Chemical Genetic and Genomic Approaches Reveal a Role for Copper in Specific Gene Activation

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Unbiased approaches toward exploring biological systems can yield unexpected, but useful results. Genetic screening of random mutations is a powerful tool for uncovering the molecular basis of biological processes.1 Chemical genetic screening of small molecules may prove to be similarly useful.2 Genetic and chemical genetic screens identify mutations and small molecules, respectively, that affect a biological process of interest.3,4 However, a potential barrier to the widespread use of the chemical genetic approach is the need to identify the molecular mechanisms by which small molecules affect biological systems.

Genomics-based techniques provide a possible solution to this problem. Transcriptional profiling with cDNA microarrays reveals which small molecules affect biological systems.5,7,8-quinolinols 1b (Figure 1) were used to measure the amount of luciferase that had been enzymatic activity. We determined the extent of reporter gene activation by measuring the amount of luciferase, which has a readily detectable enzymatic activity, illuminating the small molecule-sensitive pathway.

We developed a primary screen for small molecules that activate the reporter gene p3TPLux2 in a stably transfected mammalian cell line.3 This reporter is composed of a regulatory DNA sequence of DNA optimized for its sensitivity to TGF-β,2 and the coding sequence for luciferase, which has a readily detectable enzymatic activity. We determined the expression levels of a large number of genes simultaneously.5,7,8 For example, this technique can measure the change in expression of nearly every gene in the model organism Saccharomyces cerevisiae under various conditions,5 such as in response to treatment with a small molecule.7 The pattern of such transcriptional changes may reveal the mechanism of action of a compound’s activity, illuminating the small molecule-sensitive pathway.

We wondered if transcriptional profiling in S. cerevisiae would reveal anything about the mechanisms by which 1a and 2 act.

We purified messenger RNA (mRNA) from cultures of S. cerevisiae treated with either nothing or a small molecule, then converted this mRNA to complementary DNA (cDNA) labeled with one of two fluorescent dyes. We simultaneously hybridized these cDNA populations to a 2 cm × 2 cm microarray containing over 5800 distinct S. cerevisiae genes (> 90% of the total number of genes in S. cerevisiae). By comparing the amount of the two fluorescent dyes at each spot in the array, we determined the effect of a particular compound on the expression of each gene in S. cerevisiae.

Compound 2 had no significant effect on the expression of any gene in S. cerevisiae (data not shown). On the other hand, 1a reproducibly increased the expression of five genes (HSP26, ZRT1, FET3, YDR534C, and YOL155C), out of 5800, more than 2-fold. Of these five genes, only three have been previously characterized (HSP26, ZRT1, and FET3). HSP26 is a heat shock protein induced by osmotic stress,12 ZRT1 is a high-affinity zinc transporter,13 and FET3 is a multicyclop copper oxidase required for iron transport.14 Thus, these genes are all involved in metal ion homeostasis. Given our results (see below), it is likely that the two uncharacterized genes (YDR534C and YOL155C) are somehow regulated by the level of intracellular copper, iron, or zinc.

To determine whether the effect of 1a in S. cerevisiae is also involved in its ability to activate the TGF-β responsive regulatory sequence of the reporter in mammalian cells, we tested the effect of various metal ions on reporter activity in the presence of 1a or 2. Addition of 1 mM alkali or earth alkali metal (Na+, K+, Ca2+, Mg2+, and Ba2+) did not affect reporter activation by 1a or 2 (Table 1). However, 30 μM Fe2+ completely suppressed the activity of both 1a and 2 (Table 1 and Figure S1), but had no effect on TGF-β-induced activation of the reporter (data not shown). In addition, 7.5 μM Zn2+10, 10 μM Cu2+, and 20 μM Al3+ potently suppressed the activity of 1a but failed to suppress the activity of 2 or TGF-β at these or higher concentrations (Table 1 and Figure S1).

Surprisingly, Cu2+ alone activated the reporter (Figures 3 and S1). Moreover, 2 synergized potentially with Cu2+, but not other metal ions (Figures 3 and S1). These observations are consistent with our results and suggest that Cu2+ potently activates the reporter in the presence of the TGF-β-responsive regulatory sequence of the reporter in mammalian cells.

(11) Purchased from Chembridge Corporation (San Diego, CA).
Figure 1. Small molecule activators of a TGF-β-responsive reporter gene.

Figure 2. Dose-responses of 1a and 2 for activating a TGF-β-responsive reporter gene. 20,000 6F mink lung cells were seeded in 384 well plates, allowed to attach for 16 h in 10% mink medium, and treated with the indicated concentrations of 1a or 2 in the absence (black circles) or presence (white circles) of 400 pM TGF-β1.

Table 1. Metal Binding by 1a and 2 Correlates with Sensitivity to Metal Antagonism

<table>
<thead>
<tr>
<th>Metal</th>
<th>EC50 UV (μM)</th>
<th>EC50 Su (μM)</th>
<th>EC50 UV (μM)</th>
<th>EC50 Su (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn2+</td>
<td>&lt;7.5</td>
<td>0.5</td>
<td>500</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Fe2+</td>
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<td>2.0</td>
<td>&lt;10</td>
<td>9</td>
</tr>
<tr>
<td>Cu2+</td>
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<td>&lt;10</td>
<td>10</td>
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<tr>
<td>Al3+</td>
<td>30</td>
<td>100</td>
<td>200</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Mn2+</td>
<td>80</td>
<td>200</td>
<td>&gt;1000</td>
<td>&gt;300</td>
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<tr>
<td>Co2+</td>
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</tr>
<tr>
<td>Ni2+</td>
<td>280</td>
<td>10</td>
<td>500</td>
<td>200</td>
</tr>
<tr>
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<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<td>Ba2+</td>
<td>&gt;1000</td>
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</tbody>
</table>

All metals were used in the form of the chloride salts, except for NiSO4 and Ba(OAc)2.

Fe3+ may suppress the activity of 2 by binding to 2 and thereby preventing copper from binding to 2. To measure the ability of various metal ions to bind directly to 1a and 2, we measured ultraviolet light absorbance of each compound from 240 to 320 nm in the absence or presence of metal ions. We found a reproducible shift in the λmax of a shoulder in the UV spectrum of 1a from 292 to 273 nm and of 2 from 297 to 310 nm, in the presence of certain metal ions, likely due to binding of the metals (data not shown). Alkali and earth alkali metals (Na+, K+, Ca2+, Mg2+, and Ba2+) did not cause this perturbation in the UV spectrum of 1a or 2, at concentrations up to 1 mM (Table 1).

Furthermore, the metal ions that suppressed 1a and 2 also induced this perturbation at metal concentrations in the micromolar range, resulting in a correlation between the metal binding affinity and suppression ability of metal ions in the two assays. Interestingly, 1a and 2 had different metal-binding specificities (Table 1). Most notably, 1a binds Zn2+ with high affinity while 2 binds Zn2+ poorly. In addition, we found that Zn2+ suppressed the activity of Cu2+ (Zn2+ EC50 = 10 μM, Figure 3) while other metal ions did not (data not shown).

Compound 1a is a bis(8-quinolinol) (Figure 1). Monomeric 8-quinolinol itself had no activity in the reporter gene assay, neither activating on its own nor suppressing or enhancing the activity of 1a (data not shown). 2,2’-Dipyridylidine, which is a partial structural mimic of 1a, had no effect in the reporter gene assay (data not shown). Thus, simple structural analogues of 1a do not mimic the properties of this compound.

We determined that 1a, 2, and Cu2+ do not activate the reporter by affecting free radical formation or upregulating TGF-β itself, since H2O2, 2,2’-azobis(2-methylpropionitrile) (AIBN), and a neutralizing antibody to TGF-β did not affect the activity of 1a, 2, or Cu2+ (data not shown). Activation of the reporter by 1a, 2, and Cu2+ is selective, since none of these reagents activated a stably transfected NFAT-lacZ reporter gene (data not shown). However, we have not yet determined whether 1a, 2, and Cu2+ activate a component of the TGF-β signaling pathway, or whether the reporter gene that we used contains a previously unidentified copper-responsive element.

Remarkably, all of the active compounds identified in our screen, including 1a, bind to metal ions. This property of 1a is consistent with the observation that all three of the characterized genes induced by 1a in yeast are likely to be involved in metal homeostasis. Thus, if 1a depletes Zn2+, Fe3+, and Cu2+ in vivo, then induction of a zinc transporter (ZRT1), a copper-dependent iron oxidase required for iron transport (FET3), and an osmotic stress response (HSP26) is a reasonable response of yeast to 1a. The genomic approach has the added benefit of revealing potential functions for uncharacterized genes, such as YDR334C and YO1155C. This strategy of using small molecules, cell-based screens, and transcriptional profiling is complementary to the classical bioinorganic approach of studying a single metal–protein interaction, and furthermore may reveal new and interesting roles for metal ions in biology.

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Supporting Information Available: Detailed experimental procedures and an additional figure (Figure S1) showing metal ion suppression of compound-induced reporter gene activity (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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