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Control of Gene Expression with Small Molecules: Biotin-Mediated Acylation of Targeted Lysine Residues in Recombinant Yeast

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Summary

Chemical inducers of dimerization (CIDs) are powerful tools for controlling diverse cellular processes. These small molecules typically form strong noncovalent interactions with proteins. We report a related approach involving covalent acylation of a specific lysine residue of a target protein by the small molecule biotin. To control protein-protein interactions with biotin, the biotin protein ligase BirA from *E. coli* was coexpressed in yeast with a streptavidin-LexA fusion protein and Avitag or BCCP biotin acceptor peptides fused to the B42 activation domain. The addition of biotin (10 nM) resulted in BirA-mediated biotinylation of the biotin acceptor protein, recruitment to LexA DNA sites, and maximal activation of reporter gene expression in this yeast tribrid system. The high potency, low toxicity, and low molecular weight of biotin as a covalent CID are attractive properties for controlling cellular processes.

Introduction

Interactions between small molecules and proteins are critical regulators of gene expression in eukaryotic cells. Representative noncovalent small molecule-protein interactions include activation or repression of transcription controlled by ligands of members of the nuclear hormone receptor family of transcription factors. Binding of these small molecules to their receptors triggers conformational changes that affect the stability of specific receptor-protein complexes, thereby initiating a complex series of events that modulate transcription [1, 2].

Nature widely employs ligand-mediated protein dimerization as a mechanism to regulate gene expression and signal transduction [3, 4]. Dimerization of transcription factors, such as the nuclear hormone receptors by ligands, typically promotes receptor recognition of specific DNA sites, recruitment of components of the transcriptional machinery, and activation or repression of transcription. Related chemical inducers of protein dimerization (CIDs) have been developed as small molecules capable of controlling and probing diverse aspects of cellular biology. CIDs have been used to investigate signal transduction pathways [5–12], control gene expression [13–16], regulate protein secretion [17], manipulate protein splicing [18], assay enzyme activity [19, 20], and identify protein targets of small molecules [21–23].

CIDs can control gene expression by inducing protein-protein interactions that reconstitute functional transcription factors [24, 25]. This approach typically involves cellular expression of two ligand binding proteins as separate transcription factor elements. One ligand binding protein is expressed fused to a DNA binding domain (DBD). The second ligand binding protein is fused to a transcriptional activation domain (AD). Addition of the CID, which dimerizes these two proteins, recruits the transcriptional activation domain and associated transcriptional machinery to transcriptional enhancer elements occupied by the DNA binding protein. This interaction between two proteins, now bridged by the small molecule CID, can reconstitute a functional transcription factor to activate gene expression.

In addition to noncovalent small molecule-protein interactions, the covalent modification of proteins by small molecules also plays key regulatory roles in gene expression. For example, the activation domains of many transcription factors are associated with histone acyltransferase enzymes (HATs) that covalently modify histones to enable access of proteins to DNA packed tightly in chromatin [26–28]. This covalent modification involves acetylation of specific lysine residues by the endogenous small molecule substrate acetyl-CoA. Although acylation of specific lysines by HATs is well precedented, to our knowledge the use of exogenous agents to manipulate gene expression by acylating specific protein lysine residues has not been previously investigated.

We report here a novel method to regulate gene expression in living yeast cells involving acylation of a lysine residue with the small molecule biotin (vitamin H). In contrast to related noncovalent CID-based systems for controlling gene expression, biotin was used to induce protein heterodimerization after covalent biotinylation of a specific lysine residue of a target protein. Biotin can be employed in this fashion because this vitamin normally functions as a covalently linked cellular coenzyme involved in CO_2 transport [29]. Because biotin biosynthesis is restricted to only certain plants and fungi, many organisms, including the yeast *Sacchromyces cerevisiae*, require external sources of biotin for optimal growth, and these organisms possess active biotin uptake machinery [30]. Cellular enzymes termed biotin protein ligases (BPLs) covalently attach biotin to proteins by first condensing the substrates biotin and ATP to form biotin-AMP [31]. This BPL-bound cofactor enables nucleophilic attack by the ϵ-amine of a conserved lysine residue on the activated carboxy group of biotin to form an amide bond [32].

Biotinylation of lysine is a rare and highly specific posttranslational modification [33]. One of the best-characterized proteins biotinylated in this way is the biotin carboxy carrier protein (BCCP) domain of *E. coli* acetyl CoA carboxylase [34]. This protein is biotinylated in vivo by the *E. coli* biotin protein ligase BirA [35]. Truncation analysis of BCCP has identified a minimal 87 amino acid substrate (BCCP87) [34]. Proteins fused to BCCP87 are biotinylated in vivo in both *E. coli* and *S.*

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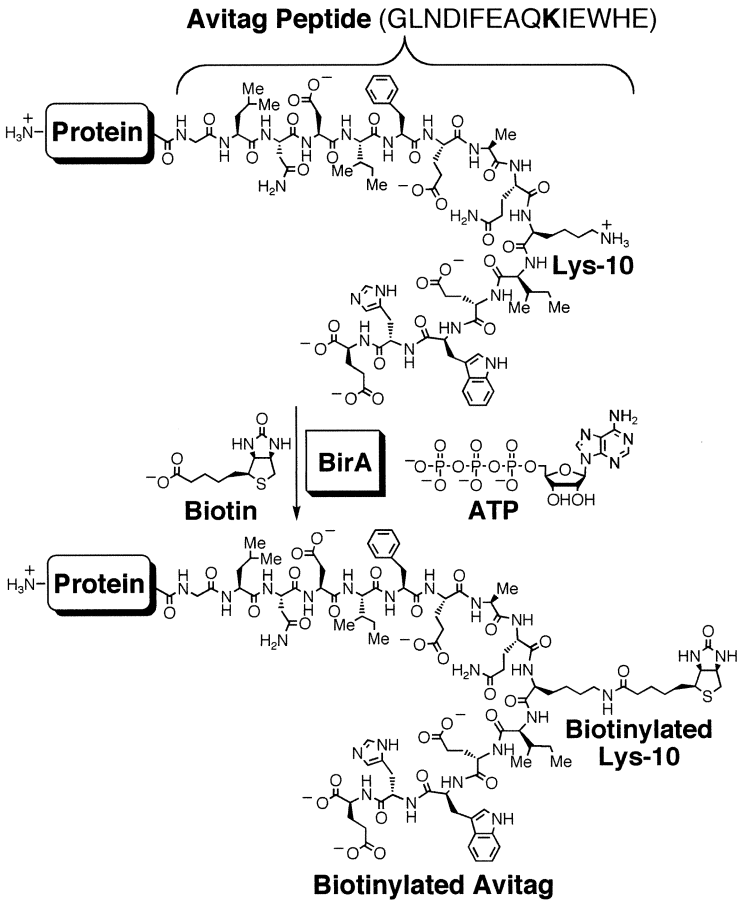

Figure 1. A Molecular Illustration of BirA-Mediated Biotinylation of a C-Terminal Avitag Peptide

The single-letter amino acid sequence and chemical representation of the Avitag peptide is shown.

cerevisiae [36]. However, a much shorter 15 amino acid peptide substrate of BirA termed Avitag has also been identified by screening peptide libraries [37]. As shown in Figure 1, this short peptide undergoes efficient BirA-mediated biotinylation of a key lysine residue, but this substrate bears little sequence homology to BCCP87 [38, 39].

By expressing BirA, Avitag, and streptavidin (SA) [40] fusion proteins in yeast cells, we constructed a yeast tribrid system that enables protein dimerization to be controlled by exogenously added biotin. Biotin was employed as a cell-permeable and nontoxic small molecule that covalently modifies a specific lysine residue of a target protein. This system comprises a novel method to manipulate reporter gene expression. Furthermore, BirA-mediated biotinylation of proteins fused to the short Avitag peptide expressed in yeast allowed isolation of labeled proteins on SA (or avidin) matrices to facilitate purification, identification, and analysis of biotinylated target proteins. Similar approaches for purification of proteins from *E. coli* [39] and mammalian cells [41] have been previously reported.

The strategy described here for affecting intracellular interactions is conceptually related to recent studies of small molecule adducts of the DNA repair enzyme hAGT [42, 43]. In living cells expressing hAGT fusion proteins, this enzyme can be covalently modified on a cysteine residue with small molecules linked to O6-alkylguanine to install fluorescent tags, biotin, or immobilize proteins on solid support. Biarsinical ligands have also been used to covalently modify specific peptide sequences in living cells [44].

Results

Biotin-Mediated Gene Expression in Yeast Tribrid Systems

Modifications of the yeast two-hybrid system [45] termed "tribrid systems" have been used to detect post-translational modification of proteins, such as tyrosine phosphorylation [46, 47]. As illustrated schematically in Figure 2, we employed the interaction trap two-hybrid approach [48] to construct a related yeast tribrid system in which reporter gene expression was designed to be dependent on exogenous biotin. The substrate "prey" protein to be biotinylated comprised the bacterial B42 transcriptional activation domain fused to green fluorescent protein (GFP) as a spacer and fluorescent tag that was in turn fused to a C-terminal 15 amino acid Avitag peptide. This protein was coexpressed in yeast with the BirA biotin protein ligase from *E. coli*. The biotin binding SA protein was also coexpressed in this system fused to the DNA binding domain of the bacterial LexA protein as the "bait" component of the tribrid assay. The LexA DNA binding domain functions to occupy four dimeric LexA DNA sites upstream of a *lacZ* reporter gene. We hypothesized that growth of these recombinant yeast in media supplemented with biotin would enable BirA

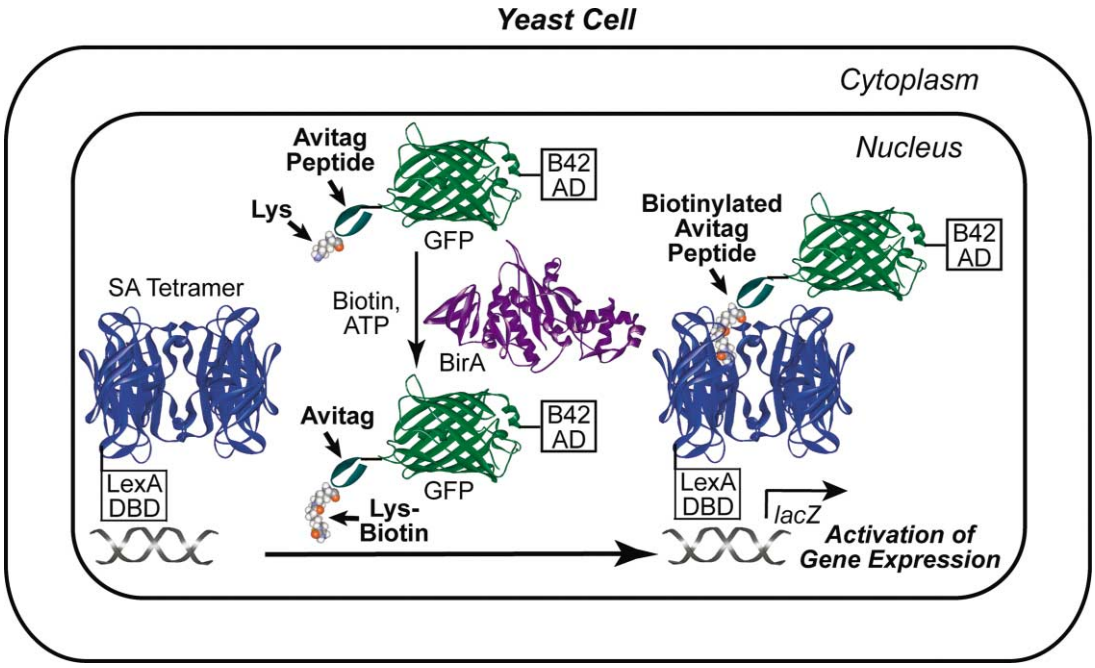

Figure 2. Schematic Representation of Biotin-Mediated Gene Expression in a Yeast Tribrid System

X-ray crystal structures of BirA (PDB 1HXD), tetrameric SA (PDB 1SWR), GFP (PDB 1EMA), and a 15 amino acid fragment of biotinylated BCCP (PDB 1BDO) as a model of Avitag were rendered with WebLab Viewer Lite 3.2 (Molecular Simulations Inc.). The SA-Avitag complex model was constructed by overlaying the SA-bound biotin with biotinylated lysine in an extended conformation from the Avitag model using Macromodel 6.5 (Schrödinger).

to catalyze the biotinylation of the key lysine-10 residue of the Avitag peptide. The resulting biotinylated lysine should bind tightly to SA on DNA, thus recruiting the B42 activation domain to DNA enhancer sites controlling the reporter gene (Figure 2). Production of the reporter gene product β-galactosidase could be assayed with a chromogenic substrate.

Previous studies of SA expressed in bacteria [49] and yeast [23] have revealed that fusion of a T7 peptide to the SA N terminus facilitates efficient protein folding in living cells. Hence, this peptide was fused to the N terminus of SA-LexA fusion proteins to enable functional expression of this biotin binding protein. The high-affinity SA wild-type protein (biotin $K_d \sim 100$ fM) [50] was compared with the lower-affinity SA Y43A (biotin $K_d \sim 100$ pM) [51] and SA W120A (biotin $K_d \sim 100$ nM) [52] mutants in yeast tribrid assays. To circumvent any potential effects on growth associated with expression of SA proteins in yeast [23], the inducible *GAL1* promoter was employed to regulate protein expression [48]. In contrast, the BirA and B42-GFP-Avitag constructs were expressed constitutively from plasmids bearing the strong *ACT1* (actin) promoter [53]. This strategy allowed us to compare biotin-mediated gene expression driven by continuous expression of SA with induced expression after preequilibration of the system with biotin.

Reporter gene expression was substantially activated by the addition of biotin to yeast expressing BirA, the Avitag fusion protein, and the SA fusion protein (Figure 3). Under conditions of continuous expression of these three protein components, addition of biotin (10 nM) to cells expressing wild-type (wt) SA protein afforded the greatest transcriptional response, enhancing gene expression by 7-fold above levels observed in the absence of added biotin (Figure 3A). Concentrations of biotin greater than 100 nM dramatically inhibited reporter gene expression, presumably by saturating the biotin binding sites of the intracellular SA proteins. Under these conditions, the lower-affinity Y43A and W120A SA mutant proteins were significantly less effective than the high-affinity SA wt as detectors of intracellular biotinylation.

Preequilibration of the Avitag fusion substrate and BirA with biotin prior to induction of expression of SA proteins was examined as an approach to maximize biotin-mediated gene expression. As shown in Figure 3B, this strategy increased reporter gene expression with enhancements of 10- to 20-fold observed at 10 nM biotin. Surprisingly, the lower-affinity SA Y43A and SA W120A mutants were similar to SA wt under these inducible conditions. These results suggest that the higher intracellular concentration of biotin likely to accumulate in the absence of SA expression enables a greater fraction of the Avitag protein to be biotinylated. This could explain the greater overall response when SA expression is induced. The resulting greater abundance of biotinylated Avitag protein may diminish the importance of very high-affinity interactions between SA and the target protein. Alternatively, inducible expression of SA may transiently generate a higher concentration of functional intracellular SA, which could enhance the activity of the lower-affinity mutant proteins.

Control experiments run under conditions of inducible expression of SA (Figure 3C) confirmed the importance of the three protein components and biotin in the yeast

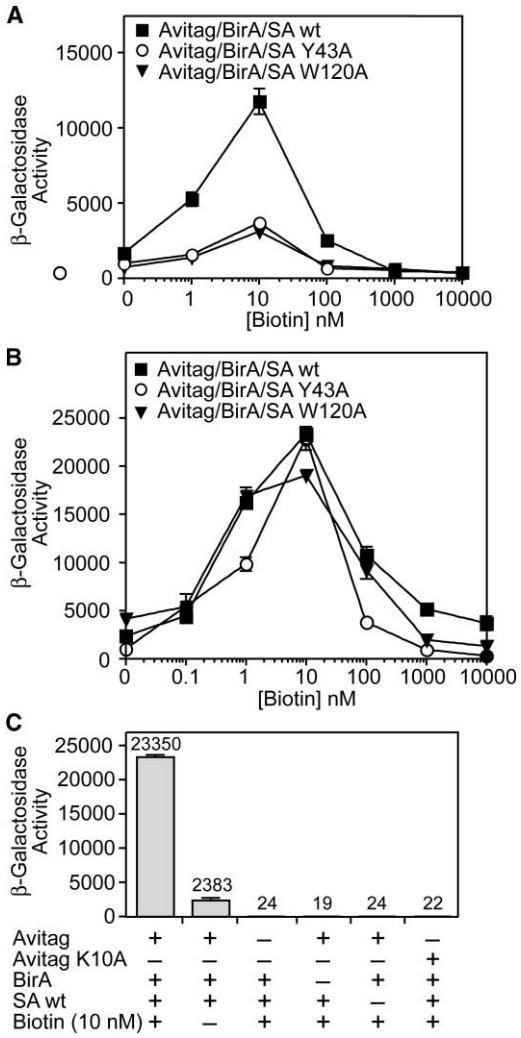

Figure 3. Analysis of Biotin-Mediated Gene Expression in Avitag-Expressing Yeast

(A) Dose-dependent reporter gene expression with constitutive expression of SA.

(B) Dose-dependent reporter gene expression with inducible expression of SA.

(C) Omission control experiments.

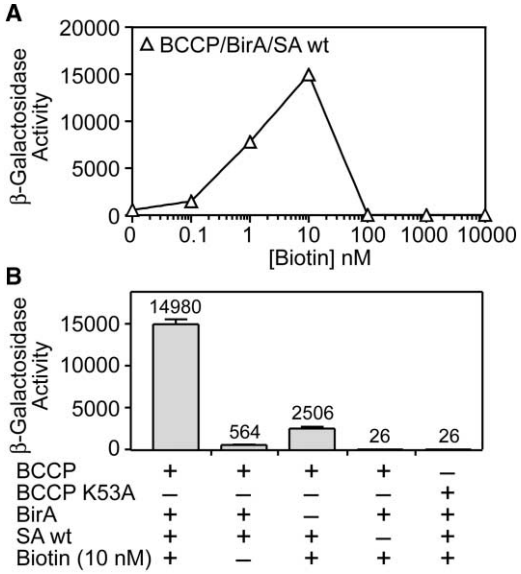

Figure 4. Analysis of Biotin-Mediated Gene Expression in BCCP87-Expressing Yeast

(A) Dose-dependent reporter gene expression with inducible expression of SA.

(B) Omission control experiments.

tribrid system. Moreover, site-directed mutagenesis to alanine of the key Avitag lysine-10 (K10) residue, predicted to be biotinylated by the BirA enzyme, abolished reporter gene expression. Under identical assay conditions, reporter gene expression driven by the Avitag K10A mutant at 10 nM biotin was diminished by 1000-fold compared with the unmodified Avitag fusion protein. These results provide strong evidence that reporter gene expression in this tribrid system results from biotin-mediated acylation of the Avitag K10 residue, resulting in interactions between the B42AD-GFP-Avitag and SA-LexA fusion proteins. The partial activation observed in the absence of exogenous biotin indicated that BirA transfers the limited endogenous biotin present in the biotin-free liquid yeast media employed or contained within intracellular stores resulting from earlier growth on biotin-containing solid media to the Avitag substrate.

To compare the efficiencies of different peptide substrates in yeast, the artificial 15 amino acid Avitag peptide substrate was substituted with the natural *E. coli*-derived 87 amino acid biotinylation substrate (BCCP87) in the yeast tribrid system. As shown in Figure 4A, under inducible conditions biotin potently activated dose-dependent reporter gene expression in yeast expressing the BCCP-derived substrate. However, the longer BCCP87 fusion protein provided a somewhat lower overall magnitude of response compared with the Avitag-derived substrate. Alanine mutagenesis confirmed that this activation required lysine-53 of BCCP87, which is the key residue biotinylated by BirA (Figure 4B) [38]. In contrast to the Avitag substrate, control experiments revealed that BCCP87 partially activated reporter gene expression in the absence of BirA. This result is consistent with previous reports demonstrating that BCCP87 can be biotinylated by endogenous yeast biotin protein ligase activity [36].

Analysis of Protein Expression and Biotinylation In Vitro

Immunoblotting of yeast cell extracts was employed to verify expression of the protein components of the yeast tribrid system. As shown in Figure 5A, expression of these proteins was confirmed by detection of HA epitope tags fused to the N terminus of each protein. These experiments further confirmed that the transcriptionally inactive Avitag K10A mutant was expressed at similar levels as the functional wild-type protein. This approach also enabled estimation of the extent of biotinylation of the Avitag substrate by BirA using a gel-shift assay (10 nM biotin). This assay examined the migration in an SDS page gel of the Avitag substrate fusion protein in

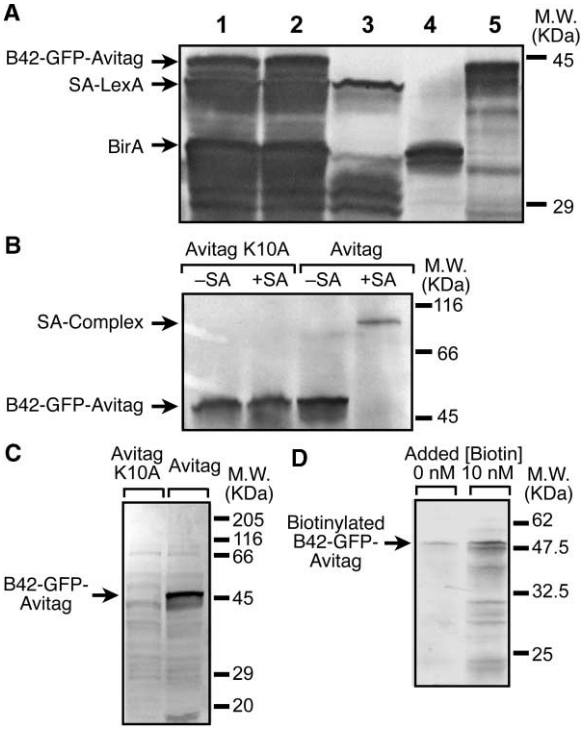

Figure 5. Detection of Protein Expression in Yeast Cell Extracts by Immunoblotting

(A) Yeast expressing the following proteins were probed to detect fused HA epitope tags. Lane 1: B42-GFP-Avitag, BirA, and T7SA-LexA; lane 2: B42-GFP-Avitag K10A, BirA, T7SA-LexA; lane 3: T7SA-LexA; lane 4: BirA; lane5: B42-GFP-Avitag.

(B) Gel-shift assays involving SA (12 μg per lane) added to extracts of yeast expressing the biotinylated B42-GFP-Avitag or nonbiotinylated B42-GFP-Avitag K10A.

(C) Detection of biotinylated proteins from extracts of biotin-treated (10 nM) yeast expressing BirA and Avitag or Avitag K10A fusion proteins. The blot was probed with SA-alkaline phosphatase.

(D) Analysis of residual biotinylation in cells expressing BirA and Avitag fusion proteins. Extracts of untreated and biotin-treated cells were probed with SA-alkaline phosphatase.

the presence or absence of excess purified SA protein added to cell extracts. This gel-shift assay revealed that all of the B42-GFP-Avitag protein isolated from BirA-expressing biotin-treated yeast formed a higher-molecular weight complex with SA (Figure 5B). Control experiments showed that added SA did not affect the analogous Avitag K10A mutant under identical conditions. These results demonstrated that the Avitag substrate is completely biotinylated by BirA under these conditions. In addition, exclusive biotinylation of the Avitag fusion protein but not the K10A mutant protein was detected by probing nitrocellulose-immobilized protein with SA conjugated to alkaline phosphatase (Figure 5C). These in vitro results demonstrated the high specificity of BirA-mediated biotinylation of the Avitag substrate. Furthermore, by comparing biotinylation in the presence and absence of added biotin, the extent of background biotinylation of Avitag was determined to be ca. 20% (Figure 5D). This result is consistent with the level of background transcriptional activity observed in the absence of added biotin. To improve the signal-to-noise

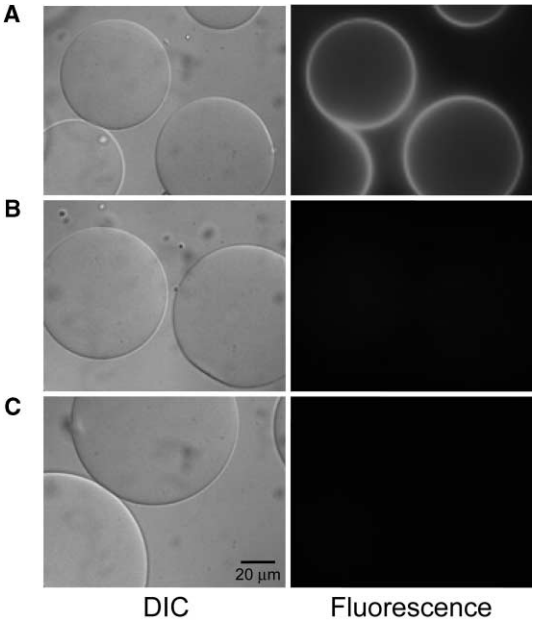

Figure 6. Differential Interference Contrast and Epifluorescence Micrographs of Streptavidin-Agarose Beads Treated with Crude Extracts from Yeast Expressing Biotinylated Avitag or K10A Mutant Avitag Fusion Proteins

(A) Treatment with B42-GFP-Avitag extract.

(B) Treatment of beads with biotin (2 mM) as a competitor followed by the B42-GFP-Avitag extract.

(C) Treatment with B42-GFP-Avitag K10A extract.

ratio in this system, it may be possible to screen libraries of mutant BirA proteins to identify mutants that do not efficiently transfer these low levels of residual biotin. Directed evolution might also identify BirA mutants capable of transferring synthetic biotin derivatives to target substrate proteins. This approach was recently used to optimize the enzyme hAGT to improve covalent labeling of a fused protein tag with small molecule substrates [54].

To examine the possibility of isolating biotinylated Avitag fusion proteins with an affinity matrix, extracts of yeast expressing BirA and B42-GFP-Avitag and treated with biotin (8 nM) were added to streptavidin-agarose beads. As shown in Figure 6, epifluorescence microscopy of these beads revealed bright green fluorescence corresponding to immobilized GFP-Avitag fusion protein. This interaction was specific as evidenced by the absence of green fluorescence upon competition with excess biotin. Furthermore, analogous extracts of yeast expressing the B42-GFP-Avitag K10A mutant protein did not confer bead-associated green fluorescence, confirming the importance of the biotinylated K10 residue (Figure 6). These results indicate that biotinylated Avitag fusion proteins expressed in yeast can be readily isolated using SA (or avidin) proteins immobilized on a solid support. This approach may be useful for isolation of recombinant proteins requiring eukaryotic posttranslational modifications from crude yeast extracts.

Discussion

Small molecules that dimerize proteins in cells are powerful tools for controlling diverse biological processes.

Typical CIDs function by forming strong noncovalent interactions with target proteins. As an alternative, we report here the covalent acylation of specific lysine residues by the small molecule biotin. This strategy provides a novel approach for regulating gene expression. In addition, in contrast to most noncovalent small molecule-protein interactions, the covalent modification of proteins by small molecules has the potential to enable pulse-chase experiments that allow tracking of proteins as a function of time.

Construction of a yeast tribrid system enabled biotin to function as an activator or inhibitor of gene expression dependent on the concentration of this small molecule. This system employed yeast engineered to coexpress the *E. coli* biotin protein ligase BirA, a SA-LexA DNA binding domain fusion protein, and the Avitag peptide substrate fused to the B42 activation domain. Treatment with biotin allowed the BirA enzyme to catalyze the intracellular formation of biotin-AMP [55] and execute highly specific biotinylation of the lysine-10 residue of the Avitag substrate [38]. This precise protein modification allowed capture of the fused B42 activation domain by the DNA-tethered SA protein. This DNA-bound protein was positioned to control activation of a *lacZ* reporter gene.

Biotin proved to be a highly potent activator of gene expression in this yeast tribrid system. Addition of 10 nM biotin to yeast media conferred maximal levels of reporter gene activity. Remarkably, this concentration is substantially below the Michaelis constant (K_m) and K_d for binding of biotin to *E. coli*-expressed BirA (K_m, 300 nM; K_{cat}, 0.3 s^{-1}; $K_{d\ BIOTIN}$, 45 nM) [56–58]. Because *S. cerevisiae* sequesters free biotin, enhancing the intracellular concentration by as many as three orders of magnitude above the concentration in growth medium [30], the high potency of biotin presumably relates in part to the high efficiency of biotin uptake by yeast. In addition, the use of tetrameric SA as the DNA-bound LexA fusion protein is likely to have significantly promoted biotin-mediated activation of gene expression. Each tetrameric SA protein could potentially capture up to four biotinylated activation domains to improve recruitment of the transcriptional machinery to enhancer sites controlling the reporter gene. Although this approach is likely to work well with monomeric biotinylated proteins, the tetrameric nature of the SA protein may complicate or prevent the capture of some oligomeric proteins.

Exogenous biotin mediated highly specific activation of gene expression by enabling biotinylation of only the lysine-10 residue of the Avitag substrate. This specific modification was confirmed by mutagenesis of this lysine residue to alanine, which reduced biotin-mediated gene expression by 1000-fold to basal levels of reporter gene activity. Moreover, although both the Avitag fusion protein and the K10A mutant were expressed at similar levels in yeast, only the functional Avitag fusion protein was observed as the major biotinylated species present in yeast cell extracts.

Biotin dramatically inhibited reporter gene expression at concentrations of 100 nM or higher. This is predicted to result from the binding of the actively accumulated free biotin to unoccupied biotin binding sites of SA. Competition by free biotin would block access to DNA of the B42 activation domain fused to the Avitag substrate. Similar but less effective autoinhibition has been previously observed in yeast three-hybrid systems regulated by high-affinity noncovalent chemical inducers of dimerization [23].

The minimalist artificial Avitag substrate was compared with the natural 87 amino acid BCCP substrate from *E. coli*. These experiments revealed that Avitag engenders higher levels of biotin-mediated gene expression than its natural counterpart. Furthermore, consistent with previous reports [36], the BCCP87 substrate was at least partially biotinylated by endogenous yeast biotin protein ligase. In contrast, Avitag was biotinylated exclusively by the BirA enzyme.

We demonstrate that biotin can be employed as a highly potent small molecule regulator of gene expression in recombinant yeast. This high potency derives in part from the efficient uptake and rapid intracellular accumulation of biotin. The availability of high concentrations of cytoplasmic biotin enables rapid and highly specific covalent biotinylation of the key lysine-10 residue of the Avitag substrate protein by BirA. The high efficiency and specificity of this process suggests that BirA or related mutants screened against this yeast tribrid system might enable transfer of synthetic derivatives of biotin to Avitag peptides. This approach has potential for highly specific labeling of proteins with molecular probes appropriately linked to biotin. Additionally, the facile isolation of site specifically biotinylated target proteins on SA or avidin affinity matrices may be useful for purification of recombinant proteins requiring eukaryotic patterns of posttranslational modification. Given the importance of noncovalent interactions between CIDs and target proteins for regulation of diverse cellular processes, intracellular biotinylation may provide an effective covalent alternative.

Significance

Small molecules that control protein dimerization in living cells provide powerful probes of cellular biology. We demonstrate here that the vitamin biotin can control protein dimerization after covalently acylating a specific lysine residue of a target protein expressed in yeast. This covalent modification of an activation domain fused to an Avitag peptide substrate, mediated by the biotin protein ligase BirA, potently activated gene expression in recombinant yeast expressing a DNA-bound streptavidin protein. Biotin maximally activated gene expression at a concentration of 10 nM, and this high potency relates in part to the efficient intracellular accumulation of this vitamin from the extracellular environment. The BirA-mediated transfer of biotin to the 15 amino acid Avitag substrate was highly specific; modification of only the key lysine-10 residue was observed. In contrast to most noncovalent small molecule-protein interactions, covalent modification by small molecules has the potential to enable pulse-chase experiments that allow proteins to be tracked as a function of time. This approach also provides a novel platform for the control of numerous cellular

processes and may enable the identification of biotin analogs capable of highly specific covalent labeling of proteins fused to the short Avitag peptide substrate.

Experimental Procedures

General

Standard protocols were employed for microbiological techniques and plasmid construction [59]. *Sacchromyces cerevisiae* FY250 (*MAT*α, *ura3–52*, *his 3Δ200*, *leu2Δ1*, *trp1Δ63*), a generous gift from Prof. M. Ptashne (Memorial Sloan Kettering), was used for cellular assays. *Escherichia coli* DH5-α (Clontech) was employed for plasmid construction. Oligonucleotides were from IDT Technologies Inc. Microtiterplate absorbance measurements utilized a Perkin Elmer HTS-7000 instrument.

Plasmid Construction

Genes were constructed using the polymerase chain reaction (PCR) with either *Pfu* polymerase (Stratagene) or platinum *Taq* polymerase (Gibco). Genes encoding the C-terminal Avitag peptide were amplified using a modified GFPuv gene [47] as the template and 3′-Avitag or 3′-Avitag K10A primers to append the peptide sequence GGGLNDIFEAQ**K**IEWHE [38] to the GFPuv C terminus. The biotinylated K10 residue is shown in bold, and the two underlined glycine residues were incorporated as a spacer at the N terminus of the Avitag peptide. The 87 amino acid BCCP-derived biotinylation substrate was amplified by PCR from a thermal (95°C, 5 min) lysate of *E. coli*. PCR megaprimer mutagenesis [60] was used to introduce the BCCP87 Lys53Ala mutation. The GFPuv-Avitag and GFPuv-BCCP87 genes were expressed from plasmid pDCWA B42, a yeast shuttle vector that fuses the bacterial B42 activation domain to the N terminus of GFPuv-Avitag. The pDCWA expression vector is a derivative of pRF4-6 (a vector related to pJG4-5 [48] bearing a *TRP1* selection marker and 2 μ origin but lacks the B42 activation domain and SV40 NLS) modified by D. Clark to substitute the inducible *GAL1* promoter with the constitutive *ACT1* (−663 to −4) promoter [53] from pJP190 (a gift of Prof. M. Ptashne). Genes encoding the T7-streptavidin (T7SA, 14–159) wild-type, Y43A, and W120A proteins were previously reported [23]. A 5′-MfeI-T7SA-EcoRI-XhoI-3′ cassette was cloned into MfeI/XhoI-cut pDC1 (a derivative of pLM [61] modified by D. Clark to include the following in-frame amino acids, unique in-frame restriction sites, and in-frame stop codons downstream of the T7 promoter: XbaI-MRGSHHHHHHPGSRIPMA-KpnI-MfeI-EcoRI-STOP-XhoI-STOP-SalI-STOP-HindIII) to afford pDC1 T7SA vectors. A second DNA cassette comprising MfeI-HA tag-SV40NLS-LexA-XhoI was ligated to EcoRI/XhoI-cut pDC1 T7-SA vectors to afford pDC1 MfeI-T7SA-HA tag-SV40NLS-LexA-Stop-SalI. This insert was isolated by digestion with MfeI/SalI and ligated to EcoRI/XhoI-cut pPA1 (a variant of pBC103 [62], containing an inducible *GAL1* promoter, *LEU2* selection marker, and 2 μ origin, but modified by P. Andrade to replace the ATG-HA tag-SV40NLS with EcoRI-XhoI restriction sites). The gene encoding BirA (GENBANK# NP_418404) [63] was amplified by PCR from *E. coli* and flanked with in-frame 5′EcoRI and 3′SalI restriction sites. This gene was ligated to EcoRI/SalI cut pSM1, a derivative of pLM [61] modified by S. Martin to add a hemagglutinin (HA) epitope tag (MfeI-MASYPYDVP DYASP-EcoRI-XhoI-SalI) to fused proteins. The MfeI-HA tag-BirA-XhoI fragment was ligated to EcoRI/XhoI-cut pDCHA (a derivative of pAM423 [64] bearing the HIS3 selection marker and 2 μ origin but modified by D. Clark to replace the *GAL1* promoter with the constitutive *ACT1* promoter). Plasmid pSH18-34 (Invitrogen), containing the *lacZ* gene under the control of four dimeric LexA binding sites, was used as the reporter. All the new constructs were confirmed by automated dideoxynucleotide sequencing at the Pennsylvania State University Biotechnology Institute.

Oligonucleotide Sequences

5′EcoRI-BirA: 5′-ACTCCGGAATTCATGAAGGATAACACCGTGCCA-3′; 3′SalI-Stop-XhoI-BirA: 5′-ACTCCGGTCGACTTACTCGAGTTTT TCTGCACTACGCAGGGA-3′; 3′SalI-Stop-Avitag-GG-XhoI-GFP: 5′-ACTCCGGTCGACTTATTCGTGCCATTCGATTTTCTGAGCCTCGAA GATGTCGTTCAGACCGCCACCCTCGAGTTTGTAGAGCTCATCCA TGCC-3′; 3′SalI-Avitag-Lys10Ala: 5′-ACTCCGGTCGACTTATTCGT GCCATTCGATAGCCTGAGCCTCGAAGAT-3′; 5′EcoRI-BCCP87: 5′-ACTGAATTCGGTCTCGAGGGTGGTGGTATGGAAGCGCCAGCA GCA-3′; 3′SalI-Stop-BCCP87: 5′-ACTGTCGACTTACTCGATGACGA CCAGCGG-3′; 5′BCCP-K53A: 5′-GTTGAAGCCATGGCTATGATGAA CCAG-3′.

Microtiterplate β-Galactosidase Reporter Gene Assays

S. cerevisiae FY250 was transformed by the lithium acetate method [65] with reporter gene pSH18-34 and plasmids encoding BirA, B42-GFP-Avitag, and T7SA-LexA. Yeast transformants derived from multiple combined colonies were grown overnight at 30°C in selection media comprising Brent supplement mixture ($Ura^-His^-Trp^-Leu^-$, 1.144g/L, Qbiogene), yeast nitrogen base lacking both amino acids and biotin (6.7 g/L, Qbiogene), raffinose (1%, Qbiogene), and penicillin/streptomycin (100 μ/mL, Gibco). Biotin (Sigma) was added to the media in DMSO (final DMSO conc, 1%). For the inducible experiments, aliquots of saturated (16 hr) cultures (OD_{590}, 0.7) were resuspended in biotin-free selection media (100 μL) containing raffinose (1%), galactose (2%, Qbiogene), and penicillin-streptomycin (100 u/mL) for 3 hr at 30°C to induce SA expression. The constitutive expression experiments employed analogous yeast cultures grown in the presence of raffinose (1%) and galactose (2%) for 16 hr. The plate was centrifuged (4300 rpm, 10 min) and the supernatant removed by aspiration. Z lysis buffer (Z buffer [59] containing EtOH [2%], $CHCl_3$ [1%], 100 μl) was added to the cell pellet and the plate was shaken for 5 min. Aliquots (20 μl) of suspended cells were transferred to wells containing Z lysis buffer (130 μl) to afford the final volume of 150 μl. The absorbance at 570 nm ($OD570_{cells}$) was measured to determine the cell density followed by the addition of 30 μl of chlorophenol red-β-galactopyranoside (Calbiochem, 15 mM in 0.1 M sodium phosphate buffer, pH 7.5). The plate was shaken at 23°C followed by absorbance measurements at 570 nm (OD570) over a period of 4 to 60 min (in 2 to 20 min intervals depending on the activity level). β-galactosidase activity was calculated as follows: activity = $1000 \times (OD570 - OD570_{cells} - Blank1)/(Time \times (OD570_{cells} - Blank2))$. The Blank1 value represents the absorbance at 570 nm of chlorophenol-red-β-galactopyranoside alone (30 μl). The Blank2 value corresponds to the absorbance at 570 nm of Z lysis buffer (150 μl). The time value was expressed in minutes. β-galactosidase activity values represent the mean of three independent experiments. Error bars represent standard errors of the mean. Omission control experiments employed yeast transformed with corresponding unmodified expression vectors.

Immunoblotting

Yeast transformants from multiple combined colonies were grown to saturation (16 hr) in appropriate selection media containing biotin (8 nM), raffinose (1%), and penicillin/streptomycin (100 u/mL). Streptavidin expression was induced by the addition of galactose (2%) for 3 hr. Cells were harvested by centrifugation (4300 rpm, 10 min), resuspended in one volume of 2X SDS loading buffer (125 mM Tris, pH 6.8, 4% SDS, 300 mM β-mercaptoethanol), frozen at –80°C for 10 min, and the tubes were boiled in water for 10 min. Cell debris was removed by centrifugation (8000 rpm, 5 min), and the supernatant was analyzed by SDS-PAGE (Tris-glycine 15%, CAMBREX). Proteins were transferred to nitrocellulose (PALL Life Sciences) using a semi-dry immunoblotting apparatus (Hoefer). The membrane was blocked using bovine serum albumin (2%, Sigma) in TBST (20 mM Tris, 150 mM NaCl, pH 7.5, 0.05% Tween-20) followed by incubation with mouse anti-HA antibody (Sigma, 4 μl in 20 ml final volume TBST). Goat-anti-mouse-alkaline phosphate conjugate (Zymed Laboratories Inc., 10 μl in 20 ml final volume) was used as the secondary antibody. Proteins were visualized with Western blue stabilized alkaline phosphatase substrate (Promega). Alternatively, biotinylated proteins were detected using a streptavidin-alkaline phosphatase conjugate (Sigma, 7.5 μl in 15 ml final volume) as the probe. To estimate the extent of biotinylation by gel-shift assay, yeast transformants expressing B42-GFP-Avitag constructs and BirA were grown for 16 hr (OD_{590}, 0.8) in selection media containing biotin (10 nM), raffinose (1%), and penicillin/streptomycin (100 u/mL). Cell extracts were prepared as previously described but were resuspended in one volume of "lite" 2X SDS loading buffer (containing

only 2% SDS) to prevent SA denaturation. Aliquots of these samples were incubated with excess SA protein (12 μl, 1 mg/mL, Sigma) at 23°C for 1 hr and analyzed by SDS-PAGE (Tris-glycine 15%, CAMBREX). Proteins were detected by immunoblotting against fused HA tags as previously described. The extent of residual biotinylation was determined by image analysis (NIH image 1.6) of the intensity of biotinylated Avitag bands (probed with SA-alkaline phosphatase) from cells grown in the presence or absence of added biotin.

Immobilization of Biotinylated B42-GFPuv-Avitag on SA-Agarose Beads

Yeast expressing B42-GFP-Avitag and BirA were grown for 16 hr in selection media containing biotin (8 nM). Cultures (10 mL) were centrifuged (4300 rpm, 10 min) and resuspended in yeast protein extraction reagent (YPER, Pierce, 100 μL). The cells were shaken at 30°C for 1 hr, centrifuged (8000 rpm, 5 min), and the supernatant (50 μL) was shaken with streptavidin-agarose beads (Sigma, 10 μL) in PBS (200 μl) at 23°C for 2 hr. The beads were washed with PBS containing 0.5% Tween-20 (250 μl × 3) and resuspended in PBS (50 μL) prior to analysis. Capture of B42-GFPuv-Avitag was assessed by examination of beads by DIC and epifluorescence microscopy (FITC filter set, Chroma Technologies Corp.) using a Zeiss Axiovert S100TV microscope fitted with a Zeiss Fluar (100×) objective. Images were obtained with a Zeiss Axiocam digital camera. Competition experiments employed beads preincubated with biotin (2 mM, in PBS containing 1% DMSO) for 1.5 hr.

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