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Induced Protein Dimerization in Vivo through Covalent Labeling

Susanne Gendreizig,[§,†] Maik Kindermann,[§,†] and Kai Johnsson*[,§,†]

Institute of Molecular and Biological Chemistry, Institute of Biomolecular Sciences, École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

Received August 12, 2003; E-mail: kai.johnsson@epfl.ch

The dynamic dimerization and dissociation of pairs of proteins plays an important role in various biological processes.[1] As a chemical approach to study processes that depend on protein dimerization the groups of Schreiber and Crabtree have introduced "chemical inducers of dimerization" (CIDs).[2] CIDs are cell-permeable molecules which can bind simultaneously to two different proteins, thereby inducing their dimerization. Various biological processes have been controlled and studied with this approach, including signal transduction and control of transcription in eukaryotic and prokaryotic cells.[1−3] Currently used CIDs rely on noncovalent interactions, and we present here a new approach to induce protein dimerization in vivo which is based on the covalent labeling of fusion proteins with ligands capable of interacting with other proteins. The high specificity of the covalent labeling reaction should allow the approach to become an important tool for studying and controlling protein dimerization in biological processes.

The specific labeling of one of the partners participating in the induced dimerization is based on the unusual mechanism of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT). In its natural DNA repair function, hAGT irreversibly transfers the methyl group from O^6-alkylated guanine-DNA to a reactive cysteine.[4] Using cell-permeable O^6-benzylguanine (BG) derivatives of the type **1**, we have shown that hAGT fusion proteins can be labeled in living cells with a variety of different reporter groups (Figure 1A).[5] To convert **1** into a CID, we envisioned the use of BG−methotrexate heterodimers **2** or **3** in which BG is coupled to methotrexate (Mtx) using different linkers (Figure 1B). Mtx is a tight-binding inhibitor of dihydrofolate reductase (DHFR), and heterodimers of Mtx and dexamethasone, a ligand of the glucocorticoid receptor (GR), have been used as CIDs to control transcription in yeast.[6] In this so-called three-hybrid system, a DNA-binding domain and a transcriptional activation domain were expressed as DHFR and GR fusion proteins, respectively, and transcription initiated through the addition of the CID. To test if BG derivatives **2** or **3** can act as CIDs in such a three-hybrid system, we envisioned the construction of fusion proteins of hAGT with the DNA-binding domain LexA and of DHFR with the transcriptional activation domain B42 (Figure 1C). The in vivo labeling of the hAGT fusion protein with methotrexate using **2** or **3** should then induce the dimerization of the hAGT and DHFR fusion proteins, leading to stimulation of transcription of a reporter gene (Figure 1C).

We synthesized the different BG−Mtx heterodimers **2** and **3** (Supporting Information), as it is known that the nature of the linker can significantly influence the efficiency of CIDs.[6b] For the construction of hAGT fusion proteins, we used a previously described hAGT mutant with increased activity against BG compared to wild-type.[5b] In addition, we introduced three mutations that have been shown to disrupt DNA binding of hAGT: Lys125Ala, Ala127Thr, and Arg128Ala.[7] The resulting hAGT mutant carrying the five mutations described above was abbreviated as ^{3HY}AGT. To verify that ^{3HY}AGT possesses activity against **2** and **3**, we expressed ^{3HY}AGT as fusion protein with glutathione S-transferase (GST−^{3HY}AGT) in *Escherichia coli*, purified the protein, and determined its activity in an in vitro assay (Supporting Information). GST−^{3HY}AGT reacts rapidly with both **2** and **3**, possessing second-order rate constants of about (2600 ± 460) s^{-1} M^{-1} for the reaction with **2** and of about (1800 ± 430) s^{-1} M^{-1} for the reaction with **3**. For control experiments we also constructed a hAGT mutant in which the reactive Cys145 was mutated to alanine (^{A145}AGT).

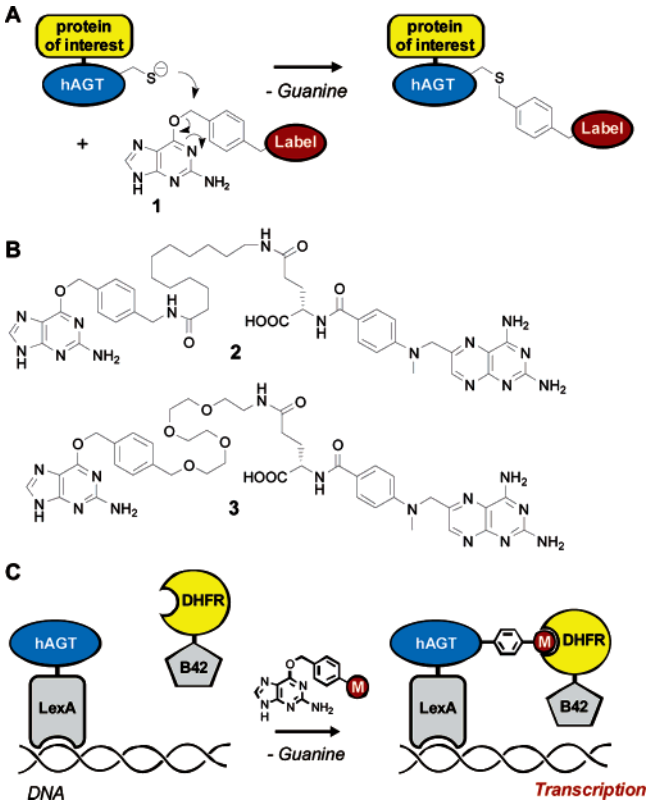

Figure 1. Labeling of hAGT fusion proteins with synthetic molecules. (A) Mechanism of labeling reaction; (B) BG derivatives used for the labeling of hAGT fusion protein with Mtx; (C) hAGT-based three-hybrid system. Mtx in the BG-based CID is represented as a red ball.

Pairs of plasmids encoding LexA and B42 fusion proteins were transformed into the yeast strain L40 in which the dimerization of LexA and B42 fusion proteins leads to transcription of the reporter genes *HIS3* and *lacZ* (Supporting Information). The expression of each fusion protein after transformation in L40 was verified by Western blotting using appropriate antibodies. We then tested if the expression of different combinations of fusion protein in the presence of either **2** or **3** complemented the histidine auxotrophy of the yeast L40 (Table 1).

§ Institute of Molecular and Biological Chemistry.
† Institute of Biomolecular Sciences.

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Table 1. Transcriptional Activation of *HIS3* and *lacZ* in Yeast L40

protein pair	CID	growth rate[a]	ONPG hydrolysis[b]
LexA-^{3HY}AGT/ B42-DHFR	1 μM **2**	++	870 ± 410
	1 μM **3**	+	39 ± 19
	−	−	4 ± 2
	100 μM BG	−	4 ± 4
	100 μM Mtx	−	6 ± 4
	1 μM **2**, 100 μM BG, 100 μM Mtx	+	19 ± 2
	1 μM **2**, 100 μM BG	+	41 ± 16
	1 μM **2**, 100 μM Mtx	++	820 ± 210
LexA-^{A145}AGT/ B42-DHFR	1 μM **2**	−	1 ± 0.3
LexA-Fos/ B42-Jun	−	++	260 ± 130

[a] ++: detection of colonies within 3 days; +: detection of colonies within 6 days; -: no colonies within 7 days. [b] nM of *o*-nitrophenol formed per min per mg total protein. Each value represents the average of at least three independent experiments.

Coexpression of LexA−^{3HY}AGT and B42−DHFR allowed yeast L40 to grow on plates lacking histidine but containing either **2** or **3**, indicating transcription of *HIS3*. No growth was observed in the absence of **2** or **3** or in the presence of only BG or Mtx. For yeast L40 expressing LexA−^{3HY}AGT and B42−DHFR, **2** proved to be more effective as a CID than **3**, most likely due to differences in cell permeability of the two CIDs. Yeast L40 coexpressing LexA−^{A145}AGT and B42−DHFR did not grow on plates lacking histidine and supplemented with **2**, indicating that the growth of the yeast depends on the labeling of the hAGT fusion protein. For CID **2**, we also investigated if the growth of yeast expressing LexA−^{3HY}AGT and B42−DHFR on plates lacking histidine but containing **2** could be suppressed by the addition of free Mtx and BG (Table 1). Although the growth rate was significantly lower in the simultaneous presence of excess Mtx and BG, growth was not completely suppressed. Addition of only Mtx did not significantly affect the growth rate. The competition experiments indicate that the intracellular concentration of the small molecules, and in particular that of Mtx, is below that of the fusion proteins.

We then examined the activation of transcription of *lacZ* by the BG-based CIDs in yeast L40 expressing LexA−^{3HY}AGT and B42−DHFR fusion proteins (Table 1). In this assay, the activity of the product of the *lacZ* gene, β-galactosidase, was determined by measuring the hydrolysis rate of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in cell extracts of liquid cultures.[8] The data obtained for the transcriptional activation of *lacZ* confirm those observed for *HIS3* described above. At 1 μM CID, **2** leads to about 20-fold higher levels of β-galactosidase activity compared to **3**, and no significant activity was measured in the absence of CID or when CID was replaced by BG or Mtx. No transcriptional activation in the presence of **2** was observed when ^{3HY}AGT was replaced by ^{A145}hAGT. The addition of free Mtx and BG to the culture medium did not lead to complete suppression of β-galactosidase activity. As observed in the *HIS3* assay, adding only Mtx to the medium did not significantly affect the activity of CID **2**, whereas the addition of only BG leads to a reduction of β-galactosidase activity by a factor of 20. Again, the lack of (complete) suppression of transcription of *lacZ* indicates that the covalent and noncovalent labeling of the ^{3HY}AGT and DHFR fusion proteins under these conditions is not quantitative. This hypothesis is supported by the observation that the transcriptional activation of *lacZ* in this system increases with the concentration of **2** and **3**, whereas a quantitative labeling would result in a decrease of transcriptional activation of *lacZ* with increasing concentration of CID.[9] At 1, 10, and 100 μM of **3**, the measured ONPG hydrolysis in cell extracts was 39, 830, and 8100 nM min^{-1} mg^{-1}, respectively (Supporting Information). For **2**, the measured β-galactosidase activity in cell extracts was 13, 865, and 2600 nM min^{-1} mg^{-1} at concentrations of 0.1, 1, and 10 μM of **2**, respectively (Supporting Information). The relatively low solubility of **2** made measurements at higher concentrations impossible.

To evaluate the achieved transcriptional activation using CID **2**, we measured the transcriptional activation of *lacZ* by coexpression of LexA−Fos and B42−Jun fusion proteins, a protein pair known to yield a strong interaction signal in the two-hybrid system.[10] β-Galactosidase activity in cell extracts of L40 expressing LexA−Fos and B42−Jun was below the activity measured for LexA−^{3HY}AGT and B42−DHFR in the presence of 1 μM CID **2** (Table 1). Furthermore, the observed 200-fold transcriptional activation at 1 μM of **2** over background is comparable to those achieved with other noncovalent CIDs in yeast.[6,9]

The work presented here demonstrates that BG derivatives can be used as CIDs to control transcription in yeast. The specificity of the labeling of hAGT fusion proteins and its independence of the nature of the ligand should make the approach a valuable tool to control protein dimerization in vivo.

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Supporting Information Available: Detailed procedures for synthesis of **2** and **3** and biochemical assays (PDF). This material is available free of charge via the Internet at http:// pubs.acs.org.

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