[38] Identifying Small Molecule Inhibitors of the Ubiquitin-Proteasome Pathway in *Xenopus* Egg Extracts

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Abstract

Small molecule inhibitors of the proteasome have been crucial for dissecting the mechanism of proteasome-dependent protein degradation and identifying substrates of the ubiquitin-proteasome system (UPS). To identify small molecules that block ubiquitin-dependent protein degradation through other mechanisms, we have developed pathway-based screening approaches in *Xenopus* egg extracts. The regulated degradation of UPS substrates can be reconstituted in these extracts, providing an excellent system in which to perform forward chemical genetic screens. The ability to manipulate extracts biochemically and to compare the activity of small molecules across different assays facilitates the identification of potential target proteins. Here we describe methods for identifying inhibitors of the proteolytic pathways that regulate cell cycle progression and Wnt signaling in *Xenopus* extracts.

Introduction

Small molecule inhibitors of the proteasome have been essential for understanding the mechanism of proteasome-dependent degradation and identifying substrates and functional roles of the ubiquitin-proteasome pathway (Kisselev and Goldberg, 2001). The recent approval of a proteasome inhibitor for the treatment of multiple myeloma also highlights the importance of this pathway as a target for the development of new cancer therapies (Adams, 2004). Despite the utility of proteasome inhibitors, the biochemical complexity of ubiquitin metabolism suggests that small molecules that inhibit other steps in the pathway, such as ubiquitin chain formation or removal, will also be valuable, both as tools and as therapeutics (Pray *et al*., 2002; Robinson and Ardley, 2004). Recently, a targeted screen has identified a class of small molecules (nutlins) that block the interaction between MDM2 and p53, thereby preventing the ability of MDM2 to catalyze p53 ubiquitination (Vassilev *et al*., 2004). Inhibitors of ubiquitin C-terminal hydrolases have also been discovered through high-throughput screening (Liu *et al*., 2003). Although such targeted approaches

METHODS IN ENZYMOLOGY, VOL. 399

0076-6879/05 $35.00
DOI: 10.1016/S0076-6879(05)99038-1

with purified proteins have been successful, it remains difficult to know which steps in the ubiquitin-proteasome pathway are most amenable to small molecule inhibition with the chemical libraries that are available today.

Chemical Genetics

Forward chemical genetics provides an opportunity to identify small molecules that inhibit a biochemical pathway without making assumptions about which step is likely to be most sensitive to inhibition (Lokey, 2003; Mayer, 2003). In addition to identifying new inhibitors, the approach can provide tools that illuminate new components of a pathway or identify unexpected steps in the pathway that are sensitive to inhibition by small molecules. We have taken a forward chemical genetic approach to identify small molecules that inhibit the UPS in *Xenopus* egg extracts. These screens, combined with reconstituted biochemical assays, led to the identification of ubistatins, small molecules that inhibit ubiquitin-dependent degradation by binding to the ubiquitin chain (Verma *et al*., 2004).

Xenopus egg extracts provide a convenient system for performing chemical genetic screens for inhibitors of the UPS. Complex biochemical pathways can be reconstituted in these extracts, enabling many potential targets to be screened simultaneously. Unlike cell-based assays, compounds do not need to be membrane-permeable to be active. Extracts can be fractionated and biochemically manipulated to facilitate target identification, which is often the rate-limiting step in forward chemical genetics (Tochtrop and King, 2004). The ability to generate large quantities of extracts facilitates high-throughput screening of large chemical libraries (Verma *et al*., 2004). Several screens have been performed in *Xenopus* extracts, including screens for inhibitors of actin assembly (Peterson *et al*., 2001, 2004), spindle assembly (Wignall *et al*., 2004), and cell cycle progression (Verma *et al*., 2004). Targets of active molecules have been identified either by affinity purification (Wignall *et al*., 2004) or biochemical reconstitution and candidate testing (Peterson *et al*., 2004; Verma *et al*., 2004).

Here we describe the application of chemical genetic methods to identify small molecule inhibitors of cyclin B degradation or β-catenin degradation. Both of these substrates are degraded by ubiquitin-dependent proteolysis, yet are targeted for ubiquitination by distinct ubiquitin ligases. Comparison of the activity of molecules in these two assays provides a convenient method for identifying potential targets.

Studies of Cell Cycle Progression and Cyclin B Proteolysis in Xenopus *Extracts. Xenopus* egg extracts have been especially useful for understanding

the mechanism and regulation of cyclin B proteolysis during the cell cycle. In this system, anaphase onset and exit from mitosis require the activation of a multisubunit ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC/C), which is responsible for ubiquitinating cyclin B and targeting it for destruction (Peters, 2002). The APC/C can work in concert with one of two different E2 enzymes, UbcH5 or UbcH10 (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996). The APC/C is activated by mitotic phosphorylation catalyzed by cyclin B/cdc2 and also polo kinase and requires the participation of an activator protein called Fizzy or Cdc20 (Peters, 2002). Cyclin degradation requires the presence of a 9-amino acid sequence in its N-terminal domain, called the destruction box, which targets the protein to the APC/C (Glotzer *et al.*, 1991; King *et al.*, 1996). Deletion of the destruction box, or the N-terminal domain from cyclin B, results in a stable protein that cannot be recognized or ubiquitinated by the APC/C. However, this nondegradable protein remains capable of binding and activating Cdc2 and can be used to generate stably arrested mitotic extracts in which APC/C is constitutively activated (Glotzer *et al.*, 1991).

Xenopus extracts are an ideal system to screen for compounds that directly target the core cell-cycle machinery, because egg extracts lack the checkpoint pathways that normally respond to DNA damage (Dasso and Newport, 1990) or spindle damage (Minshull *et al.*, 1994) unless exogenous nuclei are added. Because small molecule libraries typically contain a large number of compounds that can perturb DNA replication or microtubule function (Mayer *et al.*, 1999), this is a great benefit, because these compounds do not inhibit cell cycle progression in *Xenopus* extracts as they would in mammalian cells.

Studies of the Wnt Pathway and β*-Catenin Proteolysis in* Xenopus *Extracts.* The Wnt pathway, which takes its name from the secreted Wnt signaling proteins, is one of the most ancient signaling pathways in metazoans, conserved from Hydra to humans (Logan and Nusse, 2004; Moon *et al.*, 2004). It is used repeatedly during embryonic development in many different contexts, regulating cell fate and tissue specification in embryos from the unicellular stage to late organogenesis. Wnt signaling is also important in cancer (Polakis, 2000), because most colon cancers express increased levels of β-catenin. A few dozen genes are involved in Wnt signaling, but a core module transduces extracellular Wnt signals to control the rate of degradation of β-catenin. Normally β-catenin is unstable in the absence of Wnt stimulation, because of a futile cycle of synthesis and ubiquitin-dependent degradation. To be targeted for degradation, β-catenin must be first phosphorylated on conserved serine and threonine residues clustered close to the N-terminus of the protein. A casein kinase 1 (CK1) site is phosphorylated first (''priming''), which then triggers phosphorylation by

glycogen synthase kinase 3 (GSK3) (Amit *et al*., 2002; Liu *et al*., 2002). These phosphorylations occur in a large, multi-subunit complex built around two scaffold proteins: axin and the adenomatous polyposis coli protein (APC, not to be confused with the anaphase-promoting complex that degrades cyclin), both required for β-catenin degradation in *Xenopus* extracts (Salic *et al*., 2000). Axin concentration is limiting for β-catenin phosphorylation and degradation in extracts. Once phosphorylated, β-catenin is recognized by the F-box protein β-TRCP (Hart *et al*., 1999; Kitagawa *et al*., 1999; Liu *et al*., 1999; Winston *et al*., 1999) and polyubiquitinated by the SCF complex, followed by proteasomal degradation. Although the exact mechanism is still unclear, a Wnt signal inhibits β-catenin phosphorylation and degradation, resulting in β-catenin accumulation and transcriptional activation of target genes. For more details, the reader is referred to several recent reviews (Logan and Nusse, 2004; Moon *et al*., 2004; Seidensticker and Behrens, 2000).

The Wnt pathway is active in early frog development, where it plays critical roles in axis formation (Heasman *et al*., 1994). Early embryos respond dramatically to perturbations of Wnt signaling: Wnt stimulation results in embryos with exaggerated dorsal structures, whereas Wnt inhibition generates embryos with expanded ventral structures (Heasman *et al*., 1994; McMahon and Moon, 1989; Sokol *et al*., 1991). We have taken advantage of the responsiveness of the egg cytoplasm to Wnt signaling and reconstituted the cytoplasmic steps of Wnt signaling in a physiological, unsimplified context (Salic *et al*., 2000). The rate of β-catenin degradation in *Xenopus* extracts is very similar to that in embryos. β-Catenin degradation in extracts requires axin, GSK3, APC, and β-TRCP, and is inhibited by Dishevelled, consistent with the genetics of Wnt signaling. Experiments in egg extracts facilitated the biochemical dissection of the mechanism by which Dishevelled signals (Salic *et al*., 2000) and uncovered a role for Tcf3 in β-catenin turnover (Lee *et al*., 2001). More recently, the ability to manipulate Wnt pathway components and to make precise biochemical measurements of Wnt signaling in extracts was used to develop a mathematical model of Wnt signaling. The model accurately describes signal propagation through the Wnt pathway and has predicted several interesting features of Wnt signaling (Lee *et al*., 2003).

Preparation of Extracts and Reporter Proteins

Preparation of Reporter Proteins

Most analyses of ubiquitin-dependent protein degradation in *Xenopus* extracts have detected endogenous substrates by immunoblotting or relied

on addition of exogenous radiolabeled substrates. To develop reporter proteins suitable for high-throughput screening, we generated fusions of cyclin B or β-catenin to firefly luciferase (Deluca, 1976; Gould and Subramani, 1988). These fusion proteins permit simple determination of reporter protein level using a well-established luciferase assay that can be easily adapted to a high-throughput screening format. For small-scale screens, we have found expression of reporter proteins in reticulocyte lysate to be a convenient approach. To construct a cyclin-luciferase fusion protein (cyc-luc) for expression in reticulocyte lysate, the N-terminal sequence of *Xenopus laevis* cyclin B1, including amino acids 2–97, was amplified by PCR, digested with *BstE*II, and ligated into the pSP-lucNF expression vector (Promega). The fusion protein was expressed by coupled *in vitro* transcription and translation in reticulocyte lysate using the SP6-TNT Coupled Reticulocyte Lysate System (Promega), flash frozen in liquid nitrogen, and stored at −80° until the time of use. The parental pSP-lucNF vector was used to express unmodified luciferase as a stable control protein. A similar approach was used to generate a luciferase β-catenin fusion protein (Salic *et al.*, 2000).

To express higher amounts of reporter proteins for large-scale screens, expression in *Escherichia coli* or baculovirus can be performed. A vector for expression of cyclin-luciferase in *E. coli* (pET cyc-luc) was constructed, and the *E. coli*–expressed protein was found to behave identically in all assays to the protein expressed in reticulocyte lysate. To generate this reporter protein, pSP cyc-luc was digested with *Hind*III and *Xho*I. The resulting 1949-bp fragment containing the cyclin B1-luciferase sequence was ligated into the pET 28b expression vector (Novagen) containing an N-terminal hexahistidine tag for protein purification. To express this fusion protein, 1 liter of LB containing *E. coli* strain BL21(DE3) was grown at 37° to an OD600 of 0.6. Expression was induced for 3 h with 1 m*M* IPTG. The cells were pelleted and lysed and protein purified by Ni-NTA batch purification under native protein conditions (Qiagen). This procedure typically yields approximately 500 μg of protein per liter of culture.

For large-scale screens of β-catenin degradation, we expressed luciferase-β-catenin through baculovirus-mediated expression in Sf9 cells (Salic *et al.*, 2000). A hexahistidine-tagged luciferase-β-catenin fusion was built in the pFastBac vector, and a baculovirus was generated using the Bac-to-Bac system (Invitrogen). The recombinant protein was purified from insect cells in high yield by standard Ni-NTA affinity chromatography. Yields were typically 10 mg per liter of cultured Sf9 cells. After dialysis against the desired buffer (XB, see later) the protein was concentrated to 1 mg/ml and aliquots flash frozen in liquid nitrogen. Although the protein remains soluble at this concentration, we have noted that precipitation occurs at higher concentrations.

Preparation of Proteins to Stimulate Protein Degradation

An important feature of the *Xenopus* system that makes it especially valuable for small molecule screening is that proteolysis can be specifically stimulated by addition of critical regulatory proteins to the extract. For example, in studies of cyclin degradation, we can activate APC/C in interphase extracts in one of two ways. The extracts can be induced to enter mitosis by addition of nondegradable cyclin B, which activates Cdc2 and stimulates mitotic phosphorylation, resulting in APC/C activation. Alternately, APC/C activity can be stimulated by adding recombinant Cdh1 to interphase extracts, which can induce cyclin proteolysis in the absence of mitotic phosphorylation (Pfleger and Kirschner, 2000). The ability to stimulate APC/C-dependent destruction by two different mechanisms provides a useful way for characterizing the mechanism of action of inhibitors discovered in the screen (Verma *et al.*, 2004).

To express nondegradable cyclin B, we generated a fusion of the maltose-binding protein to *Xenopus* cyclin B lacking its N-terminal 90 amino acids (MBP-Δ90). This protein can be expressed in a soluble form and purified according to standard procedures (New England Biolabs). We have found that MBP-Δ90 preparations are more reproducible than inclusion body preparations that use untagged sea urchin cyclin Δ90 (Glotzer *et al.*, 1991) and yield 2–3 mg/liter of culture. To express Cdh1, we use his-tagged human protein expressed in baculovirus (Pfleger and Kirschner, 2000). We have found this protein difficult to purify to homogeneity, with yields less than 1 mg/liter of culture, but even in impure form it is capable of stimulating cyclin proteolysis in interphase extracts.

In a similar manner, β-catenin degradation in *Xenopus* extracts can be significantly accelerated by supplementing the extract with recombinant axin and/or GSK3. For small-scale experiments, supplementing the extracts with axin expressed by *in vitro* translation in reticulocyte lysates works well. For large-scale screens, MBP-tagged full-length mouse axin can be purified in soluble and active form from either bacteria or Sf9 cells. For GSK3, we obtain the active his-tagged *Xenopus* protein by expression in insect cells. Alternately, the protein can be expressed and purified from bacteria as an MBP fusion.

Preparation of Extracts

Xenopus egg extracts are prepared from eggs laid overnight according to the protocol of Murray (1991) with several modifications.

A. Solutions

Extract buffer (XB): 100 m*M* KCl, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* potassium HEPES (pH 7.7), 50 m*M* sucrose.

MMR (1X): 100 m*M* NaCl, 2 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 0.1 m*M* EDTA, 5 m*M* HEPES (pH 7.8). We typically prepare a 25× solution, pH to 7.8, with NaOH and dilute to 1× just before use.

Energy mix: For a 20× stock, prepare a solution of 150 m*M* creatine phosphate, 20 m*M* ATP, 2 m*M* EGTA, and 20 m*M* $MgCl_2$ in water and adjust pH to 7.7.

Dejellying solution: Dissolve 3% w/v cysteine HCl (Sigma C-7880) in water. Titrate to pH 7.8 with NaOH. Prepare within 1 h of use.

Protease inhibitors: For a 1000× stock, prepare a mixture of leupeptin (Calbiochem NC9267778), chymostatin (MP Biomedical 15284550), and pepstatin (MP Biomedical 19536825) dissolved to a final concentration of 10 mg/ml each in DMSO. Store in aliquots at −20°.

Cytochalasin B: For a 1000× stock, dissolve cytochalasin B (MP Biomedical 19511910) at 10 mg/ml in DMSO and store in aliquots at −20°.

Calcium ionophore: For a 5000× stock, dissolve A23187, free acid form (Calbiochem 100105) at 10 mg/ml in DMSO and store in aliquots at −20°.

Cycloheximide: For a 100× stock, dissolve at 10 mg/ml in water and store in aliquots at −20°.

Pregnant mare serum gonadotropin (PMSG): 100 U/ml PMSG (Calbiochem, 367222) made up in water and stored at −20°.

Human chorionic gonadotropin (hCG): 500 U/ml hCG (Sigma CG-5) made up in water and stored at 4°.

B. Induction of Ovulation

Frogs are primed with 50 U of PMSG on day 1 and 25 U of PMSG on day 3. Ovulation can be induced by administration of 250 U of hCG on days 5–12. After injection of hCG, frogs are placed in separate 6-liter buckets containing 2 liters of 1× MMR. Frogs are allowed to lay eggs overnight (12–18 h) at 18°.

C. Preparation of Extracts

1. Frogs are removed from containers and eggs examined. If more than 10% of the eggs are white in color, have an abnormal morphology, or are bound together in strings, all of the eggs laid by the frog are discarded.

2. The remaining eggs are pooled and washed three times in 1× MMR (prechilled to 16°) to remove debris. Excess buffer is removed, and the eggs are incubated with three volumes of 3% cysteine to remove the jelly coat. Eggs should be gently swirled during the dejellying procedure, which should take approximately 5 min. Dejellying is complete when the eggs pack as dense spheres.

3. Wash eggs thoroughly in 1× MMR until the buffer remains clear, typically 5 times. Remove dead eggs (white or puffy in appearance) with a

Pasteur pipette that has been modified such that the mouth opening is wide enough to accommodate the eggs without lysing them.

4. To prepare interphase extracts, dilute calcium ionophore to 2 μg/mL in 1× MMR with rapid mixing, and immediately incubate eggs with a twofold volume excess of the diluted ionophore solution. Continue incubation until cortical contraction is observed (contraction of the pigmented area of the egg into a smaller circle), approximately 5 min. For β-catenin degradation, egg activation with ionophore is not necessary.

5. After egg activation, eggs are washed three times in XB (prechilled to 16°) and then into XB containing 1 × protease inhibitors (10 μg/ml each). For interphase extracts, the eggs are allowed to incubate for 25 min after activation. During this period, dead eggs can be removed with a pipette.

6. After the incubation period, eggs are transferred to centrifuge tubes that have been prechilled on ice. For a large preparation involving more than 25 ml of eggs, we use 50-ml centrifuge tubes (Nalgene 3110-0500) that contain 2 ml of XB with 100 μg/ml cytochalasin B and 10 μg/ml protease inhibitors. Mix cytochalasin rapidly with buffer to prevent precipitation. Eggs should be transferred to tubes in a minimum of buffer. For smaller-scale preparations, eggs can be transferred to 1.5-ml microcentrifuge tubes that contain 200 μl of buffer with diluted cytochalasin and protease inhibitor. Allow eggs to settle and remove excess buffer with a pipette.

7. Spin tubes at low speed (for 50-ml tubes, we use a Sorvall HB-6 rotor at 860 rpm) for 1 min to pack the eggs. For small preparations, a brief spin at 600 rpm in a refrigerated Microfuge is sufficient to pack the eggs. Aspirate excess buffer.

8. For interphase extracts for studies of cyclin proteolysis, we crush the eggs by spinning for 15 min at 4° in an HB-6 rotor at 12,000 rpm (23,000*g*) or at 14,000 rpm in a Microfuge (21,000*g*) for small preparations. The cytoplasmic layer (the middle layer between the yellow lipid on top and dark yolk at the bottom) is then removed by needle puncture and aspiration. Heating the needle in a flame facilitates puncture of thick-walled tubes. The extracts are placed on ice, cytochalasin and protease inhibitors are each added to 10 μg/ml, and cycloheximide is added to 100 μg/ml, and the extracts mixed thoroughly by pipetting. The extracts are then spun a second time under the same conditions, and the cytoplasmic layer is harvested as before. The extract is then supplemented with 1× energy mix and 4% glycerol, mixed well, and aliquots snap frozen in liquid nitrogen and stored at −80°. If the eggs are good quality, we generally obtain 1–2 ml of extract from the eggs laid overnight by one PMSG-primed frog.

9. For preparation of extracts to study β-catenin degradation, eggs are packed as previously but crushed by spinning for 5 min at 21,000*g* in a refrigerated Microfuge. For larger-scale extract preparations, eggs can be

spun at 21,000*g* in either an HB-6 (swinging bucket) or an SS-34 (fixed angle) rotor. The middle layer of cytoplasm is removed by using a P-1000 Pipetman (Rainin) to pierce the top lipid layer. The lipid that sticks to the sides of the pipette tip is removed with a Kimwipe before expelling the contents into a fresh chilled tube. The crude extract is supplemented with cytochalasin B (10 μg/ml), mixed well, and then spun again for 5 min at 21,000*g*. The cytoplasmic layer is again removed and spun a third time under identical settings. The final extract should be a clear yellow-gold color. Protease inhibitors (10 μg/ml) and energy mix are added, and aliquots are snap frozen in liquid nitrogen and stored at -80°. We did not find it necessary to add cryoprotectants (sucrose or glycerol) to the extracts before freezing for studies of β-catenin degradation. A good batch of extract retains its ability to degrade cyclin or β-catenin for at least 1 y, and even 2- to 3-year-old extracts can degrade β-catenin if supplemented with axin.

Assessment of Extract Quality

Extracts can vary significantly in their ability to degrade cyclin B or β-catenin. For reproducible cyclin B proteolysis, we have found it essential to adhere rigorously to the priming protocol used to induce ovulation. Although frogs can be induced to ovulate without prior PMSG priming, we have found that extracts prepared from eggs laid from unprimed frogs often do not degrade cyclin B efficiently. Also, we have found that efficient egg activation with calcium ionophore is essential for extracts to reproducibly degrade cyclin B.

Earlier protocols (Murray, 1991) use lower-speed centrifugation (10,000 rpm or 16,000*g*) for crushing the eggs. We have noted that such lower-speed extracts are often plagued by caspase activation that can obscure *bona-fide* ubiquitin-dependent β-catenin degradation (Fig. 1; A. Salic and E. Lee, unpublished). β-Catenin is a very good substrate for caspases (Brancolini *et al*., 1997), and proteolysis of β-catenin by caspases generates cleavage products that are no longer degraded in the Wnt pathway. The same pattern of β-catenin cleavage is seen when extracts that do not have high caspase activity are supplemented with dATP (10 m*M*) or with purified cytochrome *C*, two known apoptotic triggers (Kluck *et al*., 1997; Liu *et al*., 1996). Also, caspase 3 is cleaved and activated in a similar manner in these *Xenopus* extracts (Fig. 1), further supporting the idea that β-catenin clipping in these extracts is due to caspase activation. We found that a more clarified extract produced by higher-speed centrifugation has negligible caspase activity, thus allowing robust reconstitution of β-catenin degradation without caspase interference. We speculate this is due to reduced amounts of mitochondria, which are required for triggering apoptosis in *Xenopus* extracts.

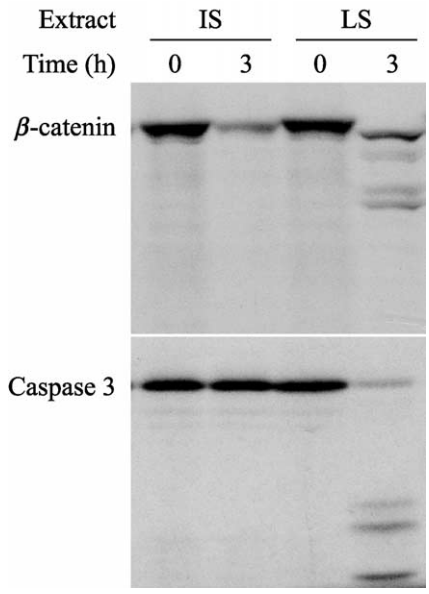

FIG. 1. Intermediate speed extracts are devoid of caspase activity. Extracts were prepared using low-speed centrifugation (LS, 16,000*g*) or intermediate-speed centrifugation (IS, 21,000*g*). Extracts were incubated with [^{35}S]-labeled β-catenin or procaspase 3 for 3 h. Aliquots were removed at the indicated times and processed by SDS-PAGE and radiography. In low-speed extracts (LS), pro-caspase 3 is activated, and β-catenin is cleaved, but the cleavage products remain stable. In intermediate speed extracts (IS), pro-caspase 3 is not activated, β-catenin is degraded by ubiquitin-dependent proteolysis.

Before embarking on a large-scale screen, we recommend making a large batch of extract and testing it for activity before initiating the screen. Extracts that generate β-catenin cleavage products indicative of caspase activity should be discarded. If the β-catenin degradation rate is too slow (half-life longer than 1–1.5 h), an extract batch can usually be ''rescued'' for screening by supplementing it with recombinant axin.

High-Throughput Screening

Screening for Inhibitors of Cyclin Proteolysis

We have performed small molecule screening in 384-well plates, which use about 5–10 μl of extract per well, and also in 1536-well plates, which use 2 μl of extract per well. Here we describe detailed procedures for screening

in 384-well plates, but methods for screening in 1536-well plates are available in other articles (Verma *et al*., 2004; Walling *et al*., 2001).

1. Interphase extracts are thawed rapidly and diluted to a final concentration of 75% in extract buffer (XB) just before assay. Extracts are kept on ice and supplemented with 1 mg/ml bovine ubiquitin (Sigma), 10 μg/ml MBP-cyclin BΔ90, and cyclin-luciferase (cyc-luc) reporter protein. If using an *in vitro* translated reporter protein, the reticulocyte lysate is mixed directly into the *Xenopus* extract at a dilution of 1:200. If bacterially expressed cyc-luc is used instead, it is added to a final concentration of 0.1 μg/ml. It is important to mix all components well.

2. Extract is then distributed to individual wells of chilled 384-well plates. We use white, low-volume 384-well plates, such as Cliniplates (Thermo Labsystems, 95040010). Extract can be distributed using a multichannel pipetter or a plate dispenser such as the Multidrop Dispenser (Labsystems).

3. Compound libraries are stored as 5 mg/ml stocks in DMSO in 384-well polypropylene plates. For high-throughput screening, compounds are transferred to plates using an array of stainless steel pins attached to a robotic arm (Walling *et al*., 2001). The amount of compound transferred is determined by pin size and the speed at which the pin array is removed from the compound stock solution. We use pin arrays that transfer approximately 100–200 nl of compound per well to yield a screening concentration of 50–100 μg/ml. High compound concentrations are used because of the high protein (30–50 mg/ml) and lipid concentration of *Xenopus* extracts, resulting in a large fraction of added compound to be nonspecifically sequestered by protein binding or partitioning into membrane compartments. The contents of the wells are mixed using an orbital plate shaker followed by a brief spin in a tabletop centrifuge equipped with microplate carriers (Sorvall Legend RT). For more accurate transfer of compounds for retesting and determination of dose–response, compounds are first diluted at varying concentrations in DMSO. Compounds are then diluted 10-fold in XB and mixed well. The compound stocks are then diluted 10-fold again in extracts, adding 1 μl of compound solution to 9 μl of extract that has already been dispensed into the plate. The contents of each well are mixed with a multichannel pipette.

4. Plates are then warmed to room temperature, allowing the extracts to proceed into mitosis, activating the APC/C, and initiating cyclin degradation. After 60–75 minutes, 30 μl of luciferin reagent (20 m*M* tricine, pH 7.8, 470 μM D-luciferin [Molecular Probes]), 270 μM coenzyme A, 0.1 m*M* EDTA, 33 m*M* DTT, and 530 μM ATP) is added using a multidrop

dispenser (Labsystems). Luminescence is then measured on a plate reader, such as the Analyst (Molecular Devices).

5. For each screen we also prepare interphase extracts that contain the cyclin-luciferase reporter protein but lack MBP-cyclin BΔ90. These extracts remain in interphase and thus do not degrade the cyclin-luciferase reporter protein. We calculate percent inhibition as 100*(T–M)/(I–M), where *T* equals the luminescence value for the test compound in mitotic extract, *M* equals the value in a mitotic extract lacking inhibitor, and *I* equals the value in an interphase extract lacking inhibitor.

6. To characterize inhibitors identified in the screen, the preceding protocol can be modified in several ways. First, extracts can be preactivated to enter mitosis before addition of compound and reporter protein. In this case, interphase extracts are mixed with MBP-cyclin BΔ90 and allowed to incubate for 40–60 minutes at room temperature. The extract is then chilled on ice and mixed with cyc-luc. The extract is distributed to chilled plates, and compounds are then added as described previously. The plates are allowed to warm to room temperature, incubated for 30–60 min, and luciferin reagent is added and luminescence measured. In this assay, only compounds that directly interfere with the cyclin degradation machinery remain active, whereas compounds that act by blocking the transition from interphase into mitosis lose activity. Alternately, interphase extracts can be stimulated to degrade cyclin B by addition of the APC/C activator protein Cdh1. This activation step does not require mitotic phosphorylation of the APC/C, and, therefore, compounds that remain active in this assay are likely to function by direct inhibition of the cyclin proteolysis machinery rather than by blocking the transition from interphase to mitosis.

Screening for Inhibitors of β*-Catenin Proteolysis*

1. Extracts are thawed rapidly and placed on ice. If desired, the extract can be diluted with cold XB. Extracts are supplemented with bovine ubiquitin (0.3 mg/ml), protease inhibitors, and energy mix. Axin and GSK3 are added to 20–50 n*M* and 200 n*M*, respectively. β-Catenin-luciferase is then added at a concentration of 50 n*M*, and the extract is mixed thoroughly. The extract is then distributed to chilled 384-well plates as described for the cyclin degradation assay. Compounds are transferred and the plates mixed as described previously.

2. The plates are then incubated at room temperature in a closed, humidified chamber (such as a large Tupperware container) for 3 h. Luciferin solution is then added and the assay read as described previously.

Characterization of Active Compounds

Comparison of the activity of compounds across a variety of assays in *Xenopus* extracts has proved to be a useful method for determining the mechanism of action of inhibitors (Verma *et al.*, 2004). Figure 2 illustrates how known inhibitors of cell cycle progression or the Wnt pathway affect degradation of the reporter proteins in the two different assays described previously. Figure 3 demonstrates how comparison of compounds in multiple assays can be used to characterize the activity of unknown compounds discovered through high-throughput screening. Using the cyclin-luciferase assay in which degradation was stimulated from interphase extracts with nondegradable cyclin B, we screened more than 100,000 compounds to identify 22 inhibitors (Verma *et al.*, 2004). These compounds were subsequently retested in the four assays described previously. Figure 3 shows the activity of 12 of these inhibitors in the four assays. The compounds were tested in assays in which extracts were first allowed to enter mitosis before compound addition or were stimulated to degrade cyclin B-luciferase by Cdh1 addition. Finally, the compounds were tested in a β-catenin-luciferase degradation assay. As shown in Fig. 3, compounds have unique patterns of activity across each of these four assays, allowing compounds to be grouped into different functional classes. For example, compounds in class I blocked degradation in interphase but not mitotic extracts, suggesting they acted by inhibiting the transition from interphase to mitosis. A subset of these compounds (class IB) also inhibited degradation in the β-catenin assay, which may be due to inhibition of kinases such as GSK3, CK1, and cyclin B/cdc2 kinase. Other compounds, such as those in Class IIA, seem to be specific inhibitors of the cyclin proteolysis machinery, because they did not inhibit β-catenin degradation. Compounds in Class IIB inhibited both β-catenin and cyclin degradation, suggesting they inhibited a common component of the ubiquitin-proteasome pathway. Two of these compounds (C59 and C92) were identified as ubistatins, compounds that inhibit proteasome-dependent degradation by binding to the ubiquitin chain (Verma *et al.*, 2004).

Surprisingly, this screening method did not identify small molecules that directly inhibit the peptidase activity of the proteasome. We have found that inhibitors such as MG132 that target proteasome peptidase activity scored only weakly in our assay (Verma *et al.*, 2004) when we measure luciferase activity. However, immunoblotting of the reporter protein indicated that proteasome inhibitors indeed stabilize cyclin-luciferase fusion proteins in *Xenopus* extracts. One potential explanation for this finding is that the luciferase reporter may become unfolded by the proteasome when the peptidase activity is blocked, rendering the reporter protein

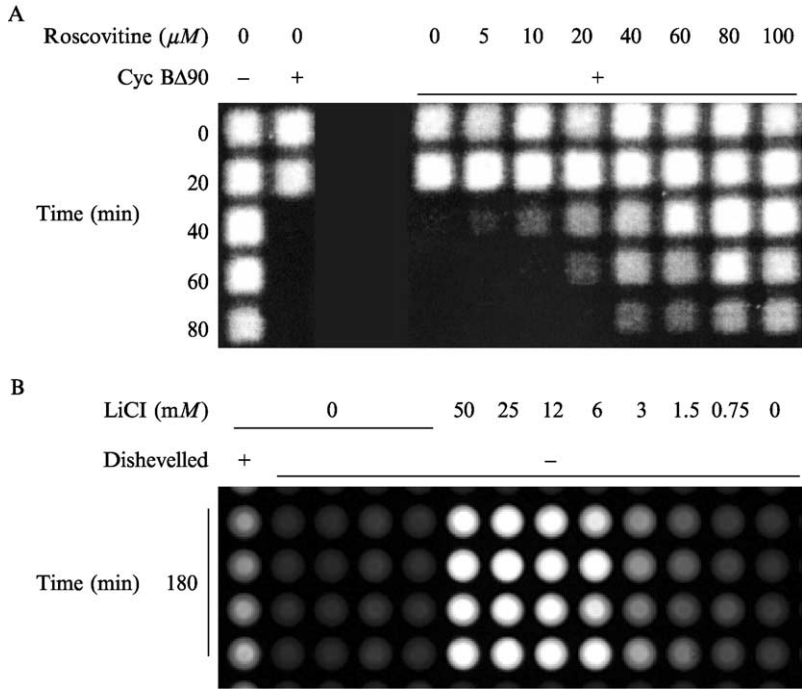

FIG. 2. Known inhibitors block the regulated degradation of cyc-luciferase and luciferase–β-catenin reporter proteins in *Xenopus* extracts. (A) Roscovitine, a small molecule inhibitor of Cdc2 kinase, blocks cyclin degradation by preventing mitotic entry. Interphase extracts containing the cyc-luciferase reporter protein were stimulated to enter mitosis by addition of nondegradable cyclin B (cyclin BΔ90), in the presence or absence of varying concentrations of roscovitine. At the indicated time points, aliquots were snap frozen. At the end of the experiment, samples were thawed and processed for luminescence imaging in a 384-well plate. The reporter protein is stable in interphase extracts that lack cyclin BΔ90, or in the presence of roscovitine, which blocks mitotic entry by inhibiting Cdc2 kinase activation. (B) Luciferase–β-catenin degradation by axin-supplemented extracts in a 384-well plate. Addition of recombinant mouse Dishevelled1 (1 μM) or lithium chloride (LiCl), a GSK3 inhibitor, block the degradation of the reporter protein in a dose-dependent manner.

inactive. Compounds such as ubistatins, which block recruitment of ubiquitinated proteins to the proteasome, stabilize luciferase reporter proteins in an active, folded conformation.

A variety of secondary assays can be performed to characterize the mechanism of inhibition. Extracts can be probed with antibodies that monitor the phosphorylation status of extract components in the presence of inhibitor. For example, CDC27, a subunit of the APC/C, undergoes a mitosis-specific change in mobility on SDS-PAGE that is due to mitotic

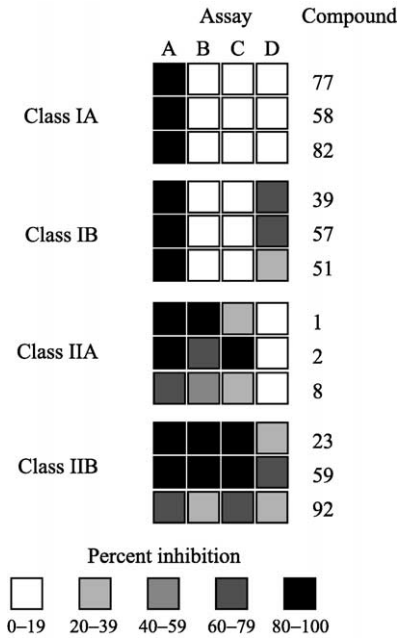

FIG. 3. Characterization of inhibitors by comparing activities in multiple assays in *Xenopus* extracts. More than 100,000 compounds were screened for the ability to block cyc-luciferase turnover in interphase extracts stimulated to enter mitosis with nondegradable cyclin B (Verma *et al.*, 2004). The most active compounds were then retested at 200 μM concentration in several different assays. (A) Activity of compounds in the original assay. (B) Extracts were allowed to enter mitosis before addition of compound and reporter protein. (C) Interphase extracts were stimulated to degrade cyc-luciferase by addition of Cdh1. (D) Determination of activity in the luciferase–β-catenin assay.

phosphorylation (King *et al.*, 1995; Peters *et al.*, 1996). Analysis of class I and II inhibitors showed that class I compounds blocked CDC27 phosphorylation, consistent with inhibition of mitotic entry, whereas class II compounds resulted in mitotic arrest with sustained CDC27 phosphorylation, consistent with specific inhibition of the cyclin proteolysis machinery (N. Peters and R. King, unpublished data). Compounds can be tested directly in a variety of reconstituted biochemical assays for direct effects on kinase inhibition, APC/C-dependent ubiquitination (King *et al.*, 1995), or proteasome-dependent degradation (Verma *et al.*, 2004).

Similar approaches can be used to further study compounds that inhibit β-catenin degradation. Phospho-specific antibodies are available to assay the status of β-catenin phosphorylation by GSK3 or CK1 (Amit *et al.*, 2002;

Liu *et al.*, 2002). To test whether a compound inhibits GSK3 or CK1, β-catenin can be first probed with phospho-specific antibodies directly in the extract. If inhibition of specific phosphorylation is detected, the compound(s) can be tested with *in vitro* kinase assays with purified components. Axin stimulates β-catenin phosphorylation by both GSK3 and CK1; it would, thus, be best to perform the kinase assay using purified kinase, axin, and β-catenin, rather than just assaying the phosphorylation of a peptide substrate by the kinase. This more complex kinase assay might also identify inhibitors that interfere with the kinase-axin or axin–β-catenin interactions that would be missed in a simple peptide phosphorylation assay. Protein–protein interactions in the β-catenin degradation complex are regulated by phosphorylation, such as the binding of β-catenin to APC that is stimulated by APC phosphorylation. To test whether a compound inhibits a protein–protein interaction, gel filtration or density gradient centrifugation can be performed to determine whether the size of the known complex is affected by the small molecule. Another interaction required for β-catenin degradation is recognition of phosphorylated β-catenin by the F-box protein β-TRCP. Compounds that stabilize β-catenin by blocking this interaction could be identified by performing binding assays between β-TRCP on beads and recombinant β-catenin phosphorylated *in vitro* with axin, CK1, and GSK3. Extracts also allow quick "epistasis" experiments to narrow down the level at which a given inhibitor acts. If a compound inhibits β-catenin degradation by blocking its recognition by β-TRCP, adding more axin and/or GSK3 would not be expected to rescue the effect; if, however a compound inhibits β-catenin degradation upstream of or at the level of the degradation complex, increasing axin levels will likely reverse the effect of the compound.

Ubiquitin conjugates of cyclin B and β-catenin are ultimately degraded by the proteasome. Therefore, small molecules that inhibit protein degradation downstream of substrate ubiquitination are likely to score in both the luciferase–β-catenin and cyclin-luciferase screens. The activity of such molecules can be characterized in peptidase assays that monitor catalytic activity of the 20S proteasome core or in reconstituted assays using ubiquitinated proteins and purified 26S proteasomes (Verma *et al.*, 2004).

Acknowledgments

A. S. was a Damon-Runyon postdoctoral fellow and is currently supported through a fellowship from the Charles King Trust of the Medical Foundation. R. W. K. is a Damon Runyon Scholar, and work in the laboratory is funded by grants from the NIH (CA78048, GM66492), the McKenzie Family Foundation, and the Harvard-Armenise Foundation.

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[39] Development and Characterization of Proteasome Inhibitors

By KYUNG BO KIM, FABIANA N. FONSECA, and CRAIG M. CREWS

Abstract

Although many proteasome inhibitors have been either synthesized or identified from natural sources, the development of more sophisticated, selective proteasome inhibitors is important for a detailed understanding of proteasome function. We have found that antitumor natural product epoxomicin and eponemycin, both of which are linear peptides containing a α,β-epoxyketone pharmacophore, target proteasome for their antitumor activity. Structural studies of the proteasome–epoxomicin complex revealed that the unique specificity of the natural product toward proteasome is due to the α,β-epoxyketone pharmacophore, which forms an unusual six-membered morpholino ring with the amino terminal catalytic Thr-1 of the 20S proteasome. Thus, we believe that a facile synthetic approach for α,β-epoxyketone linear peptides provides a unique opportunity to develop

METHODS IN ENZYMOLOGY, VOL. 399

0076-6879/05 $35.00
DOI: 10.1016/S0076-6879(05)99039-3