Principles and Applications of the Photochemical Control of Cellular Processes

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Principles and Applications of the Photochemical Control of Cellular Processes

Alexander Deiters*[a]

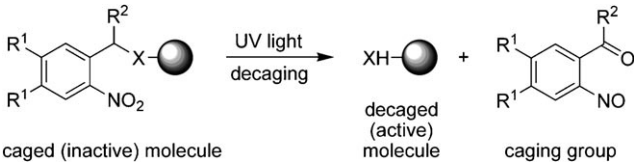
1. Introduction

Biological processes, particularly gene function, are naturally regulated with high spatiotemporal resolution in single cells and multicellular organisms. The activity of genes, proteins, and other biological molecules is precisely controlled in timing and location. This is especially evident during the complex biological processes observed in the development of an organism. In order to understand and to study these processes and their misregulation in human disease, it is imperative to control them with the same level of spatiotemporal resolution found in nature. Here, light irradiation represents a unique tool, because it can be easily and precisely controlled in timing, location, and amplitude; thus, light enables the precise activation and deactivation of biological function.

Rather than providing a comprehensive literature review, this article focuses on the basic concepts and requirements of controlling biological (especially cellular) function with light. Recent examples are used to illustrate these concepts. The interested reader can find additional excellent and comprehensive reviews regarding the photochemical regulation of biologically active molecules in the literature.[1]

2. The Photocaging Concept

A general way of achieving photochemical control over a molecule (for example, a small organic molecule or a biological macromolecule) is by rendering the biologically active molecule temporarily inactive through chemical modification with a light-removable protecting group, a so called "caging group".[1] The term "caging" was first introduced by Kaplan in 1978, in the context of a light-activated ATP molecule.[2] To date, many "light controllable" (or "caged") compounds have been devised, ranging from caged metal ions such as Ca^{II} and Cu^{II}, neurotransmitters, secondary metabolites (for example, steroid hormones), nucleic acids, and proteins.[1] Irradiation of the caged molecule, typically with nonphotodamaging UV light of >360 nm, releases the biologically active molecule (from its cage) and allows it to perform its biological function. This enables one to switch biological processes on (or off) by using light. Because light can be readily controlled with high spatiotemporal resolution, this strategy allows for the precise regulation of biological processes. Moreover, caging technologies can also be used to produce a repetitive release of the effector molecule and finely graded changes in the concentration of the active compound. Caged molecules are typically synthesized through the installation of a caging group, most often a classical *ortho*-nitrobenzyl group, on an oxygen, sulfur, or nitrogen atom, thereby inactivating the biological function of the molecule. Upon exposure of the caged compound to UV light, a photochemical fragmentation reaction is initiated that restores the biologically active molecule (Scheme 1). Several



Scheme 1. General decaging reaction of a molecule (sphere), caged with a classical *ortho*-nitrobenzyl group. Chemical and photochemical properties of the caging group can be tuned with suitable substituents, including: $R^1 = H$, OCH_3; $R^2 = H$, CH_3, CO_2H. $X = O$, CO_2, S, NH, $NHCO_2$, OPO_3H.

other caging groups are known,[1] and most recent developments were made in the area of quinoline-,[3] dibenzofuran-,[4] and coumarin-[5]based structures that enable decaging under two-photon irradiation.

There are several possibilities in how the installed caging group can abrogate biological function of the molecule. Most importantly, the steric demand of the caging group can inhibit the molecular interaction of the caged molecule with its biological partners (see 2.1 below). This steric inhibition of an interaction can potentially be enhanced through the installation of multiple caging groups. Another possibility is preventing a functional group (for example, XH = OH, SH, NH_2, CO_2H, or $OPO(OH)_2$) from performing its biologically relevant chemistry. Examples of chemistries inhibited though caging group installation include covalent bond formation through nucleophilic substitution (see 2.2 below) and acid/base reactions.

2.1 Steric blocking of interactions through photocaging

One example of steric blocking of an active molecule through caging group installation is the inhibition of oligonucleotide hybridization and its application in the photochemical regula-

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tion of antisense activity. Transient gene silencing represents an important technology in the investigation of gene function, and antisense agents provide powerful tools for the elucidation of the genetic details of the development of an organism.[6] Most antisense agents act through one of two mechanisms: 1) Degradation of target mRNA through enzymatic cleavage, for example, through the recruitment of RNase H, or 2) inhibition of translation by blocking ribosomal access to the mRNA without inducing mRNA degradation. Antisense technologies have been employed in targeting a wide range of different genes in almost every cell line and various developmental model organisms.

A fundamental characteristic of DNA is its ability to form Watson–Crick hydrogen bonds and thus undergo DNA duplex formation. The direct installation of caging groups on the bases of DNA (and RNA) has been shown to efficiently disrupt hybridization through steric blocking of hydrogen bonding.[7] The caging of phosphorothioate DNA (PS DNA) antisense agents on the nucleotide bases was recently accomplished,[8] and it was demonstrated that hybridization of a transfected 19-mer PS DNA oligonucleotide to a *Renilla* luciferase mRNA complement could be completely suppressed through the incorporation of three thymidine nucleotides caged at the N-3 position (Figure 1 A, $\phi = 0.1$, $\varepsilon = 6887\ M^{-1}cm^{-1}$). The complete inactivity of the caged PS DNA antisense agent was verified through a luciferase reporter assay in mouse fibroblast cells (NIH 3T3), in which luminescence was observed in a cell monolayer (Figure 1 C). A brief, localized irradiation with UV light of 365 nm fully restored antisense activity within the irradiated area, and thus silenced luciferase activity (Figure 1 B).

The disruption of protein–RNA interaction through the installation of a caging group on a thymidine base enables photochemical regulation of siRNA activity in mammalian cell culture. This was achieved by incorporating an O-4 caged thymidine residue at a crucial site in the central region of an RNA duplex.[9] This completely abrogated gene silencing; however, UV irradiation (366 nm, 40 min, 2.88 $J\,cm^{-2}$) initiated RNA interference, which led to the down-regulation of GFP.

A different approach to photochemically regulate antisense activity through steric blocking of oligonucleotide:mRNA hybridization involves inhibition of the activity of the antisense agent through the formation of a hairpin by using a hybridized complementary oligonucleotide linked through a light-cleavable tether.[10] This has been successfully applied to the photochemical regulation of peptide nucleic acids (PNAs), morpholinos (MOs), and PS DNAs after transfection into cultured cells or injection into zebrafish embryos.[11] An advantage of this strategy is that only one photolysis needs to occur to fully restore antisense activity; however, a careful oligonucleotide design is required to achieve complete inactivity of the antisense agent before irradiation.[12]

Chelated Ca^{II} cations that are complexed with photo-cleavable EDTA analogues[13] represent another example of a sterically blocked agent. This is one of the few examples of a truly caged molecule, and the most recent report of such a compound employs a nitrodibenzofuran (NDBF, $\phi = 0.7$, $\varepsilon = 18400\ M^{-1}cm^{-1}$) group (see **1**, Figure 2). This caging group

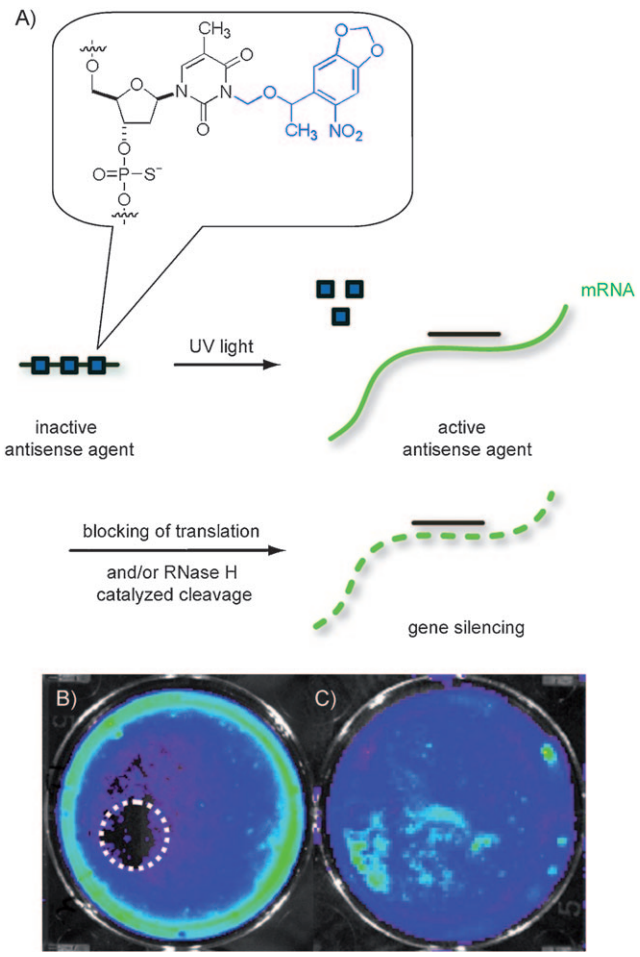

Figure 1. Light-activated phosphorothioate antisense agents. A) Caged thymidine nucleotide incorporated into an antisense PS DNA. The caging group (blue) is removed through UV irradiation; this enables hybridization of the antisense agent to the mRNA, and thus leads to gene silencing. B) Spatial regulation of *Renilla* luciferase expression by using caged PS DNA antisense agents. The cellular monolayer was irradiated only inside the white circle (365 nm, 5 min, 23 W); this led to localized silencing of luciferase in cells transfected with the caged PS DNA, compared to C) cells which have not been irradiated. Adapted with permission from: *ChemBioChem* **2008**, *9*, 2937–2940. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

enables efficient photochemical calcium release under two-photon irradiation with a pulsed 720 nm laser due to a large two-photon cross section of 0.6 GM.[4] Besides calcium, other prominent second messengers and neurotransmitters have been photocaged, including several nucleotides (AMP, ADP, ATP, cAMP, etc.), nitric oxide, glutamate, γ-aminobutyric acid (GABA), and phenylephrine.[14] The binding of Ca^{II} to the thin filament regulatory system of muscle cells leads to muscle activation and contraction. Skinned cardiac muscle fibers were subjected to the caged calcium **1** at 1 mM and exposed to two-photon excitation by using 70 mJ of energy; this produced almost full contraction of the muscle fibers (Figure 2 A). In contrast, the simple *ortho*-nitrobenzyl derivative of **1** produced a much lower level of relative tension (Figure 2 C), even after a second irradiation with 150 mJ (Figure 2 B).[4]

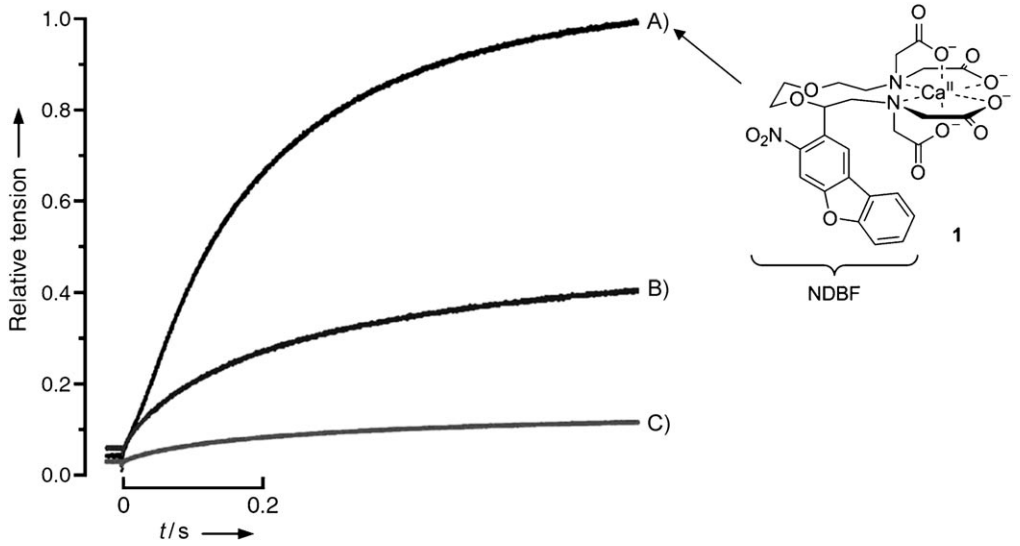

Figure 2. A) Exposure of a single-skinned cardiac trabecula to the caged calcium **1** (1 mM), followed by flash photolysis. B)–C) Identical experiments performed by using a classical *ortho*-nitrobenzyl analogue of **1** and one or two irradiations; this led to lower tension recordings. Adapted by permission from Macmillan Publishers Ltd: *Nat. Methods* **2006**, *3*, 35–40, Copyright 2006.

Other examples of the steric blocking of intermolecular interactions by caging groups include a photocaged small molecule inducer of gene expression (see 3.1), a light-activated DNA polymerase (see 3.2), and a GTPase–LOV fusion protein (see 3.3).

2.2 Inhibition of covalent interactions through photocaging

The inhibition of covalent bond formation with caging groups is much less common than the steric blocking of interactions. However, an example of the complete inhibition of enzymatic activity through caging was recently reported in the photochemical activation of Cre recombinase.[15]

A versatile approach for the site-specific caging of proteins is the unnatural amino acid mutagenesis in pro- and eukaryotic cells through the addition of an orthogonal tRNA/tRNA synthetase pair to the cellular translational machinery.[16] Here, a tRNA synthetase was engineered to accept an *ortho*-nitrobenzyl tyrosine as its substrate and to charge the corresponding tRNA with this caged amino acid.[17] This tRNA then allows for site-specific in vivo incorporation of the caged amino acid into proteins by the ribosome in response to an amber stop codon, TAG. The in vivo incorporation of the caged tyrosine was employed in the caging of Tyr324 of Cre recombinase. This residue is essential for catalytic activity, because its phenolic hydroxyl group undergoes a nucleophilic attack onto the phosphodiester backbone of DNA and forms a covalent bond between the oligonucleotide and the protein in the recombination event.[15] Installation of an *ortho*-nitrobenzyl caging group prevents the hydroxyl group from conducting a nucleophilic attack, and thus completely abrogates recombination activity. Identification of a tyrosine residue crucial for enzymatic activity is a prerequisite for the application of this caging approach. Importantly, catalytic activity of Cre recombinase could be restored through a brief UV irradiation (365 nm). This enabled the photochemical deactivation of DsRed expression and the photochemical activation of green fluorescent protein (GFP) expression after transfection into human embryonic kidney cells (Figure 3).

Other caged amino acids that have been site-specifically introduced into proteins by using the same unnatural amino acid mutagenesis methodology include caged cysteine,[18] serine,[19] and lysine.[20]

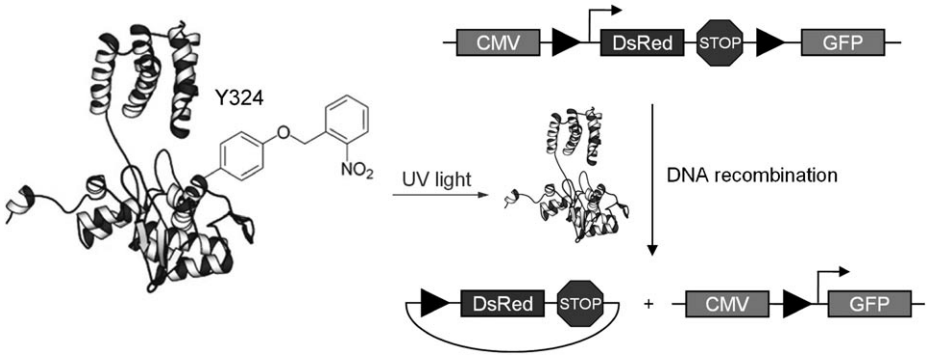

Figure 3. Cre recombinase was rendered completely inactive through site-specific incorporation of an *ortho*-nitrobenzyl-protected tyrosine residue at position Y324. Irradiation with UV light (365 nm, 5 min) restores activity and triggers DNA recombination in HEK293T cells. Photochemical activation of GFP and simultaneous deactivation of DsRed was achieved in mammalian cell culture through the excision of the DsRed gene and a stop codon through DNA recombination between two loxP sites (black triangles). CMV = cytomegalovirus promoter.

Another example of the blocking of covalent interactions by caging groups includes a small molecule–RNA interaction in mammalian cells. A full-length hammerhead ribozyme introduced into the 5′-untranslated region (UTR) of a gene leads to deactivation of translation through self-cleavage and removal of the 5′ cap from the mRNA.[21] The natural antibiotic toyocamycin inhibits ribozyme function through covalent introduction into the ribozyme, thereby inducing gene expression in mammalian cell culture.[22] Consequently, a caged analogue of toyocamycin is incapable of undergoing covalent bond formation with the RNA and does not induce gene expression until decaged through irradiation with UV light of 365 nm, as shown in a luciferase assay.[22] Another example is the site-specific covalent modification, and thus inhibition, of active-site serine residues in proteases with photocaging groups.[23] Examples that do not involve the inhibition of crucial covalent-bond formation through caging groups, but instead use covalent-bond formation after UV irradiation and activation have been reported with a caged tamoxifen molecule[24] and the LOV protein domain discussed in section 3.3 below.

3. The Activity of Caged and Uncaged Molecules Determines Biological Applicability

In an optimal scenario (Figure 4A), a caged molecule is completely inactive and biological activity is fully restored upon light irradiation. However, in real-world applications, this ideal

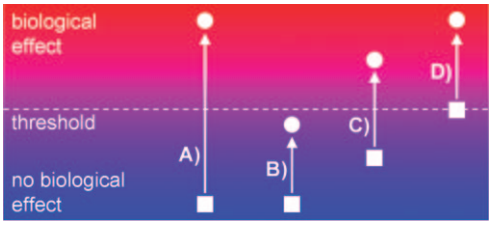

Figure 4. Potential scenarios for the biological activity of caged molecules before (■) and after (●) irradiation. The biological activity increases from blue to red; however, a cellular environment often displays a threshold of biological activity to be reached before activation of the desired biological effect.

situation is often not attainable, as decaging can lead to activity falling short of reaching the threshold necessary for induction of the desired biological effect (Figure 4B), or a caged molecule could still exhibit biological activity that might contribute to "leakiness" in the biological assay (Figure 4C–D). Several examples of light-activated molecules that fall into the different categories are discussed below, and their successful application in the light regulation of biological function is presented. All examples involve the use of caging group(s) to sterically block molecular interactions.

3.1 Scenario A): The caged molecule is completely inactive and biological activity is quantitatively restored upon irradiation

An example for scenario A) in Figure 4 is the caged antisense agent discussed in section 2.1, which is wholly inactive and does not hybridize to RNA.[8] It can be completely decaged, and this enables stringent regulation of biological activity.

Another example for a light-activation according to process A) is the control of eukaryotic gene expression by using a photocaged small molecule inducer, namely caged isopropyl-β-D-thio-galactoside (IPTG). IPTG is used to activate the *lac* operon, and thus gene expression in bacterial cells, through binding to the lac repressor protein. The lac repressor binds to the *lac* operator sequence on double-stranded DNA, and thereby inhibits gene transcription by RNA polymerase.[25] The allosteric binding of IPTG to the lac repressor releases the protein from the DNA and allows for gene transcription. A crystal structure of IPTG bound to the lac repressor[26] reveals a tight binding pocket and the ability to sterically disrupt binding through installation of a caging group. Thus, the caged IPTG (**2**, Figure 5A, $\phi = 0.1$, $\varepsilon = 4533\ M^{-1}cm^{-1}$) is completely inactive and

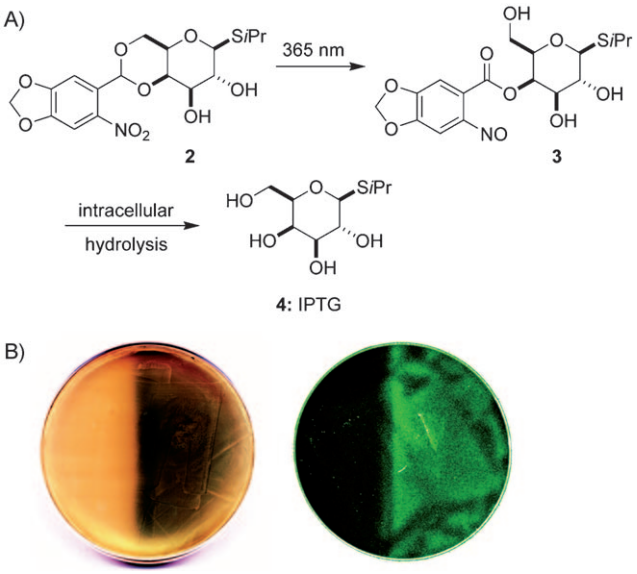

Figure 5. A) Light-irradiation of the caged IPTG (**2**) followed by intracellular hydrolysis of ester **3** to yield IPTG (**4**). B) Bacterial lithography with UV irradiation of 365 nm for 30 s while blocking the left half of a Petri dish. Two different reporter genes were employed; *lacZ* (left) and *GFP* (right) display a negative and a positive image, respectively. Adapted with permission from: *Angew. Chem. Int. Ed.* **2007**, *46*, 4290–4292. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

does not induce gene expression. UV irradiation (365 nm, 23 W, 5 min) converts **2** (which is taken up by the cells from the media) quantitatively into a 1:1 mixture of 4- and 6-carboxylates **3** (only the 4-isomer is shown), which are then intracellularly hydrolyzed (half-life of 1 h) to IPTG (**4**). The spatially restricted activation of IPTG and gene expression in a lawn of bacterial cells was visualized on plates using β-galactosidase or green fluorescent protein (GFP) reporter genes under control of the *lac* operator (Figure 5B).

Other examples of completely inactive, cell permeable caged small molecule activators and inhibitors of gene function include caged toyocamycin (see 2.2),[22] caged estradiol,[27] caged ecdysone,[28] and caged anisomycin.[29]

3.2 Scenario B): The caged molecule is completely inactive, but biological activity cannot be fully restored upon irradiation

If irradiation cannot fully restore biological activity (for example, through incomplete decaging, photochemical side reactions, or the inability of the molecule to reach a biologically active conformation after caging group removal), an increased concentration of the caged effector molecule can be used to counteract this issue. This is only possible if the caged molecule does not show any biological activity (leakiness) that prevents the higher concentration of the caged molecule from surpassing the threshold for biological activity. An example for this is a recently caged *Taq* DNA polymerase,[30] a classical DNA polymerase commonly used in polymerase chain reactions. The unnatural amino acid methodology described under section 2.2 was used to introduce an *ortho*-nitrobenzyl tyrosine amino acid at residue Tyr671. Positioning a sterically demanding caging group site-specifically on Tyr671, located in the active site of the enzyme, blocks the incoming nucleotide triphosphate from reaching the active site and thus completely inhibits the enzymatic activity of the polymerase. A brief UV irradiation (365 nm, 5 min) restored only two thirds of the enzyme's original activity, and further irradiation did not increase the activity. However, because the caged polymerase was completely inactive, a light-activated hot-start PCR could be performed by increasing the amount of the caged enzyme from 0.250 to 0.725 $\mu g\,\mu L^{-1}$, compared to wild-type *Taq*.

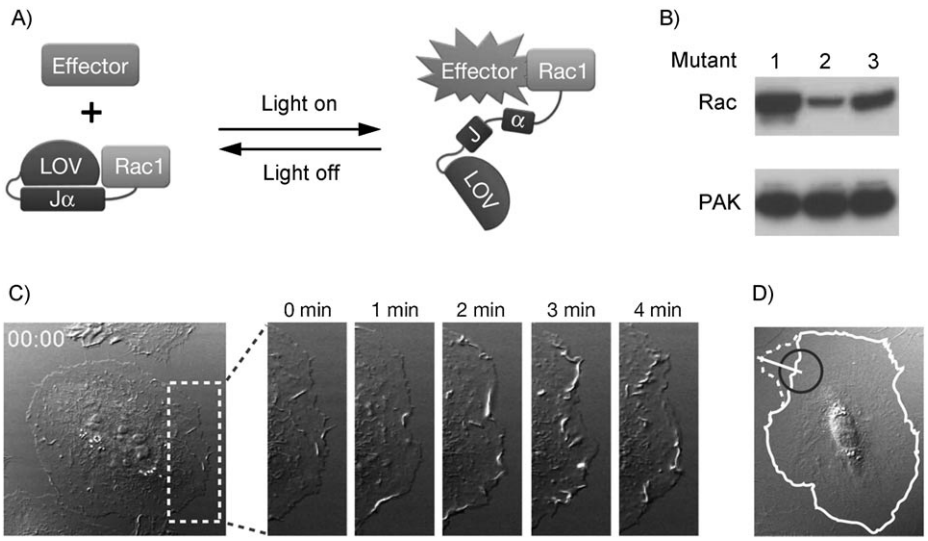

Figure 6. A) Schematic representation of the reversible LOV–Rac1 light-regulation. B) Pull-down assays of selected LOV–Rac1 mutants in the dark. C) Irradiation of HeLa cells expressing LOV–Rac1 induced lamellipodial protrusions and membrane ruffles within minutes. D) Spatially restricted irradiation enables localized control of Pac1 activity. Adapted by permission from Macmillan Publishers Ltd: *Nature* **2009**, *461*, 104–108, copyright 2009.

3.3 Scenario C): The caged molecule still exhibits biological activity, but this activity falls below a threshold necessary for the biological effect

Even if biological activity is not fully abrogated through the caging process, the molecule can still be used in a biological context if the residual activity is below the threshold of the desired biological effect. A recent example of regulating an important cellular function with a genetically encoded "caged" protein was reported in the reversible activation of the GTPase Rac1.[31] Rac1 regulates a variety of intracellular processes, including cell cycle, adhesion, and motility. Rac1 was light regulated through the generation of a fusion protein with the light oxygen voltage (LOV) domain from phototropin, which has previously been applied to the light-regulation of a histidine kinase,[32] the Trp repressor,[33] and dihydrofolate reductase.[34] Here, the LOV–Rac1 fusion protein is inactive in the dark due to steric blocking of the effector binding site of Rac1 by the LOV domain-Jα motif (Figure 6A). Light irradiation at 458 nm leads to covalent bond formation of the flavin cofactor and the protein; this induces a conformational change that results in dissociation and unwinding of the Jα helix, which subsequently induces Rac1 activity. The substantial reduction in Rac1 binding to its effector, PAK, in the absence of light irradiation was demonstrated in pull-down assays of several LOV–Pac1 mutants (only three mutants are shown in Figure 6B). Although complete inactivity was not obtained in mutant 2 (mutants 1 and 3 are more active), the observed tenfold reduction in binding affinity was sufficient for cell biological investigations. HeLa cells expressing LOV–Pac1 were quiescent until irradiated with light of 458 nm; this almost instantaneously induced lamellipodial protrusions and membrane ruffles along the cell edges (Figure 6C). Moreover, irradiation of a 20 μm spot at the cell edge generated large localized protrusions (Figure 6D). Thus, the obtained reversible light regulation of Pac1 activity was sufficient to generate polarized cell movement with high spatiotemporal resolution, although the caged protein still maintained some background activity.

3.4 Scenario D): The caged molecule is active enough to exhibit undesired background activity in the biological experiment

A caged molecule with activity that is not completely abrogated and thus produces background activity in its caged state can still be employed if its concentration is lowered below the biological threshold, and its decaging still leads to the induction of the desired biological function. An example of this situation can be found in a caged doxycycline molecule that has

been successfully used in the photochemical control of eukaryotic gene function by using the established Tet-On system.[35,36] This technology has recently been applied to the photochemical control of GFP expression in brain slices from transgenic mice.[36] Moreover, surgically removed embryos from the same mouse line were incubated with caged doxycycline ($\phi = 0.07$, $\varepsilon = 11\,400\ \text{M}^{-1}\,\text{cm}^{-1}$) for 24 h in a mouse whole-embryo culture system. Whole-embryo irradiation (290–370 nm, 15 s) was performed and GFP fluorescence was imaged (Figure 7 A). A low

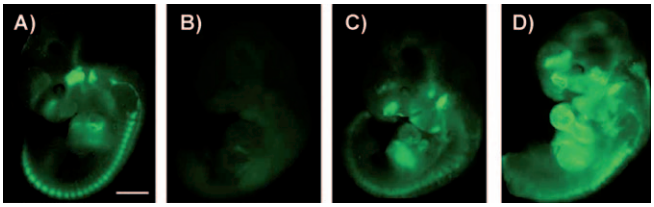

Figure 7. Photoactivated gene function in a mouse embryo by using caged doxycycline as a small molecule inducer of gene expression. A) Fluorescence image of widespread GFP fluorescence in a control embryo at day 10.5, incubated with 20 μM doxycycline. B) Fluorescence image of an embryo incubated with 2.6 μM of caged doxycycline without irradiation, C) after a single 15 s pulse of UV irradiation, and D) after a second irradiation pulse 3 h later. Adapted by permission from Macmillan Publishers Ltd: *Nat. Methods* **2009**, *6*, 527–531, copyright 2009.

level of background fluorescence in the nonirradiated embryo (Figure 7 B) was achieved only in cases in which an approximately tenfold lower concentration of the caged molecule was used (compared to the typically employed concentration of the noncaged small molecule inducer of gene expression). This leakiness may be caused by the caged doxycycline still binding to the Tet-repressor with reasonable affinity.[36] However, this issue could be resolved through the reduction of compound concentration, because the irradiated embryo displayed GFP levels comparable to an embryo that was exposed to noncaged doxycycline (Figure 7 A, C and D), especially if two subsequent irradiations were conducted.

4. Conclusions

The photochemical regulation of biological processes can be achieved through a variety of different caging methodologies. Examples of the applications of caging groups to the regulation of molecular function involve the steric blocking of molecular interactions and the inhibition of covalent bond formation. The presented studies demonstrate the application of these concepts to the photochemical regulation of biological processes in a variety of different situations. To date, the majority of the examples of caged molecules in the literature involve the regulation of simple reporter systems and other proof-of-principle examples. Further developments will have to show the applicability of photocaging concepts to the investigation of important biological questions.

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Keywords: biological activity · cage compounds · chemical biology · light regulation · photochemistry

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