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COMMUNICATION

Caged glucosamine-6-phosphate for the light-control of riboswitch activity†‡

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We have synthesized a light-activatable ("caged") derivative of glucosamine-6-phosphate (GlcN6P), which only upon irradiation becomes a cofactor for the *glmS* riboswitch. This *glmS* riboswitch maintains its activity when embedded in the 3′-untranslated region of eukaryotic mRNA molecules and caged GlcN6P reduces the amount of translated EGFP upon irradiation with light *in vitro*.

Riboswitches are non-coding RNA elements mainly located in the 5′-UTR (untranslated region) of bacterial genes.[1] They specifically bind to small metabolites, such as thiamin pyrophosphate, adenine, lysine and others. Upon metabolite binding conformational changes within the RNA structure occur, resulting in gene expression control.[2] According to their regulatory properties riboswitches are sub-divided into three functionally distinct groups: i) riboswitches that control transcription termination ii) riboswitches that control translation initiation and iii) riboswitches that act as metabolite-dependent ribozymes. It has been demonstrated that riboswitches can also control splicing in eukaryotes.[3] To date only one metabolite-dependent ribozyme, the so-called *glmS* riboswitch, has been described.[4,5] This riboswitch is found in the 5′-UTR of the bacterial mRNA that encodes the gene for glucosamine-6-phosphate synthetase (glmS). The *glmS* ribozyme is specifically activated by glucosamine-6-phosphate (GlcN6P), which is thought to act as a true cofactor rather than an allosteric effector.[6] GlcN6P is the metabolic product of the glmS-catalysed reaction and initiates the *cis*-cleavage of the corresponding mRNA, thus representing a means for the feedback inhibition of metabolic pathways in bacteria. The *glmS* ribozyme element is located upstream of the monocistronic coding region and can be found in many bacteria, including *Bacillus subtilis* and human pathogens, such as *Staphylococcus aureus*.[4,5]

Several artificial gene expression systems have been described in the past decade, which use synthetic aptamers as gene regulatory elements.[7] Other groups made use of hammerhead ribozyme variants to gain control over the expression of certain genes with exogenously applied inhibitors or activators.[8,9] However, these approaches are limited in regard to regulation efficiency and compatibility of the hammerhead variants with the intracellular environment. Recently, extended hammerhead ribozyme variants were described that are thought to be adaptable to a cellular environment and aptazymes that are based on these variants might represent ways for the artificial control of gene expression.[9,10] In contrast and due to their natural activity, riboswitches are excellently suited for the control of gene expression. Their adaptation to organisms other than bacteria might open a new route for the exogenous control of gene expression. We hypothesized that a variant of GlcN6P that bears a photolabile group, a so-called "cage",[11] at a strategic position might be incapable of inducing *glmS* ribozyme mediated *cis*-cleavage. According to our hypothesis light irradiation would cause GlcN6P release and thus induce ribozyme cleavage. This mechanism would allow the exogenous control of riboswitch activity and consequently gene expression by light. The advantage would be that well-established techniques to generate and manipulate light can then be used to irradiate at moments of choice in arbitrary regions of interest. Artificial ribozymes have been put under a light trigger before: For example, Breaker *et al.* reported on the selection and light control of a cAMP-responsive ribozyme,[12] Deiters *et al.* on a hammerhead-ribozyme derivative that responds to caged theophylline,[13] Sen *et al.* on the reversible photocontrol using a small-molecular photoswitch[14] and Perrin *et al.* on the incorporation of a caged adenosine into a DNAzyme.[15] However, our current study reports for the first time on the photocontrol of a naturally occurring riboswitch with a caged version (**1**) of its naturally occurring regulatory cofactor (**2**). GlcN6P is a major precursor for the cell-wall biosynthesis in bacteria. In contrast, eukaryotic cells use GlcN as a starting point for the synthesis of glycosaminoglycans and *N*-glycosylated proteins, whereas GlcN6P is a temporary intermediate. Protein modification with glycanes mainly takes place in the endoplasmatic reticulum and Golgi complex. Thus, due to the low levels of GlcN6P in the cytosol of eukaryotic cells the GlcN6P-dependent *glmS* riboswitch might be useful as a cytoplasmically

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located gene expression control element in eukaryotes, when embedded in a corresponding mRNA.

Based on previous findings we chose the amino-position of GlcN6P for being a suitable strategic location that allows efficient caging and thereby rendering GlcN6P incapable of initiating *glmS* ribozyme *cis*-cleavage (Fig. 1).[5,16] To synthesize the caged GlcN6P (**1**) we started from GlcN hydrochloride (**3**) and isolated the amino function for further derivatization according to a literature procedure (→**4**, Scheme 1).[17] The alcohol of the caging group was then introduced using phosgene (→**5**). Then the primary hydroxyl group was isolated (→**6**) and phosphorylated using $POCl_3$ and proton sponge. Final deprotection afforded the caged GlcN6P (**1**).

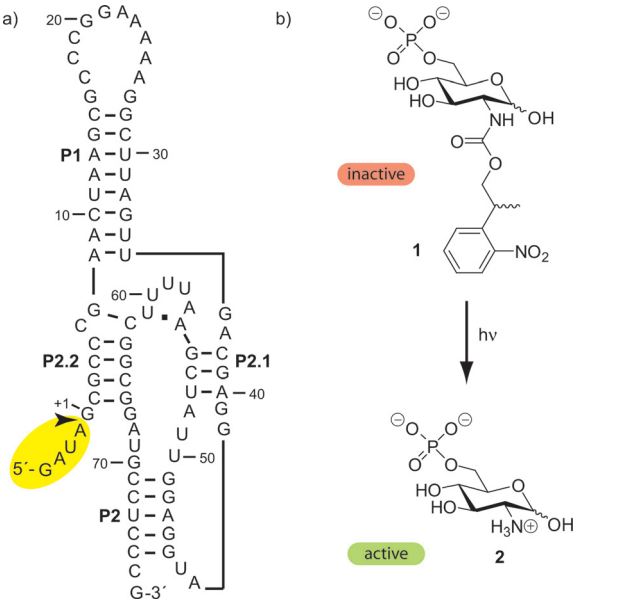
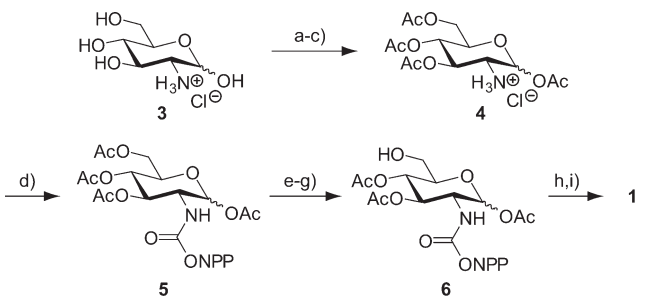


Fig. 1 a) A schematic representation of the secondary structure of the minimal core motif of the *glmS* riboswitch from *B. subtilis*. The cleavage site is indicated by an arrowhead and the sequence that is cut off is highlighted. b) Light-dependent uncaging to generate the active riboswitch effector **2** from the inactive derivative **1**.



Scheme 1 Reaction conditions: a) $MeOC_6H_4CHO$, NaOH, H_2O, 85%; b) Ac_2O, py, 89%; c) HCl, acetone, 88%; d) NPPOH, $COCl_2$, DMAP, Et_3N, py, CH_2Cl_2, 79%; e) NaOH, MeOH/THF, 75%; f) 1. TBDMSCl, DMAP, Et_3N, DMF, 2. Ac_2O, DMAP, py, 79%; g) AcOH, H_2O/THF, 95%; h) 1. proton sponge, $POCl_3$, MeCN, 2. $Et_3N{\cdot}H_2CO_3$, 75%; i) NaOH, MeOH/H_2O, 89%. NPP = 2-(2-nitrophenyl)-propyl.

Next, we analysed the synthesized caged GlcN6P for its ability to induce *glmS* ribozyme self-cleavage in a light-dependent manner (Fig. 2). Therefore, we employed a reported assay format that enables the monitoring of metabolite-dependent ribozyme self-cleavage by employing fluorescence polarization.[18] We incubated a 5′-fluorescein labelled *glmS* ribozyme with increasing concentrations of either GlcN6P (**2**), glucosamine (GlcN, **3**) or the caged GlcN6P variant (**1**).

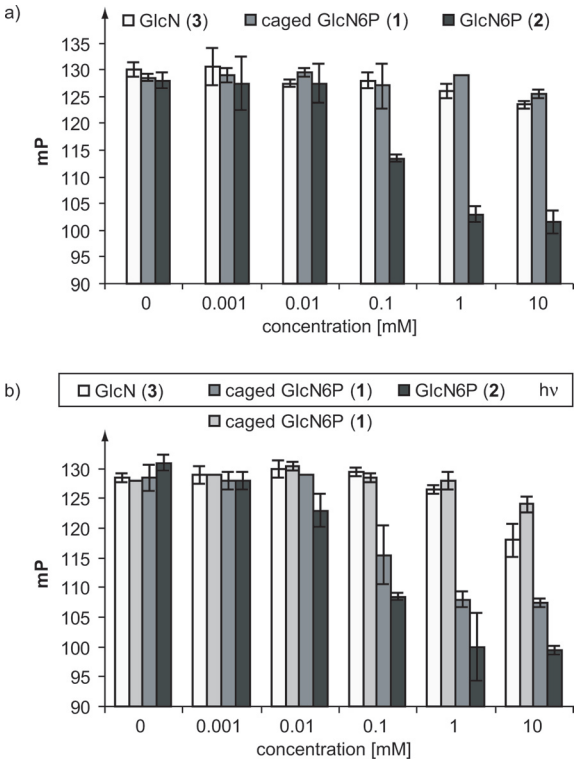


Fig. 2 Results of fluorescence polarization measurements in which the *glmS* ribozyme was incubated with the indicated glucosamine derivatives. Both sets of data are from the same microtiter plate, however, in the lower set the wells with the indicated samples (box) had been irradiated at 366 nm for 45 min with a UV hand lamp.

Metabolite-induced cleavage of the ribozyme leads to the release of a four nucleotides containing 5′-leader sequence (Fig. 1a, nucleotides highlighted in yellow), enabling visualization of the reaction by fluorescence polarization since the non-cleaved ribozyme and the released leader sequence vary considerably in length and molecular weight, resulting in significantly different fluorescence polarization values. As indicated in Fig. 2a, only GlcN6P was capable of inducing ribozyme cleavage after incubation for 15 min at 25 °C, whereas no cleavage was observed using GlcN (**3**) or the caged GlcN6P (**1**) at concentrations as high as 10 mM. In contrast, UV irradiation caused the light-induced GlcN6P release and thus induction of ribozyme cleavage was observed (Fig. 2b). Remarkably, the non-irradiated caged GlcN6P (**1**) did not induce ribozyme cleavage whereas GlcN (**3**) showed some induction of *glmS* ribozyme self-cleavage, which is in accordance with data found in the literature.[18,19]

Next, we studied the ability of caged GlcN6P to induce riboswitch activity in the context of an mRNA molecule. To achieve this we cloned the *glmS* riboswitch from *B. subtilis* into the 3′-UTR of the gene encoding for enhanced green fluorescent protein (EGFP) and generated mRNA transcripts equipped with

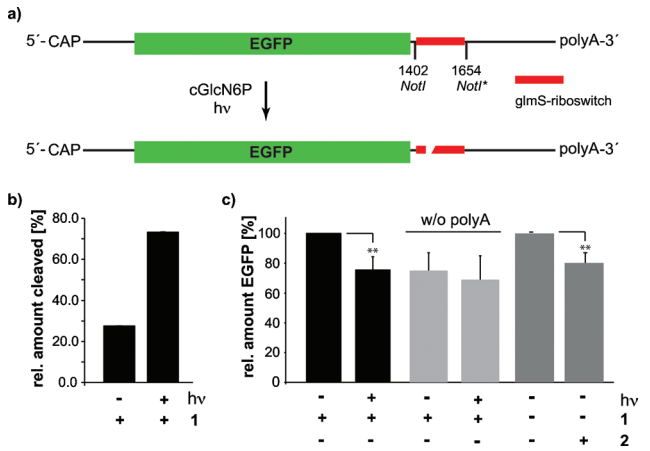


Fig. 3 Construction of an mRNA sensitive to GlcN6P. a) The *glmS* riboswitch (red) has been cloned into the NotI-site within the 3′-UTR of an EGFP (green) expressing plasmid. The corresponding mRNA equipped with a 5′-CAP and a 3′-polyA tail was generated through *in vitro* transcription. b) Addition of caged GlcN6P (**1**) and light (+) induces site-specific cleavage of the mRNA as detected by agarose gel analysis. c) *In vitro* synthesis of EGFP in the presence of caged GlcN6P (**1**) with and without light irradiation. Black bars: *in vitro* translation of EGFP from CAP-EGFP-polyA mRNA in the presence of caged GlcN6P (**1**) with (+) and without light-irradiation (−); light grey bars: *In vitro* translation of EGFP from CAP-EGFP mRNA in the presence of caged GlcN6P (**1**) with (+) and without light-irradiation (−); dark grey bars: *In vitro* translation of EGFP from CAP-EGFP-polyA mRNA in the presence (+) or absence (−) of GlcN6P (**2**). Bars represent the standard deviation of fluorescence emission intensity obtained from five replicate experiments. Statistical significance was determined using a one-tailed Student's t-test indicated by **.

a 5′-CAP and a 3′-polyA by *in vitro* transcription (Fig. 3a).[20] Transfection of a plasmid containing this construct into HeLa cells proved that the cytosolic levels of GlcN6P are not high enough to effect significant downregulation because the same bright green fluorescence was observed with or without the *glmS* ribozyme (Supplementary Information†). The mRNA construct was subsequently investigated regarding light-regulated mRNA cleavage and inhibition of translation in the presence of caged GlcN6P (**1**) and GlcN6P (**2**). As shown in Fig. 3b, irradiation of caged GlcN6P (**1**) results in enhanced cleavage of the mRNA bearing the *glmS* riboswitch in its 3′-UTR. Notably, self-cleavage activity of the *glmS* riboswitch was enhanced when embedded in the mRNA what was also observed in the absence of any metabolite (data not shown). Subsequently, we performed *in vitro* translation assays to elucidate the effect of caged GlcN6P on EGFP synthesis. As a control we used mRNA molecules lacking the polyA-tail and, thus, mimicking the product of GlcN6P-mediated mRNA cleavage. As shown in Fig. 3c, *in vitro* translation assays revealed that EGFP synthesis can be controlled by caged GlcN6P and depends on the irradiation status of the sample (Fig. 3c).

More importantly, the reduction induced by light irradiation reaches almost the same level as observed when using a mRNA molecule that lacks the polyA tail and, thus, represents the GlcN6P-mediated cleaved mRNA. Furthermore, *in vitro* translation of the latter in the presence of caged GlcN6P is not light dependent. Similarly, treatment of mRNA transcripts with GlcN6P (**2**) results in the same reduction of EGFP synthesis as observed by irradiated caged GlcN6P. This clearly indicates that caged GlcN6P provides a means for the control of protein synthesis.

Conclusions

In summary, we present a novel route for the construction of a light-dependent RNA element based on a naturally occurring metabolite-dependent ribozyme and its naturally occurring cofactor GlcN6P. We synthesized a caged variant of GlcN6P and showed that this metabolite derivative can be employed to regulate the *glmS* ribozyme self-cleavage activity by light. Furthermore, *in vitro* translation assays revealed that caged GlcN6P and the *glmS* riboswitch allow the efficient regulation of protein synthesis, exemplified by EGFP. Consequently, our system provides a means for the construction of novel gene regulatory systems with spatiotemporal control. This might be realized by constructing variants of GlcN6P equipped with additional functionalities that render the compound cell permeable, for example cell penetrating peptides or lipophilic residues. Owing to the fact that the native function of the *glmS* riboswitch is the regulation of gene expression, this system might allow access to a highly efficient expression system in other model systems than bacteria, for example, eukaryotes.

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Notes and references

1 M. Mandal and R. R. Breaker, Gene Regulation by Riboswitches, *Nat. Rev. Mol. Cell Biol.*, 2004, **5**, 451.
2 (*a*) A. Rentmeister, G. Mayer, N. Kuhn and M. Famulok, Conformational changes in the expression domain of the *Escherichia coli* thiM riboswitch, *Nucleic Acids Res.*, 2007, **35**, 3713; (*b*) U. Rieder, C. Kreutz and R. Micura, Folding of a transcriptionally acting $PreQ_1$ riboswitch, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 10804.
3 M. T. Cheah, A. Wachter, N. Sudarsan and R. R. Breaker, Control of alternative RNA splicing and gene expression by eukaryotic riboswitches, *Nature*, 2007, **447**, 497.
4 W. C. Winkler, A. Nahvi, A. Roth, J. A. Collins and R. R. Breaker, Control of gene expression by a natural metabolite-responsive ribozyme, *Nature*, 2004, **428**, 281.
5 C. E. Lünse, M. Schmidt, V. Wittmann and G. Mayer, Carba-sugars activate the *glmS*-riboswitch from *Staphylococcus aureus*, *ACS Chem. Biol.*, 2011, **6**, 675.
6 (*a*) D. J. Klein and A. R. Ferré-D'Amaré, Structural basis of *glmS*-ribozyme activation by glucosamine-6-phosphate, *Science*, 2006, **313**, 1752; (*b*) J. C. Cochrane, S. V. Lipchock and S. A. Strobel, Structural investigation of the *glmS* ribozyme bound to its catalytic cofactor, *Chem. Biol.*, 2007, **14**, 97.
7 B. Suess, S. Hanson, C. Berens, B. Fink, R. Schroeder and W. Hillen, Conditional gene expression by controlling translation with tetracycline-binding aptamers, *Nucleic Acids Res.*, 2003, **31**, 1853.
8 L. Yen, J. Svendsen, J. S. Lee, J. T. Gray, M. Magnier, T. Baba, R. J. D'Amato and R. C. Mulligan, Exogenous control of mammalian gene expression through modulation of RNA self-cleavage, *Nature*, 2004, **431**, 471.

9 M. Wieland, A. Benz, B. Klauser and J. S. Hartig, Artificial ribozyme switches containing natural riboswitch aptamer domains, *Angew. Chem., Int. Ed.*, 2009, **48**, 2715.

10 K. H. Link, L. Guo, T. D. Ames, L. Yen, R. C. Mulligan and R. R. Breaker, Engineering high-speed allosteric hammerhead ribozymes, *Biol. Chem.*, 2007, **388**, 779.

11 A. P. Pelliccioli and J. Wirz, Photoremovable protecting groups: reaction mechanisms and applications, *Photochem. Photobiol. Sci.*, 2002, **1**, 441; G. Mayer and A. Heckel, Biologically active molecules with a "light switch", *Angew. Chem., Int. Ed.*, 2006, **45**, 4900; Caged Compounds, *Methods in Enzymology*, ed. G. Marriott, Academic Press, London, 1998, Vol. 291; *Dynamic Studies in Biology*, ed. M. Goeldner and R. Givens, Wiley-VCH, Weinheim, 2005.

12 M. Koizumi, G. A. Soukup, J. N. Q. Kerr and R. R. Breaker, Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP, *Nat. Struct. Biol.*, 1999, **6**, 1062.

13 D. D. Young and A. Deiters, Photochemical hammerhead ribozyme activation, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 2658.

14 H. W. Lee, S. G. Robinson, S. Bandyopadhyay, R. H. Mitchell and D. Sen, Reversible photo-regulation of a hammerhead ribozyme using a diffusible effector, *J. Mol. Biol.*, 2007, **371**, 1163.

15 R. Ting, L. Lermer and D. M. Perrin, Triggering DNAzymes with light: a photoactive C8 thioether-linked adenosine, *J. Am. Chem. Soc.*, 2004, **126**, 12720.

16 J. Lim, B. C. Grove, A. Roth and R. R. Breaker, Characteristics of ligand recognition by a *glmS* self-cleaving ribozyme, *Angew. Chem., Int. Ed.*, 2006, **45**, 6689.

17 M. Bergmann and L. Zervas, Synthesen mit Glucosamin, *Chem. Ber.*, 1931, **64**, 975.

18 G. Mayer and M. Famulok, Hight-throughput-compatible assay for *glmS* riboswitch metabolite dependence, *ChemBioChem*, 2006, **7**, 602.

19 T. J. McCarthy, M. A. Plog, S. A. Floy, J. A. Jansen, J. K. Soukup and G. A. Soukup, Ligand requirements for *glmS* ribozyme self-cleavage, *Chem. Biol.*, 2005, **12**, 1221.

20 P. Y. Watson and Martha J. Fedor, The *glmS* riboswitch integrates signals from activating and inhibitory metabolites *in vivo*, *Nat. Struct. Mol. Biol.*, 2011, **18**, 359.

