth factor receptor β (PDGFRβ), insulin receptor (IR), and cadherins. In the cells treated with Apt-clean 2 and thrombin, the normalized band intensities of these non-target proteins were almost comparable

(90–105%) to non-treatment group, while FGFR1 band intensity was substantially decreased after the treatment (50 %, Figure 3b). These results demonstrate that Apt-clean strategy achieved the targeted protein degradation on the living cell surface.

Next, we verified whether the observed FGFR1 degradation is dependent on the proteolytic activity of thrombin. We used thrombin-FPRck (throFPRck), which was irreversibly inactivated by a covalent tripeptide inhibitor, in combination with Apt-clean 2. As a result, FGFR1 abundance was not affected by treatment with throFPRck and Apt-clean 2 (Figure 3c). Moreover, a new band with lower molecular weight, which may correspond to the cleaved FGFR1, emerged when the cells were treated with catalytically active thrombin and Apt-clean 2 (Figure S3). These results indicate that the FGFR1 degradation is attributed to the thrombin activity.

Finally, we investigated whether FGFR1 degradation could inhibit FGFR1 signaling induced by native ligands (Figures 3d and 3e). 3T3-L1 cells were treated with Apt-clean 2 and thrombin for 2 h and stimulated with FGF1 (2 nM) after removing the medium containing Apt-clean 2 and thrombin. FGFR1 phosphorylation was quantified using ELISA. The data indicates that FGFR1 was phosphorylated by adding FGF1 (Figure 3d). Notably, FGFR1 phosphorylation was substantially decreased in cells pretreated with both thrombin and Apt-clean 2. We also confirmed that Apt-clean 2 alone does not inhibit FGF2-induced FGFR1 activation, indicating the aptamer works as a non-competitive FGFR1-binder (Figure S4). Therefore, the observed FGFR1 inhibition could be attributed to FGFR1 cleavage rather than FGFR1 antagonization. Furthermore, FGF1-induced phosphorylation of ERK, which is a downstream signaling molecule of FGFR1, was also reduced by the treatment with Apt-clean 2 in the presence of thrombin (Figure 3e). These data demonstrated that the current strategy represents a promising strategy for the inhibition of receptor signaling by degrading the target receptor expressed on the cell surface.

We also verified whether Apt-clean could work in complex biological environments. 3T3-L1 cells were incubated with Apt-clean 2 and thrombin in the presence of 10% fetal bovine serum (FBS) for 2 h. As a result, FGFR1 degradation was observed even in 10% FBS condition, while the efficacy was reduced compared to that induced in a serum-free condition (Figure S5). In a PAGE analysis, Apt-clean 2 showed nuclease stability as the intact aptamer remained after 2 h incubation in 50% FBS condition, where negative control oligonucleotides were completely digested (Figure S6). Therefore, the decreased proteolytic activity in 10% FBS condition is not attributed to the degradation of aptamer by nucleases in the serum. The possible causes could be (1) the replacement of thrombin with other protein components in the serum or (2) the decrease of aptamers affinity in complex biological conditions. These potential issues would be tackled by improving affinity and selectivity of aptamer components.

Conclusions

In summary, we propose a novel TPD strategy called Apt-clean, in which a bispecific aptamer is used to direct a protease to degrade a target protein on the cell surface. Unlike previous TPD strategies targeting membrane proteins; Apt-clean does not require laborious or difficult-to-control bioconjugation processes. Additionally, diverse membrane proteins, including other growth factor receptors, can be targeted by the current strategy, considering a broad repertoire of aptamers binding to them.[34–41]

Although our initial Apt-clean design may require some improvement in terms of efficacy and selectivity in the cellular context; these issues can be addressed with optimization of the choice of protease, aptamer affinity, and biological stability.

Author Contributions

Junya Hoshiyama: investigation, methodology, validation, analysis, writing – original draft. Yuga Okada, Seojung Cho: validation, analysis. Ryosuke Ueki: conceptualization, methodology, analysis, investigation, writing – original draft, supervision, funding acquisition. Shinsuke Sando: conceptualization, methodology, writing – original draft, supervision, funding acquisition.

Conflicts of interest

There are no conflicts to declare.

Cell culture

3T3-L1 cells were obtained from Japanese collection of research bioresources (JCRB) cell bank (#JCRB9014) and cultured in DMEM (#08456-65, Nacalai Tesque) supplemented with 10% calf serum (#C8056-500ML, SigmaAldrich) and 1% antibiotic-antimycotic mixed solution (#09366-44, Nacalai Tesque), and maintained in 5% CO2 in a humidified incubator at 37 °C.

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