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Light-cleavable rapamycin dimer as an optical trigger for protein dimerization†

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Rapamycin-induced protein heterodimerization of FKBP12 and FRB is one of the most commonly employed switches to conditionally control biological processes. We developed an optically activated rapamycin dimer that does not induce FKBP12-FRB dimerization until exposed to light, and applied it to control kinase, protease, and recombinase function.

Nature requires precise control of biological processes on the cellular and multicellular level. Processes such as DNA replication,[1] cell signaling,[2] molecular transport,[3] and protein biosynthesis[4] depend on the location of proteins, DNA, and other biomolecules and the timing of their activity and interactions. In trying to elucidate the mechanisms of cellular processes, it is crucial to examine them with protein switches that allow for precise control comparable to that of the natural system.[5] Light is a unique external regulatory element that can be used to probe the regulation of protein switches that are linked to biological pathways. The simplicity and accuracy of timing, location, and amplitude associated with light exposure makes it an ideal non-invasive technique to use in biology.[6] Photochemical control of biomolecules can be achieved through the application of light-removable protecting groups, so called "caging groups". The addition of a caging group to a critical position within the biomolecule of interest renders the molecule inactive until the group is removed through light exposure, typically UV light of $\geq$365 nm. Caging groups can be installed on small molecule effectors,[7] proteins,[8] and oligonucleotides.[9] As presented here, the natural product rapamycin was chemically modified with a caging group to allow for photochemical control of FKBP12 and FRB dimerization.

The macrolide rapamycin (**Rap**) was isolated in the 1970s and has received substantial attention from the scientific community due to its immunosuppressant activity.[10] Naturally, rapamycin binds to the FK506 binding protein, also known as FKBP12, with a K_d between 0.2 and 0.4 nM.[11] The FKBP12-rapamycin complex then binds to the FRB (FKBP12-rapamycin binding) domain of mTOR (mammalian target of rapamycin), forming a ternary complex consisting of the two proteins and the small molecule.[10,11] This rapamycin-induced protein dimerization has been extensively exploited as a research tool to control a wide range of cellular processes.[12–19] FKBP12 and FRB are modular proteins that have been fused to a variety of protein targets to conditionally regulate them, including transcription factor domains,[12] kinases,[20] split enzyme systems such as Tobacco Etch Virus Protease (TEVp)[13] and Cre recombinase,[14] as well as cellular localization domains[21] in order to allow for control over protein function with the addition of rapamycin.

Manipulating rapamycin to render it light-activatable through the installation of a caging group will provide precise control over triggering the aforementioned biological processes. Recently, we reported a caged rapamycin in conjunction with an engineered FKBP domain.[20] We demonstrated that the caged **Rap** had a similar activity as wild type rapamycin in mediating the dimerization of natural FKBP and FRB. Thus, a truncated mutant of FKBP12, termed iFKBP, was developed in order to improve the sensitivity of the interaction between caged **Rap** and FKBP.

Since caged **Rap** was found to be still active in mediating the heterodimerization of FKBP12 and FRB, we hypothesized that a larger caging group was required in order to fully abrogate its function. Furthermore, we speculated that an efficient way to substantially increase the size of the caging group is by using a second rapamycin molecule as a sterically demanding structure that is able to recruit an even more sterically demanding second FKBP protein. Here we present a new caged rapamycin, the light-cleavable rapamycin dimer **dRap** (Fig. 1). This analog is fully compatible with wild-type FKBP12, thus obviating the need for protein engineering of the many established rapamycin-controlled processes.[12–19] The rapamycin dimer **dRap** was synthesized from rapamycin in just two steps (Fig. S1, ESI†).

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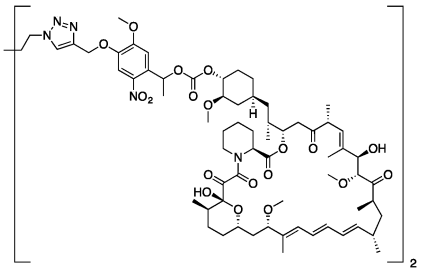

Fig. 1 The structure of the photo-cleavable rapamycin dimer **dRap**.

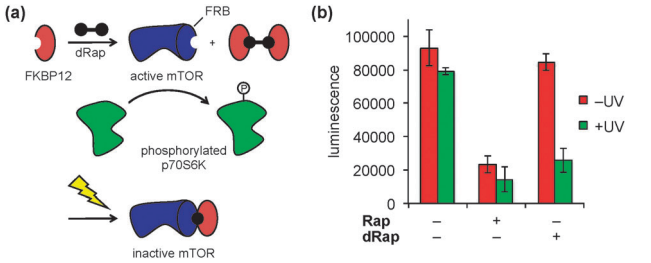

Fig. 2 K-LISA mTOR assay of **Rap** and **dRap**. (a) Cartoon representation of K-LISA mTOR Activity Assay. (b) In the absence of **Rap** or **dRap**, mTOR is functional, leading to high luminescence. **dRap** (50 nM) is blocked from inducing the heterodimerization of FKBP12 and mTOR, as shown by the high levels of luminescence before irradiation. Irradiation with UV light at 365 nm for 3 minutes converts **dRap** into **Rap**, which leads to formation of the FKBP12-**Rap**-mTOR complex and to the same low levels of phosphorylation and thus luminescence as observed in the case of **Rap**.

Fluorescence polarization was used to investigate the capability of **dRap** to homodimerize a fluorescein-labeled FKBP12 (*via* thiourea formation through reaction with FITC) (Fig. S4, ESI†). Indeed, the addition of **dRap** to a solution of FKBP12-FITC led to an increase in fluorescent polarization, indicating homodimerization of FKBP12-FITC. Moreover, further addition of a 10-fold excess of **dRap** led to a decrease in fluorescence polarization due to the saturation of FKBP12 binding sites with **dRap** molecules, effectively preventing protein dimer formation. Thus, we speculated that the FKBP12-**dRap**-FKBP12 complex could prevent binding of FRB, until it is fragmented through photochemical cleavage of **dRap**. This hypothesis is supported by the comparison of complex formation energies calculated in extensive molecular dynamics simulations (see the ESI† for a detailed discussion). Several different scenarios were computationally explored to explain the experimentally seen FKBP12 homodimers instead of FKBP12-FRB heterodimers (see mTOR assay below). First, we excluded that steric hindrance imposed by the second rapamycin molecule may block complex formation, since it appears that FRB binding would be allowed in an outstretched linker conformation. Next, the binding poses of rapamycin and **dRap** to FKBP12 were examined to determine if it is possible that **dRap** is found in a position that in unfavorable for FRB binding. However, it was found that **dRap** frequently assumes FRB favorable binding positions. The final set of simulations determined the energies necessary for formation of the **dRap** mediated FKBP12 homodimer and the **dRap** mediated FKBP12-FRB heterodimer. These showed that the formation of **dRap** mediated FKBP12 homodimers is more favorable than FKBP12-FRB heterodimers. The large free energy of **dRap**-mediated homodimerization produces highly stable FKBP12-**dRap**-FKBP12 complexes that are virtually inert to FRB, until activated by **dRap** photolysis. This tightly controlled **dRap** complex formation makes it well suited for light-regulation of biological functions.

In order to validate **dRap** as an effective light-activation tool, a K-LISA mTOR Activity Assay (Calbiochem), Fig. 2a, was performed. mTOR phosphorylates the target protein p70S6K. However, when mTOR forms a ternary complex with FKBP12 and rapamycin, it cannot maintain kinase activity. The presence of phosphorylation was monitored through an α-phosphorylation-HRP conjugated antibody. As seen in Fig. 2b, phosphorylation is indicated by high levels of luminescence in the absence of rapamycin as well as in the presence of **dRap** before UV irradiation. This is due to the inability of **dRap** to dimerize FKBP12 and mTOR, allowing mTOR to retain kinase activity. Phosphorylation is reduced by nearly 80% in the presence of rapamycin as a result of the mTOR-**Rap**-FKBP12 complex formation. The decaging of **dRap** after exposure to UV light of 365 nm releases native rapamycin and FKBP12-mTOR heterodimerization is observed by a decrease in luminescence to the same level as in case of rapamycin. This shows that only upon irradiation and photolysis, but not before, **dRap** successfully dimerizes FKBP12-mTOR *in vitro*.[22]

The ability to tightly control **dRap** makes it well suited for light-regulation of biological functions in live cells, as demonstrated by two important biological processes – protein cleavage and DNA recombination – that were investigated in tissue culture. Tobacco Etch Virus NIa protease (TEVp) is a widely used protease that recognizes a specific seven amino acid sequence, ENLYFQG. Cleavage occurs between the glutamine and glycine residues, and this recognition sequence has been genetically engineered into various proteins to either activate or deactivate their function.[13] TEVp has been used to intracellularly cleave proteins from purification tags,[23] to cleave stabilization proteins from bioactive glycopeptides,[24] to aid in the discovery of protein–protein interactions,[25] and in the inactivation of proteins *in vivo*.[26] Conditional control of TEVp activity was achieved through the application of an inducible TEV expression system[23] and the development of a split TEVp system.[13] In the latter case, TEVp was divided into N-(amino acids 1–118) and C-(amino acids 119–241) terminal fragments and each fragment was fused to FRB and FKBP12, respectively. Co-expression of both fusion proteins did not display any protease activity until the addition of rapamycin, which induces dimerization and restores activity of TEVp.

To investigate the photochemical control of protease activity (Fig. 3a), **dRap** was tested in HEK293T cells expressing the FKBP12-TEV C-terminus and the FRB-TEV N-terminus,[13] as well as a circularly permuted luciferase reporter containing a TEVp cleavage site (GloSensor, Promega).[27] Active TEVp will cleave the GloSensor protein, causing a conformational change that leads to an activation of the luciferase reporter and luminescence. As seen in Fig. 3b, addition of rapamycin to the transfected cells led to the generation of a luminescence signal, while in the absence of rapamycin only a very low background level of luminescence was detected. Importantly, only background luminescence was

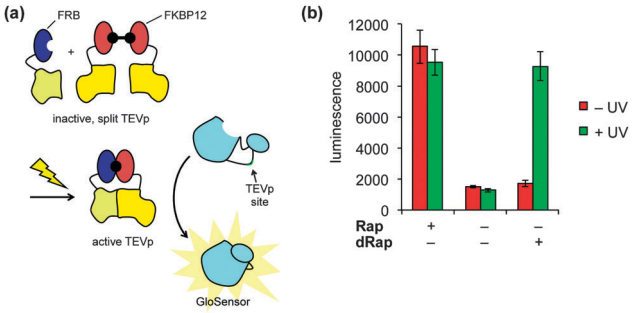

Fig. 3 (a) Photochemical activation of split TEV using **dRap**. FKBP12-TEV C-terminus (blue) and FRB-TEV N-terminus (red) dimerize only after irradiation of **dRap** and release of native rapamycin. Active TEVp then cleaves the TEVp recognition site of a circularly permutated luciferase protein, inducing catalytic activity and thus luminescence. (b) Luminescence data from the split TEVp/GloSensor assay using **dRap**. HEK293T cells expressing FKBP12-TEV C-terminus, FRB-TEV N-terminus, and the GloSensor reporter were treated with rapamycin (100 nM) or **dRap** (50 nM) and irradiated for 5 minutes at 365 nm. Luminescence readings were taken 24 hours after irradiation.

observed when the light-cleavable rapamycin dimer **dRap** was added to the media in the absence of UV irradiation. This demonstrates the inability of **dRap** to heterodimerize FRB and FKBP12. Upon a 5 minute irradiation with UV light of 365 nm, the luminescence signal is restored to the same level as rapamycin induction. Thus, irradiation efficiently cleaves the light-sensitive linker, releasing active rapamycin molecules from **dRap**. The released rapamycin stimulates dimerization of FBKP12 and FRB, leading to TEVp activity. Importantly, in addition to being fully inactive *in vitro*, the rapamycin dimer is also fully inactive in cell culture and a brief UV irradiation restores TEVp activity to the same level as the addition of rapamycin itself.

Light-activation of **dRap**, and thus photochemical control of TEVp, allows for non-invasive activation of site-specific protein cleavage in live cells. In addition to protein manipulation, the application of the light-activated rapamycin dimer was also investigated in the manipulation of DNA, specifically DNA recombination (Fig. 4). Cre recombinase is an important tool in cell and developmental biology, as it recognizes palindromic sequences of DNA, known as *loxP* sites, and is able to delete, insert, or invert any DNA sequence that is located between those sites, depending on their orientation.[14] Cre-catalyzed DNA recombination has been extensively used for a wide range of applications starting from simple DNA recombination in *E. coli*,[28] to genetic engineering in yeast,[29] plants[30] and mice.[31]

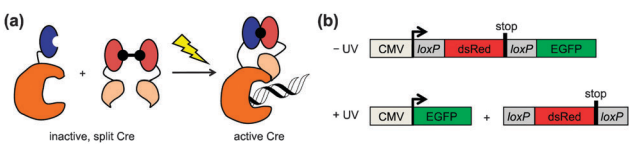

Fig. 4 Split Cre induction using **dRap**. (a) FRB-Cre C-terminus (red) and FKBP12-Cre N-terminus (blue) are dimerized after irradiation of **dRap**. (b) The Cre stoplight reporter expresses dsRed before irradiation, but in the presence of reconstituted, active Cre, the dsRed gene is excised and EGFP is expressed.

Genetic engineering in mice is widely applied and has allowed for the conditional control of genes by a *loxP* flanked stop region,[32] as well as specific chromosomal rearrangements to mimic human diseases.[33] One of the limitations of Cre is the inability to tightly control the recombination process with external regulatory elements. Methodologies have been developed for the spatiotemporal control of Cre recombinase, including the regulation of Cre catalysis with a photocaged tamoxifen,[34] by caging the catalytic tyrosine residue within the active site,[35] and by fusing each fragment of a split Cre enzyme to a blue light-absorbing photoreceptor and its binding partner.[36] However, there are limitations to each of the current methodologies. When using photocaged tamoxifen there is need for multiple irradiations and limited restoration of Cre activity is seen.[34] There is a requirement of continuous pulses of light for 24 hours when using the split-Cre fused to a natural photoreceptor,[36] and in case of the active-site caged Cre recombinase genetic encoding in mammalian cells has not been achieved.[35]

Here, we are reporting a solution to these problems by utilizing a split Cre system composed of amino acids 19–59 (N-terminus) and amino acids 60-343 (C-terminus) that was fused to FKBP12 and FRB,[14] respectively, enabling the photochemical control of Cre-catalyzed DNA recombination by **dRap** (Fig. 4a). To examine the ability of **dRap** to photochemically control Cre-catalyzed DNA recombination, HEK293T cells expressing a split Cre system and the Cre Stoplight reporter were used (Fig. 4b). The Cre Stoplight plasmid expresses dsRed when Cre recombinase is absent or non-functional. In the presence of active Cre, the gene coding for dsRed is excised from the plasmid due to flanking *loxP* sites and CMV-driven EGFP expression is activated.[37] In the presence of **dRap** but the absence of UV irradiation, only dsRed is expressed, an indicator that **dRap** is not capable of heterodimerizing FKBP12 and FRB leading to an inactive Cre recombinase (as seen by red fluorescence present in Fig. 5a). This is in perfect agreement with the results obtained from cells that are not treated with rapamycin. DNA recombinase is active through the decaging of **dRap** with a brief 5 minute irradiation at 365 nm producing native rapamycin molecules to participate in the FKBP12-**Rap**-FRB ternary complex and thus complementation of Cre fragments. The reconstituted

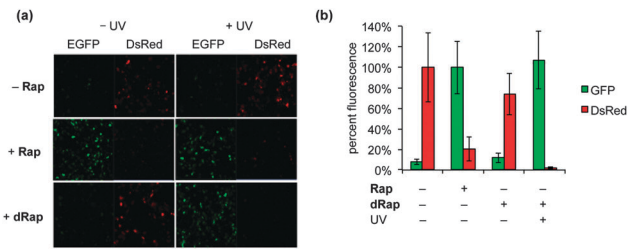

Fig. 5 Light-activated Cre DNA recombination. Plasmids encoding each fragment of the split Cre enzyme and the Cre Stoplight reporter were transfected into HEK293T cells. (a) Cells were treated with rapamycin (5 nM) or **dRap** (10 nM) and imaged for fluorescence. (b) Micrographs were quantified by integrating randomly selected areas using Zen imaging software. Error bars are standard deviations generated from three independent integrations.

Cre enzyme excises the dsRed gene and induces EGFP expression (Fig. 5a). Quantification of the micrographs through fluorescent cell counting further confirms these results (Fig. 5b).

We have successfully developed a light-cleavable rapamycin dimer (**dRap**) that does not induce FKBP12 and FRB heterodimerization until it is activated through UV exposure. Previously reported, caged rapamycin analogues have been applied to photochemically control protein phosphorylation;[20,38,39] however, these molecules have limitations. They require protein engineering of the FKBP domain or mislocalization of the rapamycin molecule outside of the cell, since the presence of the synthetic caging groups alone did not prevent rapamycin-induced FKBP12-FRB dimerization. Thus, the caged rapamycin analogues could not be directly interfaced with the wide range of biological processes that have been placed under conditional control through the construction of FKBP12-**Rap**-FRB based biological switches in cells and organisms. We envisioned developing an optochemical approach that utilizes recruitment of an entire protein[40] in order to dramatically increase the steric hindrance imposed by the caging group installed at position C-40 of rapamycin. Moreover, we planned to keep the design as simple as possible and to utilize a naturally occurring, endogenous protein for this purpose. Thus, the light-cleavable rapamycin dimer **dRap** was synthesized and we found that **dRap** was completely inactive as a heterodimerizer, until irradiated. Because of the modularity of FRB and FKBP12, **dRap** has implications in the light-regulation of protein dimerization and protein activity in biological processes that have already been rendered responsive to rapamycin, but could not be readily placed under optochemical control until now.[15–20]

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