Protein Dimerization

DOI: 10.1002/anie.201409196

Light-Induced Protein Dimerization by One- and Two-Photon Activation of Gibberellic Acid Derivatives in Living Cells**

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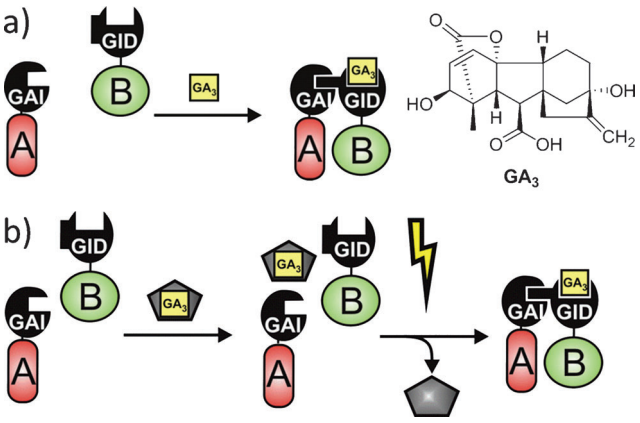
Abstract: *We developed a highly efficient system for light-induced protein dimerization in live cells using photo-caged derivatives of the phytohormone gibberellic acid (GA_3). We demonstrate the application of the photo-activatable chemical inducer of dimerization (CID) for the control of protein translocation with high spatiotemporal precision using light as an external trigger. Furthermore, we present a new two-photon (2P)-sensitive caging group, whose exceptionally high two-photon cross section allows the use of infrared light to efficiently unleash the active GA_3 for inducing protein dimerization in living cells.*

Cellular events are spatiotemporally regulated by the dynamic interplay between biomolecules within highly complex networks. The ability to rapidly perturb specific molecular interactions within such networks is of major importance in investigating the role of individual biomolecules in the temporal and spatial context of a living cell or organism. Chemical inducers of dimerization (CIDs) are small molecules that bring proteins in close proximity by binding to protein tags fused to the proteins of interest (POI).[1] Induced dimerization can be used to force interaction between proteins or to remove the protein of interest from its subcellular place of action and thereby allows the specific manipulation of cellular processes. Recently, a new CID system based on the diterpene gibberellic acid (GA_3) was reported.[2] Gibberellins are plant hormones that control diverse aspects of growth and development in plants. The bioactive gibberellic acid GA_3 is binding to the receptor "gibberellin insensitive dwarf" (GID1)[3] and thereby induces a conformational change that results in the strong binding of the GID1-GA_3 complex to the protein GAI ("gibberellin insensitive") (Scheme 1 a).[4] It was shown that a cell permeable acetoxymethyl derivative of gibberellic acid (GA_3-AM) gets converted into active GA_3 upon de-esterification by endogenous esterases in the cytosol of mammalian cells. Furthermore it has been shown that the first 92 amino acids of GAI (GAI_{1-92}) are sufficient to form a stable complex with GID1-GA_3.[2b] However, the use of CIDs is limited, as it does not provide precise control in time and space. Photoremovable protecting groups, also known as photo-caging groups, can be attached to small effector molecules and can influence the biological activity of the effector molecule.[5] Light provides a high level of temporal and spatial control[6] and is an attractive non-invasive external trigger that has successfully been used to manipulate localization,[7] interaction,[8] or activity[7b,9] of biomolecules in living cells. The work presented herein combines the properties of the GA_3 as a CID with the use of light-sensitive caging groups resulting in



Scheme 1. General scheme of a) gibberellic acid induced protein dimerization of the GA_3-receptor GID1 and GAI brings proteins A and B in close proximity (GA_3: structural formula shown top right); b) photo-caged gibberellic acid can be converted into active gibberellic acid, inducing protein dimerization upon decaging with light.

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[**] R.W. acknowledges funding from the Deutsche Forschungsgemeinschaft DFG (SPP1623, WO 1888/1-1). K.M.S. thanks the Chemical Industry Fund of the German Chemical Industry Association (VCI) for a scholarship. We thank Prof. U. Müller as well as the Nikon Imaging Center Heidelberg for access to microscopes. We gratefully acknowledge Dr. C. Tischer and the Advanced Light Microscopy Facility (ALMF) at the European Molecular Biology Laboratory (EMBL) for support with 2P-activation microscopy. We thank M. Best, S. Hauke, and M. Petzoldt for experimental support.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201409196.

a photo-activatable CID that enables the control of its dimerization activity with high spatiotemporal precision (Scheme 1 b).

Until now, only two other photo-activatable CIDs have been reported,[9a,10] both based on the established CID rapamycin[1c,11] with one of them limited to extracellular photo-release.[10b] Though, applications of rapamycin in mammalian cells are problematic because of the endogenous target mTOR (mammalian target of rapamycin). For the plant hormone GA_3 however, no target in mammalian cells has been reported to date.

To identify molecular residues in GA_3 that are preferable for a photo-cleavable modification, we analyzed the available crystal structure of GID1 in complex with the effector GA_3.[12] From the molecular interactions between GA_3 and its receptor we identified the C-6 carboxy function of GA_3 to be of particular importance. Nevertheless, GID1 encloses GA_3 into a very narrow binding pocket which means other modifications are likely to interfere with binding as well. Inoue et al. could demonstrate that GA_3-AM remains without biological effects unless cleavage by esterase results in the release of the bioactive GA_3.[2b] The synthesis of the gibberellin GA_4, caged at its carboxylic function has been described in the 1990s, but to our knowledge has not found any application.[13] To test whether photo-protection at this position can be used for photo-induced protein dimerization, we synthesized the three GA_3-esters $pcGA_3$-1, $pcGA_3$-2, and $pcGA_3$-3 containing different photo-caging groups with different spectral properties (Figure 1 a, Table S1). $pcGA_3$-1 is caged with the established 2-(4,5-dimethoxy-2-nitrophenyl)propyl caging group (DMNPP),[14] $pcGA_3$-2 with the recently reported (2-(4′-bis((2-methoxyethoxy)ethyl)amino)-4-nitro-[1,1′-biphenyl]-3-yl)propan-1-ol) caging group (EANBP),[15] and $pcGA_3$-3 is furnished with a newly developed π-extended 2-(*o*-nitro-phenyl)propyl caging group with an absorption maximum at around 400 nm (Figure 1 b).

The π-extended caging groups were synthesized starting from 1-bromo-4-nitrobenzene and *N*,*N*-bis(2-(2-methoxyethoxy)ethyl)aniline, respectively, with aryl coupling in the last step by cross-coupling procedures providing a flexible method for derivatization. Coupling of the caging groups to gibberellic acid was finally achieved by Yamaguchi esterification. The full synthetic scheme to photo-caged gibberellic acid derivatives is in the Supporting Information (Scheme S1–S4).

The one-photon photo-chemical properties of all the photo-caged compounds were investigated by UV/Vis spectroscopy (Figure 1 b, Figure S1). UV/Vis-spectra of $pcGA_3$-3 were recorded during photolysis by irradiation at 412 nm and showed absorbance bands appearing at 350 nm and 450 nm to 550 nm while the initial absorbance band at 400 nm gradually diminished. The two isosbestic points at 366 nm and 450 nm indicate a selective and direct photolysis reaction (Figure 1 b). Moreover, the extended π-system makes the caging groups of $pcGA_3$-2 and $pcGA_3$-3 suitable for decaging by two-photon absorption (2PA) upon irradiation at 800 nm.[16] The EANBP caging group of $pcGA_3$-2 has been reported to be an efficient two-photon(2P)-caging group for the neurotransmitter γ-aminobutyric acid (GABA).[15] The 2-(*o*-nitro-phenyl)propyl caging group of $pcGA_3$-3 with the π-extended diphenylacetylene core structure bearing a functionalized dialkylamino moiety in *para*-position has not been reported previously. The 2P-decaging efficiencies of $pcGA_3$-2 and $pcGA_3$-3 were compared by irradiation at 800 nm with subsequent reversed-phase HPLC analysis monitoring the disappearance of the starting material (see Supporting Information). The determined decaging efficiency for the new 2P-caging group of pcGA3-3 was about 1.8 ± 0.4 times higher than for the EANBP caging group used in pcGA3-2.

Having successfully synthesized a series of photo-caged GA_3 derivatives we tested their ability to induce protein dimerization in live cells upon light irradiation. We used a fluorescent read out to show the sequestration of cytosolic EGFP-GID1 (EGFP = enhanced green-fluorescent protein) to the mitochondria. Therefore we co-transfected COS-7 cells with the cytosolic EGFP-GID1 and the outer mitochondrial membrane localized TOM20-mCherry-GAI_{1-92}. In the presence of GA_3 the EGFP-GID1-GA_3 complex is formed and binds to GAI_{1-92} resulting in sequestration to the outer mitochondrial membrane. The binding

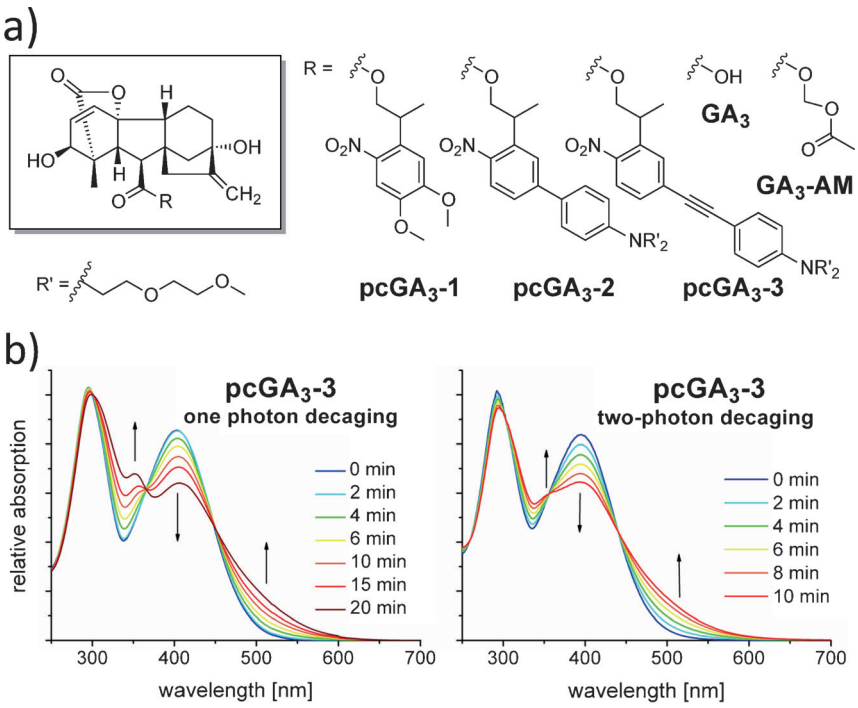

Figure 1. a) Structural formula of GA_3, its acetoxymethyl ester GA_3-AM, and the photo-caged GA_3-derivatives $pcGA_3$-1, $pcGA_3$-2, and $pcGA_3$-3 used in this study. b) Left: Changes in the UV/Vis spectrum of $pcGA_3$-3 during photolysis at 412 nm (60 μM, phosphate buffer pH 7.4 with 3 vol% DMSO). Right: changes in the UV/Vis spectrum of $pcGA_3$-3 during two-photon photolysis at 800 nm (120 μM, phosphate buffer pH 7.4 and acetonitrile 1:1 vol%).

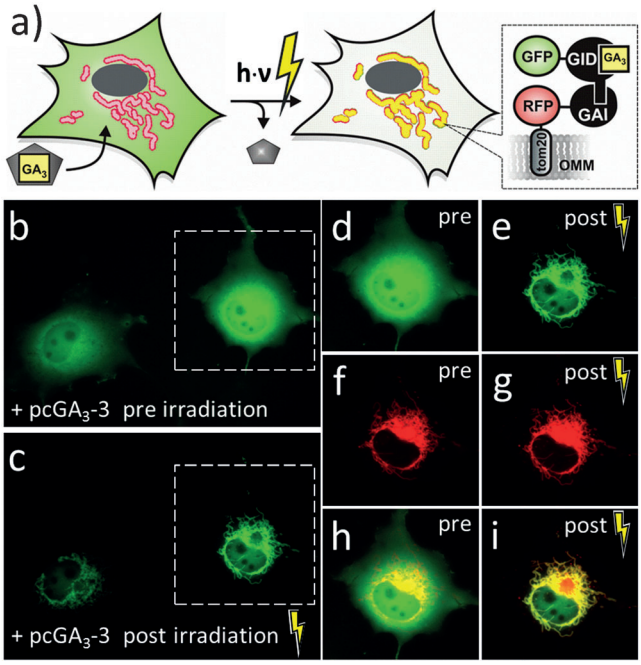

Figure 2. Photo-induced protein dimerization using the photo-caged gibberellic acid derivative pcGA$_3$-3. a) Schematic presentation of the fluorescence readout for photo-induced protein dimerization. Addition of caged gibberellic acid remains without effect until light of a suitable wavelength is used to release the active dimerizer GA$_3$. b–i) Epifluorescence microscopy images of photo-induced protein dimerization in COS-7 cells expressing EGFP-GID1 and TOM20-mCherry-GAI$_{1–92}$ incubated with pcGA$_3$-3 for 15 min with (1 μM, in complete medium (0.001% DMSO) at 37°C). Images show the localization of EGFP-GID1 after incubation with pcGA$_3$-3 before and after 10 s irradiation with DAPI filtered light (377/50 nm). b) Image of GFP-channel after incubation with pcGA$_3$-3 before irradiation with DAPI-filtered light and c) after irradiation with DAPI filtered light. d), f), h) Magnification of the white box in (b); d) green channel, before irradiation, f) red channel, before irradiation, and h) both channels merged, before irradiation. e), g), i) Magnification of the white box in (c); e) green channel, after irradiation, g) red channel, after irradiation and i) both channels (e) and (g) merged, after irradiation.

can be observed by co-localization of the fluorescent signals from EGFP and mCherry at the mitochondria (Figure 2a). Addition of GA$_3$-AM, the cell-permeable acetoxymethyl derivative, led to efficient co-localization within seconds (even using concentrations as low as 100 nM in complete medium) as shown previously. GA$_3$ however, did not result in co-localization at the mitochondria, indicating that GA$_3$ itself is not able to pass the cell membrane.[2b] This result led us to the assumption that, in general, esters of GA$_3$ possess good cell permeability and we expected similar good cell permeability for the photo-caged esters. GA$_3$-AM is converted into the active GA$_3$ by endogenous esterases within the cell on a very short timescale. To investigate the esterase-mediated release of GA$_3$ from the caged GA$_3$-esters, we synthesized the 2-phenylpropan-1-ol ester of gibberellic acid (GA$_3$-PP, Figure S3). The compound's structure is closely related to the structure of pcGA$_3$-1, missing the *o*-nitro group thus rendering the molecule GA$_3$-PP light-insensitive. Incubation of COS-7 cells expressing EGFP-GID1 and TOM20-mCherry-GAI$_{1–92}$ with GA$_3$-PP (1 μM) for 3 h did not result in observable co-localization suggesting that the photo-caged GA$_3$-esters are poor substrates for endogenous esterases. Moreover, we can exclude the possibility that spontaneous hydrolysis results in significant amounts of GA$_3$ within the timescale of our experiments (Figure S3).

Most notably, the bioactivity of GA$_3$ in plants is regulated by a number of different pathways among which is the methylation of the C-6 carboxylic acid function.[17] It has been shown that *Arabidopsis thaliana* encode gibberellin methyltransferases (GAMTs) catalyze the methylation of the C-6 carboxy group of gibberellins[18] to convert gibberellic acid into the biologically inactive C-6 methyl ester. By caging the C-6 carboxy group we created a photo-activatable CID simply by utilizing the naturally evolved structure–activity relationship of gibberellins.

As we did not observe co-localization of GID1 and GAI$_{1–92}$ upon addition of the photo-caged gibberellic acid derivatives, we tested whether we can recover the GA$_3$'s bioactivity by irradiation with light. Therefore we incubated COS-7 cells expressing EGFP-GID1 and TOM20-mCherry-GAI$_{1–92}$ with the DMNPP caged pcGA$_3$-1 for 5 min and irradiated for 3 s with standard DAPI (DAPI = 4′,6-diamidino-2-phenylindole) illumination (377/50 nm). Promptly after irradiation we observed translocation of EGFP-GID1 to the mitochondria (Movie S1, Figure S4) and complete sequestration was achieved within seconds. The co-localization of the EGFP and mCherry signals at the mitochondria clearly indicates that we efficiently recovered substantial amounts of biological active GA$_3$.

After having shown the general applicability of pcGA$_3$-1 as a photo-activatable CID in live cells, we wanted to demonstrate the spatial resolution of this method by activating pcGA$_3$-1 in a single cell using a 405 nm laser for photoactivation in a defined region of interest (ROI). We chose two positively transfected cells in the field of view, first irradiating a ROI within only one of the cells, resulting in translocation of EGFP-GID1 to the mitochondria in this specific cell but not in the neighboring one. Subsequent activation of pcGA$_3$-1 in the second, neighboring cell likewise led to sequestration of EGFP-GID1 to the mitochondria which demonstrates the high spatial control of the photo-activatable CID (Figure S5).

Next, we tested the photo-caged gibberellins pcGA$_3$-2 and pcGA$_3$-3 in the same experimental setup. Both compounds can be efficiently activated with epifluorescent DAPI illumination within seconds (Figure 2b–i). However, both compounds have red-shifted absorption spectra compared to pcGA$_3$-1 (Figure S1) and we noted that even irradiation with GFP-filtered light for 10 s resulted in noticeable photo-mediated release of GA$_3$ (Figure S6). This process has to be taken into account when using these compounds in combination with a green fluorescent protein (GFP) read out. Nevertheless, the red-shifted absorption is of advantage, as it allows chromatically orthogonal photo-activation of pcGA$_3$-2 or pcGA$_3$-3 when combined with other photo-activatable effectors which are insensitive to light of 470 nm.[6,19] Finally we wanted to utilize the excellent 2P-absorption properties of pcGA$_3$-3 to unleash the active GA$_3$ in live cells by near-infrared laser irradiation at 800 nm. For

this purpose we used a microscope setup which offers the separate activation of a given ROI using a femtosecond Ti:sapphire laser, whereas image acquisition can be performed by standard one-photon confocal fluorescence microscopy.

To avoid decaging of $pcGA_3$-3 by one-photon absorption in the course of image acquisition, we replaced the EGFP in EGFP-GID1 by the far-red-fluorescent protein mPlum and removed the mCherry from the mitochondrial localized construct. The light for mPlum excitation at 594 nm is not able to remove the photo-caging group from $pcGA_3$-3. We incubated COS-7 cells expressing mPlum-GID1 and TOM20-$GAI_{1–92}$ with $pcGA_3$-3 for 5 min and irradiated a ROI in a single cell using a Ti:sapphire laser at 800 nm with 80 mW. Time-lapse imaging after 2P-activation showed that the previously evenly distributed mPlum-GID1 significantly accumulated at the mitochondria clearly indicating efficient 2P-mediated decaging of $pcGA_3$-3 under these conditions (Figure 3, Movie S2). As red light allows for deep tissue

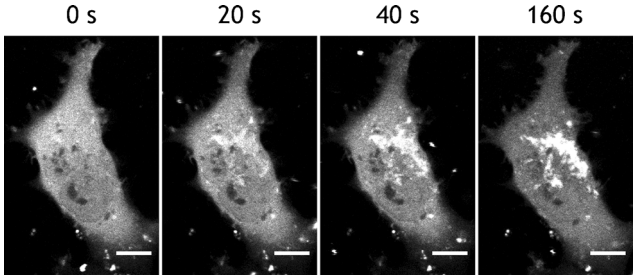

Figure 3. Efficient decaging of $pcGA_3$-3 by 2P-absorption induces translocation of mPlum-GID1 to the mitochondria within seconds. Confocal images of a COS-7 cell expressing mPlum-GID1 and TOM20-$GAI_{1–92}$ incubated for 5 min with $pcGA_3$-3 (20 μM, in complete medium (0.1% DMSO)). Activation was performed using a Ti:sapphire laser centered at 800 nm with 80 mW (see Supporting Information for additional details). From left to right images show cellular mPlum-GID1 distribution before activation as well as 20 s, 40 s, and 160 s post activation. Scale bars correspond to 10 μm.

penetration and 2PA enables high *z*-resolutions, these findings represent a major step forward for the application of photo-activatable CIDs in cell-cultures to applications inside tissues and living animals.[20]

Altogether, we presented photo-caged gibberellin derivatives for light-induced protein dimerization in live cells. The new photo-activatable CIDs can be activated by light of different wavelengths with high spatial and temporal precision. In the co-localization assay we did not observe basal activity of the caged GA_3 esters, which is an essential criterion for photo-caged effector molecules. Further, we presented the release of GA_3 by one- and two-photon absorption, which broadens the potential application of the herein presented photo-activatable CIDs.

Received: September 17, 2014
Revised: November 28, 2014
Published online: January 13, 2015

Keywords: caged compounds · chemical inducer of dimerization · photolysis · protein–protein interactions · two-photon activation

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