Method for targeting protein destruction by using a ubiquitin-independent, proteasome-mediated degradation pathway

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With the euchromatic portion of several mammalian genomes now sequenced, emphasis has turned to ascertaining the functions of gene products. A method for targeting destruction of selected proteins in mammalian cells is described, based on the ubiquitin-independent mechanism by which ornithine decarboxylase (ODC) is degraded by the 26S proteasome in collaboration with antizyme (AZ). We show that expressing whole proteins, protein domains, or peptide ligands fused to the N terminus of ODC promotes proteasome-dependent degradation of these chimeric fusion proteins and their interacting cellular target proteins. Moreover, the degradation of the interacting (targeted) protein depends on coexpression of AZ in about half of cases, providing an inducible switch for triggering the degradation process. By using 12 pairs of interacting proteins for testing, direct comparisons with several alternative strategies for achieving targeted protein destruction based on the concept of induced ubiquitination revealed advantages of the ODC/AZ system, which does not require posttranslational attachment of ubiquitin to target proteins. As proof of concept, the ODC/AZ system was used to ablate expression of specific endogenous proteins (e.g., TRAF6, Rb), and was shown to create the expected lesions in cellular pathways that require these proteins. Altogether, these findings reveal a strategy for achieving targeted destruction of cellular proteins, thus providing an additional tool for revealing the cellular phenotypes of gene products.

Abbreviations: AZ, antizyme; FL, flexible linker; ODC, ornithine decarboxylase.

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TTTGGC GCGAATTTGACTCGA-3′ were annealed and ligated into pGL3E vector cleaved with Sacl and HindIII (16). The reporter gene plasmid containing four tandem HIV-NFκB response elements has been described in ref. 17.

Transfections and Cell Culture. HEK293T cells were maintained in high-glucose DMEM containing 10% FCS, 1 mM L-glutamine, and antibiotics. Cells (2 × 10⁵) in six-well plates were transfected with plasmid DNAs by using Lipofectamine 2000 (Invitrogen). In some cases, 50 μg/ml cycloheximide was added to prevent protein synthesis.

Immunoblots. Cells were lysed in 1 ml of HKMEN solution containing 0.5% Nonidet P-40, 0.1 μM PMSF, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 20 μM MG132. Lysates were analyzed by SDS/PAGE/immunoblotting using various antibodies, allowed by HRP-conjugated goat anti-mouse or anti-rabbit Ig (Amersham Pharmacia) and detected by using enhanced chemiluminescence (Amersham Pharmacia).

Pulse–Chase Analysis. For pulse–chase analysis of ectopically expressed HA-tagged TRAF6 (271–530) or IKKβ (305–745), HEK293T cells were transiently transfected in six-well plates. After 24 h, cells were pulse-labeled for 1 h with 0.1 μCi (1 Ci = 37 GBq) of [35S]methionine and [35S]cysteine per well and then chased with cold media. Cells were lysed in RIPA buffer (0.05 M Tris·HCl, pH 7.2/0.15 M NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS) supplemented with protease inhibitors. After preclearing with 20 μl of protein G-Sepharose for 1 h at 4°C, HA-TRAF6 or HA-IKKβ were immunoprecipitated by using rat anti-HA monoclonal antibody (3F10, Invitrogen) adsorbed to protein G-Sepharose beads at 4°C for 4 h. After three washes with RIPA buffer, immunoprecipitates were subjected to SDS/PAGE. Dried gels were analyzed with a PhosphorImager (Molecular Dynamics).

Reporters Gene Assays. NFκB and E2F transcriptional activity were measured in HEK293T cells by transient transfection reporter gene assays. Cells at ~50% confluence in 24-well plates were cotransfected with 0.1 μg of reporter plasmids containing NFκB- or E2F-binding sites cloned upstream of a Luciferase gene, 0.01 μg of pCMVβ-LacZ control plasmid, and 0.1 μg of various additional plasmids, as indicated. After 24 h, cells were lysed, and the relative amount of luciferase activity was measured according to the manufacturer’s instructions (Promega), normalizing all values relative to β-galactosidase activity.

Results

To explore technologies for inducing proteasome-dependent degradation of target proteins, we engineered plasmids to express in mammalian cells various chimeric proteins in which a protein involved in ubiquitination mechanisms was fused to proteins or protein domains known to interact with specific target proteins. Twelve pairs of interacting proteins were studied, chosen randomly from reagents available in our laboratory, including: (i) the C-terminal TRAF domain (residues 271–530) of the adapter protein TRAF6, which binds TRAF6 (18), (ii) a peptide (residues 341–349) from the cytosolic domain of the TNF receptor (TNFR)-family protein RANK, known to bind TRAF6 (19); (iii) the cytosolic domain of TNFR-family member CD40, known to bind TRAF2 (20), (iv) I-TRAF, a TRAF2-binding protein (21); (v) and (vi) the leucine zipper of IKKβ, which binds IKKα and IKKβ (22); (vii) a peptide from papillomavirus E7 protein, which binds Rb; (viii) the CARD domain of proCaspase-9, which binds Apaf1; (ix) The CARD domain of Apaf-1, which binds pro-Caspase-9 (23); (x) the DED domain of adapter protein FADD, which binds pro-Caspase-8; (xi) BAG1, a Hsp70-binding cochaperone (24), and (xii) p21Waf1, a Cdk2 inhibitor (25). Protein or peptide ligands were expressed in HEK293T cells as fusion proteins with an N or C terminus appended protein known to participate in protein-ubiquitination reactions, including (i) SIP, a protein known to bind the E3 ligase Siah1 and the Skp1 protein of Skp1/cullin/F-box protein complexes (26); (ii) Siah-1, a RING-domain containing E3 ligase (27); (iii) E7, a papillomavirus protein with reported E3 ligase activity (28, 29); (iv) a fragment of the F-box protein Fbx7, in which the substrate-binding domain was substituted, leaving the Skp1-binding region; (v) ubiquitin G76V, a nonhydrolyzable variant of ubiquitin previously used to confer proteasome-sensitivity on proteins; (vi) a tandem 4′ oligomer of ubiquitin G76V (2); and (vii) S5a, a subunit of the proteasome involved in substrate recognition (30). In most cases, the protein ligand was separated from the ubiquitin-pathway domain by a FL consisting of the sequence AGGGS-(GGGGS)₃ (31). All constructs included an epitope tag, allowing verification of protein production by immunoblotting and confirmation of binding to cellular target proteins by coimmunoprecipitation assays (data not shown).

The ability of these fusion proteins to induce reductions in the steady-state levels of endogenous and plasmid-expressed interacting proteins was then explored by immunoblot analysis of HEK293T cells transfected to high efficiency (~90%) with plasmids encoding the fusion proteins. The target proteins were coexpressed with epitope tags by cotransfection by using a plasmid with a strong constitutive promoter (CMV immediate-early region promoter) to ensure continuous production of target proteins and avoid artifactual reductions that might be caused by unanticipated effects of the chimeric fusion proteins on pathways that affect endogenous gene expression and to avoid false-negatives due to

| Table 1. Summary of tested fusion proteins |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ligand          | Target          | ODC (N)         | ODC plus AZ     | SIP (N)         | Siah (N)        | E7 (C)          | Fbx7 (C)        | Ub1 (N)         |
| TRAF6-C         | TRAF6           | ↓               | ↓               | ↓               | ↓               | ↓               | ↓               | ↓               |
| RANK-pep.       | TRAF6           | –               | –               | –               | –               | –               | –               | –               |
| CD40CT          | TRAF2           | –               | –               | nd              | nd              | nd              | nd              | nd              |
| I-TRAF          | TRAF2           | –               | –               | nd              | nd              | nd              | nd              | nd              |
| IKKα (LZ)       | IKKα            | –               | –               | nd              | nd              | nd              | nd              | nd              |
| IKKβ (LZ)       | IKKβ            | ↓               | ↓               | nd              | nd              | nd              | nd              | nd              |
| E7              | Rb              | –               | –               | nd              | nd              | –               | –               | –               |
| Caspase9(CARD)  | Apaf1           | –               | –               | –               | –               | –               | nd              | –               |
| Apaf1(CARD)     | Caspase9        | nd              | –               | –               | –               | –               | –               | –               |
| FADD(DED)       | Caspase8        | –               | –               | nd              | nd              | nd              | nd              | nd              |
| BAG-1           | HSP70           | –               | –               | nd              | nd              | nd              | nd              | nd              |
| p21             | Cdk2           | ↓               | ↓               | nd              | nd              | nd              | nd              | nd              |

Success ratio All targets 3/12 5/12 0/9 0/7 0/6 0/6 0/6 0/7

N, N-terminal fusion; C, C-terminal fusion; nd, not done; ↓, decrease; –, no change.
degradation of cellular substrates, with the [Gly-Gly-Gly-Gly-Ser]3 and the protein-interaction domain can be critical for successful mimic this effect of AZ.

In this regard, the AZ protein is known to induce a conformational change in ODC that exposes a proteasome-binding domain in its C-terminus (33), raising the possibility that some fusion partners change this ODC-adapter protein by the proteasome along with the interacting target protein.

Testing of an AZ-Based System for Targeted Protein Degradation. Given the lack of efficacy of fusion proteins based on known components of the ubiquitination machinery to effectively induce degradation of interacting proteins, we turn to an alternative strategy based on knowledge of the mechanism by which ODC is degraded by the proteasome through ubiquitin-independent mechanisms. For this purpose, protein ligands were expressed as fusion proteins with ODC at their C-termini, thus exposing the C terminus of ODC, which is known to bind the proteasome independently of ubiquitin.

A FL sequence was also inserted between the protein ligands and ODC. Three types of linkers were tested, including a 63-aa segment from the Bcl-2 protein (residues 31–94), which is known from structural studies to constitute a nonstructured, flexible peptide rich in prolines and glycines (32), a Gly-Gly-Pro tripeptide, and the sequence [Gly-Gly-Gly-Gly-Ser], which binds ODC and catalyzes its degradation by the 26S proteasome (33) (Fig. 1). Again, protein targets were coexpressed from plasmids with epitope tags for initial experiments, and their levels of expression were evaluated by SDS/PAGE/immunoblotting.

Of the 12 pairs of protein interactions tested by using the ODC/AZ system, 5 resulted in specific reductions of the target protein (Table 1). For two of these five successful knock-downs, reductions of target protein were entirely dependent on coexpression of AZ with the ODC chimeric fusion protein, whereas, in another case, AZ enhanced the reduction caused by the ODC adapter protein (Table 1). For two of these five successful knock-downs, reductions of target protein were entirely dependent on coexpression of AZ with the ODC chimeric fusion protein, whereas, in another case, AZ enhanced the reduction caused by the ODC adapter protein (Table 1).

Analysis of Specificity of AZ-Based Protein Target Degradation. We performed a variety of experiments to explore the specificity of the ODC/AZ system for inducing target-protein reductions. For example, the target protein TRAF6 is a member of a family of six mammalian adapter proteins with differential specificity for peptidyl motifs located in the cytosolic domains of TNF-family receptors (18). A C-terminal region in these proteins (C-TRAF domain) is responsible for TNFR binding. These adapter proteins also form homo trimers through the proximal portion of their TRAF domains (34). We therefore contrasted the levels of TRAF6 and TRAF2 proteins in cells expressing ODC chimeric fusion proteins containing either the TRAF domain of TRAF6 or a TRAF6-binding peptidyl motif from the cytosolic domain of RANK (“RANKp”) (Fig. 3). ODC nonfusion protein served as a negative control. As shown in Fig. 3, coexpressing ODC-C-TRAF6 caused reductions in levels of target proteins, based on 40 combinations tested.

The relative levels of TRAF6 mRNA were analyzed by Northern blotting using sample normalized for total RNA content. Immunoblot analysis was performed by using detergent lysates normalized for total protein content (20 μg per lane) and analyzed by SDS/PAGE/immunoblotting using antibodies specific for HA (Rb), Myc (ODC or AZ), or HSC70 (as a control) with enhanced chemiluminescence-based detection.
after 24 h, cells were pulse-labeled with [35S]methionine and [35S]cysteine in methionine/cysteine-free medium, and chased with media lacking the labeled amino acids. Cells were lysed at the indicated times, and HA-TRAF6 was recovered by immunoprecipitation by using HA antibody. Immunocomplexes were subjected to SDS/PAGE and dried gels were analyzed by Phosphorimagery (Upper). Data from pulse–chase analysis are presented as the average (±SE) from duplicate experiments (Lower). The blots shown are representative of duplicate experiments. (C) Pulse–chase analysis of IKKβ protein-degradation rate. HEK293T cells were transiently cotransfected with plasmids encoding HA-IKKβ, with ODC or ODC-IKKβ. After 24 h, cells were pulse-labeled with [35S]methionine and [35S]cysteine in methionine/cysteine-free medium, and chased with media lacking the labeled amino acids. Cells were lysed at the indicated times, and HA-IKKβ was recovered by immunoprecipitation by using HA antibody. Immunocomplexes were subjected to SDS/PAGE, and dried gels were analyzed by Phosphorimagery (Upper). Data from pulse–chase analysis are presented as the average (±SE) from duplicate experiments (Lower). The blots shown are representative of duplicate experiments. (D) The analysis of Flag-Cdk2 protein-degradation rate after cycloheximide treatment. HEK293T cells (six wells) were transiently cotransfected with plasmids encoding Flag-Cdk2 (0.5 μg), with ODC (2 μg) or ODC-p21 (2 μg). After 24 h, cells were treated with cycloheximide (50 μg/ml), lysed at the indicated times and analyzed by SDS/PAGE/immunoblotting using antibodies specific for Flag (Upper). Data are presented as the average (±SE) from duplicate experiments (Lower). The blots shown are representative of duplicate experiments. The levels HA-TRAF6 but not HA-TRAF2 protein. Cotransfection of AZ further decreased levels of HA-TRAF6 but not HA-TRAF2. Levels of HA-TRAF6 were reduced in cells expressing ODC-RANKp only when AZ was coexpressed. ODC-RANKp did not affect levels of HA-TRAF2, confirming the specificity of these results. ODC control protein also did not alter levels of HA-TRAF6 or HA-TRAF2.

Immunoblot analysis confirmed production of the ODC-chimeric fusion proteins and AZ in the transfected cells. Note that the accumulation of ODC-C-TRAF6 was markedly reduced, compared with ODC-RANKp, suggesting that fusing C-TRAF6 to ODC promotes its proteasome-dependent degradation independent of AZ. As expected, reductions in ODC-RANKp were induced by coexpressing AZ, consistent with AZ-dependent degradation of this ODC chimeric fusion protein. Thus, we surmise that some ODC chimeric fusion proteins spontaneously associate with and are efficiently degraded by the 26S proteasome (e.g., ODC-C-TRAF6), whereas others (e.g., ODC-RANKp) require AZ as a cofactor for their degradation, like wild-type ODC.

AZ/ODC System Increases Rate of Target Protein Degradation. Next, we undertook experiments to determine the mechanism by which target protein reductions were achieved when using the ODC/AZ system. First, we determined the effect of ODC chimeric fusion proteins on the level of mRNA encoding target proteins, anticipating that mRNA levels should be unchanged. Analysis of TRAF6 mRNA levels in cells transfected with plasmids encoding AZ and either ODC-C-TRAF6 or ODC-RANKp fusion proteins confirmed no effect on expression at the mRNA level (Fig. 3). Second, we explored the effects of pharmacological inhibitors of the 26S proteasome. Fig. 4A shows an example where HEK293T cells were cotransfected with a plasmid encoding HA-TRAF6 alone or in combination with plasmids encoding AZ, and an ODC chimeric fusion protein containing a TRAF6-binding peptide from the cytosolic domain of RANK (RANKp). Coexpression of ODC-RANKp and AZ with HA-TRAF6 resulted in profound reductions in the steady-state levels of HA-TRAF6 protein, as determined by immunoblotting. Culturing these transfected cells with proteasome inhibitors MG132, epoximycin, or lactacystin restored HA-TRAF6 levels. In contrast, a trypsin inhibitor, used here as a control, was ineffective (Fig. 4A). These data demonstrate that ODC/AZ-induced degradation of target proteins is proteasome-dependent.

Third, we determined the effects of the ODC/AZ system on protein half-life using 35S-l-methionine pulse–chase methods. Fig. 4B shows results comparing the half-life of HA-TRAF6 in cells cotransfected with ODC-RANKp, with or without AZ. In cells
expressing AZ, the half-life of HA-TRAF6 was reduced from \( \approx 2 \) h to \(< 1 \) h, consistent with target protein degradation occurring via an AZ-dependent mechanism. We conclude, therefore, that the ODC/AZ system induced proteasome-dependent degradation of target proteins without affecting mRNA expression. Pulse–chase experiments were also performed for IKK\( \beta \), comparing cells transfected with plasmids encoding ODC versus ODC-IKK\( \beta \). The starting levels of IKK\( \beta \) were lower in cells expressing ODC-IKK\( \beta \) before initiating the chase, suggesting ongoing degradation. Cold \( L \)-methionine chase revealed that, indeed, the rate of degradation of IKK\( \beta \) was faster in cells expressing ODC-IKK\( \beta \), compared with ODC control (Fig. 4C). Fourth, we also used another approach to gauge the rates of target-protein degradation where cells were transfected with ODC-expressing plasmids and then protein synthesis was shut off a day later by adding cycloheximide to cultures. The ODC-TAG system induced proteasome-dependent degradation of a luciferase reporter protein, 0.01 \( \mu \)g of pCMV\( \beta \)-galactosidase as an translation-efficiency control, and 0.1 \( \mu \)g of the indicated plasmids encoding ODC, ODC-E7p, or AZ, in various combinations as indicated (total DNA amount normalized). After 48 h, lysates were normalized for total protein content and subjected to immunoprecipitation by using 1 \( \mu \)g of anti-Rb monoclonal antibody. The resulting immunocomplexes were analyzed by SDS/PAGE/immunoblotting using an anti-Rb monoclonal antibody with enhanced-chemiluminescence-based detection. (C) Effect of ODC-E7p on E2F transcriptional activity. HEK293T cells were transiently transfected with a reporter gene plasmid (0.1 \( \mu \)g) that contains an E2F-responsive element cloned upstream of a luciferase reporter gene, 0.01 \( \mu \)g of pCMV\( \beta \)-galactosidase as an translation-efficiency control, and 0.1 \( \mu \)g of the indicated plasmids encoding ODC, ODC-E7p, or AZ, in various combinations as indicated (total DNA amount normalized). Luciferase activity was measured in cell lysates 24 h later and normalized relative to \( \beta \)-galactosidase (mean \( \pm \) SD; \( n = 3 \)).

We extended these studies of effects of the ODC/AZ system on endogenous proteins to the tumor suppressor Rb. The Rb protein binds and suppresses E2F-family transcription factors, thus preventing them from activating target genes (35). We therefore expressed in cells an ODC chimeric fusion protein containing a Rb-binding peptide from the E7 protein, alone or in combination with AZ (Fig. 6A). Analysis of the levels of endogenous Rb protein by immunoblotting showed that the combination of ODC-E7p and AZ induced nearly complete disappearance of the Rb protein. In contrast, Rb protein levels were unaffected either by expressing ODC-E7p without AZ, or by expressing ODC control protein with or without AZ (Fig. 6B). Measurements of E2F activity by using reporter gene assays demonstrated a marked increase in cells coexpressing ODC-E7p and AZ (2 \( \mu \)g) in various combinations, as consistent with the observed loss of Rb protein. We conclude, therefore, that the ODC/AZ system is capable of ablat ing the function of endogenous target proteins in cells.

**Discussion**

Previous studies have demonstrated that ODC is degraded by the 26S proteasome through a ubiquitin-independent mechanism, whereby AZ binding induces exposure of the C terminus of ODC and accelerates its degradation by 50- to 100-fold. Normally, this pathway is induced in response to polyamines (spermine, spermidine, and putrescine), which triggers AZ production, thus providing a negative feedback loop for maintaining appropriate intracellular levels of these molecules (reviewed in ref. 14). We exploited the ODC/AZ system for targeting degradation of selected proteins in cells. The ODC/AZ system affords the advantage over most ubiquitin-pathway-based strategies that posttranslational modification of the target protein (by ubiquitination) is not required, thus providing a more direct means of delivering ligand/target complexes to the proteasome for degradation. Indeed, compared with a variety of ubiquitin-pathway-based approaches examined, we found the ODC/AZ system to be more effective at achieving
degradation of a test set of 12 target proteins for which interacting proteins are known. However, successful degradation was achieved in only 5 of 10 test cases, suggesting that some proteins are recalcitrant to this targeting approach. Multiple explanations could account for the intractability of certain protein targets, including (i) insufficient affinity interactions of the protein ligands with their cellular target proteins; (ii) dissociation of ligand and target during digestion of the ODC-ligand fusion by the proteasome, thus stripping the target protein off; and (iii) impeded entry of the target protein into the pore of the proteasome because of rigid protein structure, necessitating protein unfolding. Thus, the tractability of specific protein targets to degradation by the ODC/AZ system must be empirically determined.

A potential concern with the ODC/AZ system is that expression of ODC-fusion proteins or AZ in cells may alter polyamine levels, leading to artifactual changes in cell growth, chromatin structure, or other cellular events. Measurement of cell-division rates for HeLa and HEK293T cells used for our experiments revealed no apparent changes in cells overexpressing ODC-fusion proteins or AZ in cells; however, more subtle changes in cells overexpressing ODC-fusion proteins and AZ conceivably may occur and therefore should be considered in interpreting data derived from use of the ODC/AZ-based approach to targeted protein degradation. The suitability of this approach may also be dependent on the endogenous levels of AZ and AZ inhibitors in particular types of cells. Similarly, production of ODC mutants which cannot bind endogenous ODC. Therefore, should be considered in interpreting data derived from manipulations. However, more subtle changes in cells overexpressing ODC-fusion proteins or AZ in cells may alter polyamine levels, thereby ablating expression simultaneously of several redundant multigene families, where a particular protein ligand may interact with multiple members of a family of homologous gene products, thereby ablating expression simultaneously of several redundant members and revealing phenotypes that would be undetected by nucleic-acid-based methods for silencing gene expression at the mRNA level.

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