Protein–Protein Interactions

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Inducing and Sensing Protein–Protein Interactions in Living Cells by Selective Cross-linking**

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The formation and dissociation of protein complexes plays an important role in numerous biological processes. Small molecules can be used as tools to induce protein–protein interactions and to study the role of the corresponding complexes in processes such as signal transduction, transcription, apoptosis, protein degradation, and localization.[1] In these experiments, protein dimerization is achieved through expression of the protein(s) of interest as fusion protein(s) with a small-molecule-binding domain and the addition of an appropriate small molecule.[2] In addition to inducing a dimerization-dependent phenotype, the approach could potentially also be used to measure the spatial proximity of two fusion proteins as proximity should promote dimerization. However, the reversible nature of the induced dimerization prevents measurement of the dimerization efficiency. We present here small molecules that enable the covalent and irreversible dimerization of fusion proteins of O^6-alkylguanine-DNA alkyltransferase (AGT or SNAP-Tag) in living cells. The approach can thus be used to control the quaternary state of AGT fusion proteins as well as to sense the proximity and interactions of protein pairs in living cells by measuring their cross-linking efficiency through simple Western blotting.

We have previously shown that AGT fusion proteins in living cells can be labeled with a wide variety of small molecules by using O^6-benzylguanine (BG) derivatives.[3–6] For the covalent dimerization of AGT fusion proteins we envisioned the use of molecules (abbreviated as CoDis) in which two BG subunits are connected by a flexible linker (Figure 1 a).

As the nature of the linker might affect the efficiency of the cross-linking as well as cellular uptake, we synthesized three different CoDis with different linker lengths (Figure 1 b; see also the Supporting Information). All three CoDis reacted efficiently with AGT in in vitro assays in which the first of the two reactions was measured (see the Supporting Information). We then measured the covalent cross-linking of AGT at different concentrations of CoDi1–CoDi3 in vitro. Significant amounts of covalent AGT dimers were formed within six hours at nanomolar concentrations of CoDi (up to 75% as judged by Western blotting; Figure 2 a and see the Supporting Information). No significant differences between the three CoDis (less than twofold differences in dimerization efficiencies) were detected under these conditions, thus indicating that the differences in the linker length of CoDi1–CoDi3 does not affect the dimerization of AGT. To verify that the fusion

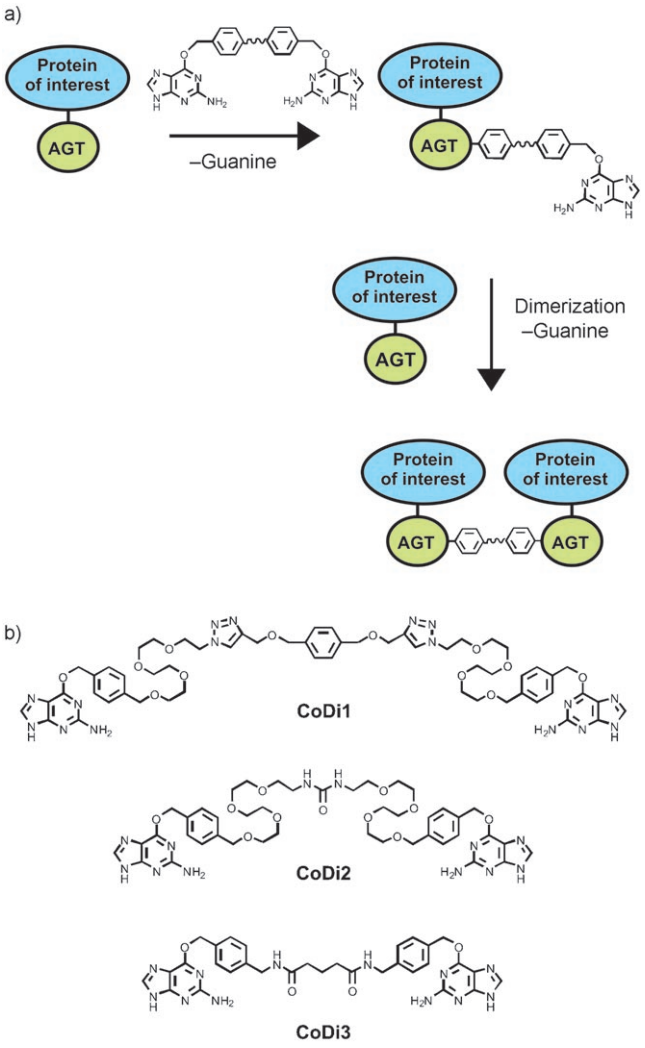

Figure 1. Covalent dimerization of AGT fusion proteins. a) General scheme for the dimerization of AGT fusion proteins using covalent dimerizing molecules (CoDis). b) Molecules used in this study.

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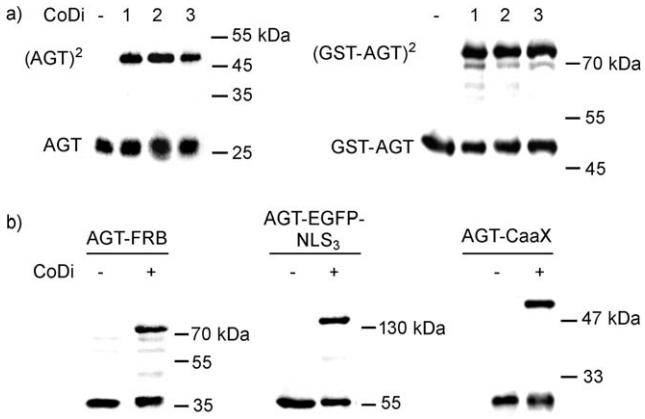

Figure 2. Cross-linking of AGT fusion proteins in vitro and in living cells using CoDis. Samples were analyzed through Western blotting. a) Incubation of purified recombinant AGT (1 μM) and AGT-GST (1 μM) with CoDi1 (0.5 μM), CoDi2 (0.5 μM), or CoDi3 (0.25 μM) for 6 h at RT. b) Incubation of HEK293 cells with CoDi1 (10 μM) for 6 h at 37 °C. The cells used were transiently expressing AGT fusion proteins with cytosolic (AGT-FRB), nuclear (AGT-EGFP-NLS_3), or plasma-membrane localization (AGT-CaaX).

of AGT to other proteins does not impede dimerization we analyzed the reaction of CoDi1–CoDi3 with a fusion protein of AGT and glutathione S-transferase (AGT-GST). As observed for the reaction with AGT alone, an efficient dimerization of AGT-GST was observed at nanomolar concentrations of CoDi1–CoDi3 (Figure 2a).

To qualitatively investigate the kinetics of the cross-linking reaction we prepared a fusion protein of AGT with acyl carrier protein (AGT-ACP) and labeled the fusion protein with the fluorophore Cy3 at ACP.[7] Incubating Cy3-labeled AGT-ACP (1 μM) with CoDi1 (0.75 μM) led to the formation of about 70% of the dimer with an apparent $t_{1/2}$ value for dimerization of about 60 minutes, as judged by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and laser-based fluorescence scanning experiments (see the Supporting Information).

We next investigated whether the CoDis could be used for the covalent dimerization of AGT fusion proteins in living cells. HEK293 cells transiently expressing different AGT fusion proteins in different cellular locations (nuclear, cytosolic, plasma membrane) were incubated with micromolar concentrations of CoDi1 for six hours, and the extent of dimerization determined through Western blot analysis of cell lysates (Figure 2b). Up to 70% of the protein was dimerized under these conditions. No interference from endogenous AGT could be observed. The CoDis were then tested for their ability to promote protein dimerization in the yeast *Saccharomyces cerevisiae*. AGT was expressed as a fusion protein with the DNA-binding domain LexA (AGT-LexA) and incubation with micromolar concentrations of CoDi1–CoDi3 led to significant dimerization (up to 50%) of AGT-LexA within two hours (see the Supporting Information). These experiments demonstrate that all three CoDis, independent of the nature of the linker, can be used for the specific and covalent cross-linking of AGT fusion proteins in living cells. In contrast to previously reported approaches, the dimerization is covalent and irreversible, and the extent of dimerization can be quantified by Western blot analysis. Quantification is important as it would allow the correlation of the extent of protein dimerization with a potential biological phenotype.

The induction of biological phenotypes through small-molecule-dependent dimerization of appropriate fusion proteins is a well-established method in chemical biology.[1] A unique feature of the approach introduced here is that the covalent cross-linking of AGT fusion proteins through the CoDis might be exploited for the detection of interactions and spatial proximity of protein pairs in living cells. This proposal is based on the assumption that the efficiency of the cross-linking of two AGT fusion proteins should depend on the spatial proximity of the two proteins, that is, the proximity of the two AGT fusion proteins should increase the "effective concentration" of the two reaction partners in the cross-linking reaction. To test the feasibility of such an approach we first transiently coexpressed AGT fusion proteins in either the same or different compartments of a cell and studied their cross-linking efficiencies upon incubation with a CoDi. Towards this end, a fusion protein of β-galactosidase with AGT (AGT-βGal) was transiently coexpressed in HEK293 cells together with AGT fused to either enhanced green fluorescent protein or nuclear-localized enhanced green fluorescent protein (AGT-EGFP and AGT-EGFP-NLS_3, respectively). AGT-βGal and AGT-EGFP are both localized in the cytosol, whereas AGT-EGFP-NLS_3 is localized in the nucleus.[4] When both proteins were localized in the cytosol, the addition of CoDi2 to the cells led to covalent homodimerization of AGT-βGal and of AGT-EGFP, as well as formation of the heterodimer of AGT-βGal and AGT-EGFP (Figure 3a). It is noteworthy that the relative yield of the heterodimer was lower than might be expected, possibly because of the oligomeric state of the fusion partners. Covalent homodimerization was favored because βGal is a tetramer and EGFP has a tendency to form dimers.[8,9]

When AGT-EGFP was targeted at the nucleus, the addition of CoDi2 yielded covalent homodimers of AGT-βGal and AGT-EGFP-NLS_3, but the heterodimer could not be detected, thereby reflecting the different localizations of the two fusion proteins. In a similar experiment, coexpression of a fusion of AGT with a farnesylation motif at the C terminus (AGT-CaaX) and AGT-EGFP in HEK293 cells followed by incubation of these cells with CoDi1 allowed detection of the two homodimers as well as the heterodimer (see the Supporting Information). However, coexpression of AGT-CaaX and AGT-EGFP-NLS_3 in the presence of CoDi1 led to very little heterodimer formation, which is in agreement with a predominant localization of AGT-CaaX at the cytosolic side of the plasma membrane and a nuclear localization of AGT-EGF-NLS_3 (see the Supporting Information).[4]

The preferential formation of homodimers over the heterodimer of AGT-βGal and AGT-EGFP indicated that the CoDis might be used to detect the formation of protein–protein interactions between AGT fusion proteins in living cells. To verify this hypothesis, we attempted to detect the rapamycin-dependent protein–protein interaction of the

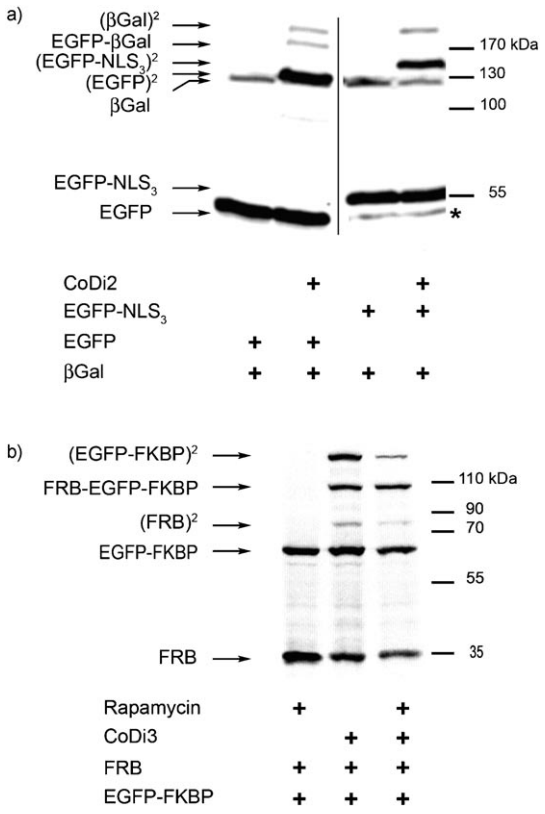

Figure 3. Cross-linking efficiency of pairs of AGT fusion proteins in living cells. Samples were analyzed as in Figure 2. a) Incubation of HEK293 cells transiently coexpressing either AGT-βGal and AGT-EGFP or AGT-βGal and AGT-EGFP-NLS_3 with or without CoDi2 at 37 °C for 6 h. All indicated proteins are AGT fusion proteins; for simplicity, only the name of the fusion partners of AGT are indicated. Degradation products of AGT-EGFP or AGT-EGFP-NLS_3 are marked with a star. A complete image of this Western blot with additional control experiments is shown in the Supporting Information. b) Incubation of HEK293 cells transiently coexpressing AGT-FRB and AGT-EGFP-FKBP with or without CoDi3 (2 μM) and rapamycin (15 μM) at 37 °C for 6 h. All indicated proteins are AGT fusion proteins; for simplicity, only the name of the fusion partners of AGT are indicated. The lower total protein concentrations in the sample of HEK293 cells treated with rapamycin is due to the effect of rapamycin on cell growth.

FK506-binding protein (FKBP) with the binding domain of FKBP rapamycin-associated protein (FRB).[10] FKBP was expressed as a C-terminal fusion of AGT and EGFP (AGT-EGFP-FKBP) and FRB as a C-terminal fusion of AGT (AGT-FRB). FKBP was expressed as a fusion protein with both AGT and EGFP to facilitate the differentiation of the different homo- and heterodimers by Western blotting. Coexpression of AGT-EGFP-FKBP together with AGT-FRB in HEK293 cells led to CoDi-inducible formation of the two homodimers and the heterodimer between AGT-EGFP-FKBP and AGT-FRB (Figure 3b). Again, the slight preferential formation of the AGT-EGFP-FKBP homodimer over the heterodimer can be interpreted as an indication of the weak dimerization of EGFP fusion proteins. Repetition of this experiment in the presence of rapamycin led to predominant formation of the heterodimer between AGT-EGFP-FKBP and AGT-FRB: The addition of rapamycin resulted in an 11-fold increase in the ratio of the FKBP-FRB heterodimer over the FKBP homodimer, as judged by Western blotting (Figure 3b). This result demonstrates how CoDis can be used for the detection of protein–protein interactions of AGT fusion proteins.

One advantage of the method is that it allows the trapping of interactions from a certain time point on, that is, from the time the CoDi is added. This distinguishes the approach from so-called split-protein sensors and could be of importance when the dissociation of protein complexes is being studied.[11,12] Furthermore, AGT fusion proteins can be employed under conditions where applications of autofluorescent proteins are limited, such as anaerobic conditions.[13] A limitation of the approach is that, in contrast to fluorescent-based assays,[14] it can not be performed on a single-cell level and in real time. Furthermore, the relative low rate of the cross-linking of the AGT fusion proteins might make the analysis of transient interactions difficult. It should also be noted that the CoDis reported here do not lead to a directional cross-linking of two different AGT fusion proteins, that is, they result in the formation of homo- and heterodimers. This is disadvantageous when the goal of the experiment is the induction of a heterodimer-dependant phenotype. However, when the goal of the experiment is the sensing of the proximity of two different proteins, the formation of homodimers can serve as an internal standard (see above). The recent development of an AGT mutant that reacts with a substrate that is not recognized by the AGT mutants used here[15] and the introduction of a self-labeling protein tag based on mutants of a bacterial dehalogenase[16] could furthermore lead to CoDis that allow a directional and covalent dimerization of fusion proteins.

In summary, the CoDis introduced here can be used for the covalent dimerization of AGT fusion proteins and to probe the spatial proximity of protein pairs in living cells. The technical simplicity as well as the broad applicability of the method makes it a welcome addition to the tool box to study and manipulate protein function in living cells.

Experimental Section

Detailed experimental procedures for the syntheses of the CoDis as well as the biochemical experiments are given in the Supporting Information. Unless noted otherwise, a recently described AGT mutant with an increased reactivity towards BG derivatives was used for the construction of the AGT fusion proteins.[17] For the labeling experiments in living cells, HEK293T cells were grown in a suspension culture in ExCell-293 medium (JRH Biosciences). Transient transfections were carried out by using standard procedures as described in the Supporting Information. To prevent the dimerization of AGT fusion proteins during sample preparation and after cell lysis, an excess of BG was added at appropriate time points. For the Western blots shown in Figures 2 and 3, a monoclonal anti-AGT antibody, an anti-mouse peroxidase conjugate as a secondary antibody, and a chemiluminescent peroxidase substrate were used.

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