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Small ubiquitin-like modifying protein isopeptidase assay based on poliovirus RNA polymerase activity

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Abstract

The ubiquitin–proteasome pathway is the major nonlysosomal proteolytic system in eukaryotic cells responsible for regulating the level of many key regulatory molecules within the cells. Modification of cellular proteins by ubiquitin and ubiquitin-like proteins, such as small ubiquitin-like modifying protein (SUMO), plays an essential role in a number of biological schemes, and ubiquitin pathway enzymes have become important therapeutic targets. Ubiquitination is a dynamic reversible process; a multitude of ubiquitin ligases and deubiquitinases (DUBs) are responsible for the wide-ranging influence of this pathway as well as its selectivity. The DUB enzymes serve to maintain adequate pools of free ubiquitin and regulate the ubiquitination status of cellular proteins. Using SUMO fusions, a novel assay system, based on poliovirus RNA-dependent RNA polymerase activity, is described here. The method simplifies the isopeptidase assay and facilitates high-throughput analysis of these enzymes. The principle of the assay is the dependence of the viral polymerase on a free N terminus for activity; accordingly, the polymerase is inactive when fused at its N terminus to SUMO or any other ubiquitin-like protein. The assay is sensitive, reproducible, and adaptable to a high-throughput format for use in screens for inhibitors/activators of clinically relevant SUMO proteases and deubiquitinases.

Keywords

Isopeptidases; Protein degradation; N terminus; 3D polymerase

The ubiquitin–proteasomal pathway regulates the cellular content and/or compartmentalization of many proteins [1,2] and is a promising, albeit underexploited, area for drug discovery. The therapeutic value of this pathway was recently validated by the introduction and clinical success of Velcade, a proteasome inhibitor, for the treatment of refractory relapsed multiple myeloma [3]. Other ubiquitin-like proteins (UBLs)¹ have recently been shown to contribute similarly to the cellular regulation of proteins, with the most extensively characterized UBL being small ubiquitin-like modifying protein (SUMO) [4].

Destruction of a targeted protein via the ubiquitin system initially involves recognition by a multienzyme system that attaches ubiquitin to the target protein. Energy is required to activate ubiquitin at its carboxy terminus. Activation is catalyzed by the enzyme E1, which links the ubiquitin C terminus to a cysteine side chain of the enzyme. This activated ubiquitin is

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¹Abbreviations used: UBL, ubiquitin-like protein; SUMO, small ubiquitin-like modifying protein; UBP/USP, ubiquitin-specific protease; UCH, ubiquitin terminal hydrolase; DUB, deubiquitinating enzyme; Ub-AMC, Ub-7-amino-4-methylcoumarin; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonylfluoride; GFP, green fluorescent protein; DTT, dithiothreitol.

transferred to a second enzyme of the series, E2 (ubiquitin-conjugating protein), as a thioester (Ub-CO-S-E1 $\rightarrow$ Ub-CO-S-E2). E2 then transfers ubiquitin to a target polypeptide, which is bound to the third enzyme in the sequence, E3 or ubiquitin ligase. Proteins tagged with polyubiquitin are delivered to the proteasome complex, which hydrolyzes the polypeptide into short oligopeptides and releases free ubiquitin that is recycled. SUMO is similarly conjugated to various cellular proteins through a similar E1–E3 sequence of enzymes [5–7].

Ubiquitin, SUMO, and other UBLs can also be deconjugated enzymically [6,8] by proteases (Fig. 1). In addition to the classical UBP/USP (ubiquitin-specific protease) and UCH (ubiquitin C-terminal hydrolase) enzymes, there are at least three distinct groups of enzymes capable of removing ubiquitin or a related UBL (e.g., SUMO) from a protein substrate. Proteases specific for SUMO conjugates include yeast Ulp1 and Ulp2 [9,10] and human SENP1 and SENP2 [11,12]. Other known ULPs include UBP43, which cleaves ISG15 conjugates [13], and DEN1 (SENP8), which deconjugates Nedd8 from protein substrates [14,15]. Two recently identified classes of deubiquitinating enzymes (DUBs) are the otubains, cysteine proteases belonging to the OTU (ovarian tumor) superfamily of proteins [16], and the JAMM family enzymes, which are quite different from most DUBs in that they are metalloproteases that cleave ubiquitin from substrates in a Zn^{2+} - and ATP-dependent manner [17]. There are approximately 100 different DUBs in humans [18] in addition to many proteases for the other ULPs. The number, structural diversity, and emerging roles as cellular regulators underscore the potential utility of these enzymes as targets for therapeutic intervention.

Although isopeptidases are clearly potential drug targets, no straightforward assays are available for mechanistic studies or high-throughput screening for drug discovery [19,20]. Most assays currently in use rely on cleavage of linear fusions produced in *Escherichia coli* or synthesized chemically [21]. Reaction products are analyzed by gel electrophoresis [19,20, 22–26] or are selectively precipitated and analyzed by liquid scintillation spectrometry [24, 27]. Gel-based procedures are labor intensive and expensive, and although scintillation counting approaches are quantitative and allow processing of larger numbers of samples than do gel-based assays, they require centrifugation and recovery of supernatant [27]. A fluorogenic substrate, Ub-AMC (Ub-7-amino-4-methylcoumarin), has been employed in some studies [28], as has a similar substrate, the tetrapeptide z-LRGG-AMC, which mimics the carboxyl terminus of ubiquitin [29]. A limitation of both fluorescent substrates is that small adducts cannot be hydrolyzed efficiently by many proteases [21]. Moreover, AMC is highly hydrophobic and may give rise to false positives in screening owing to its own interactions with test compounds.

An ideal screening assay requires simple reactions (i.e., minimal number of steps) that can be conducted using multiwell plates from which endpoints are read directly. The assay system described in this article is based on the observation that most proteases that hydrolyze ULPs can hydrolyze linear ULP fusions (α -NH–peptide bond) as well as ϵ -NH₂–isopeptide linkages. Moreover, it exploits the unique properties of certain proteins that require a free N terminus for activity. In nature, some proteins are synthesized as inactive precursors, requiring proteolytic cleavage to become active; for example, zymogens undergo cleavage and activation in settings as diverse as blood coagulation and fibrinolysis [30], sodium balance and blood pressure [31], and apoptosis [32]. Cleavage of inactive precursors could generate a positive signal based on the measurable property, catalytic or otherwise, of the released protein.

Proteolytic protein processing and activation is a strategy that is also used by many viruses. In poliovirus, for example, all proteins are initially synthesized as part of a large polyprotein [33]. The polymerase responsible for replicating the poliovirus RNA genome, 3D^{pol}, is inactive within the context of the viral polyprotein, and the addition of even a single amino acid residue to the 3D^{pol} amino terminus is detrimental to polymerase activity [34,35]. Processing of the

polyprotein generates a 3CD protein intermediate, consisting of a fusion between the 3C^{pro} protease and the 3D^{pol} polymerase, that displays only protease activity. Subsequent cleavage to remove the 3C^{pro} region results in activation of the 3D^{pol} polymerase. Thus, during the process of virus infection and production, a stepwise and coordinated activation mechanism brings the polymerase enzyme on line. The 3D^{pol} enzyme is a well-characterized, RNA-dependent RNA polymerase, and its activity is readily assayed [36]. Thus, it seemed plausible that this viral polymerase could be exploited for the development of an assay for ubiquitin or UBL isopeptidase activity.

This article describes the development of an assay for the isopeptidase enzyme SUMO protease, the catalytic domain of yeast Ulp1 [37]. This protease has been evaluated as a tool for purification of proteins from SUMO fusions expressed in *E. coli* and has been found to be extremely robust and precise in its cleavage activity [38]. SUMO protease activity is coupled to the activation of the 3D^{pol} viral polymerase.

Materials and methods

Materials

Restriction enzymes, Antarctic alkaline phosphatase, and T4 DNA Ligase were purchased from New England Biolabs. Platinum *Taq* High Fidelity DNA polymerase (Invitrogen) was employed in all of the PCR amplification reactions. Synthetic oligonucleotide primers were purchased from MWG Biotech. DNase I, egg white lysozyme, Tris buffers, ethylenediaminetetraacetic acid (EDTA), NP40, Triton X-100, glycerol, and imidazole were purchased from Sigma. The parental T7 pET24d(+) expression vector and the *E. coli* host strains BL21(DE3) [*F*⁻ *ompT* *hsdSB*(rB⁻ mB⁻) *gal dcm* (DE3)] and Rosetta(DE3) [*F*⁻ *ompT* *hsdSB*(rB⁻ mB⁻) *gal dcm* (DE3) pRARE2 (CmR)] were obtained from Novagen along with the BugBuster lysis buffer. DNA extractions and purification were performed using Qiagen system kits, and His-tagged proteins were isolated using the Ni-NTA resin from the same supplier. [α -³²P]UTP (6000 Ci/mmol) was purchased from New England Nuclear, nucleoside 5'-triphosphates (all nucleotides were ultrapure solutions) and Q Sepharose fast flow were purchased from Amersham Pharmacia Biotech, poly(rA) was purchased from Sigma, dT₁₅ was purchased from Integrated DNA Technologies, all RNA oligonucleotides were purchased from Dharmacon Research, NZCYM was purchased from Invitrogen, and phosphocellulose (P-11) and DE-81 filter paper were purchased from Whatman. All other reagents were of the highest grade available from Sigma or Fisher.

Plasmid construction

The *Smt3* gene from *Saccharomyces cerevisiae* was modified to contain an N-terminal 6×His-tag by PCR amplification using the following synthetic primer set:

5'-
GCACCATGGGTCATCACCATCATCATCACGGGTCGGACTCAGAAGTCAATC
A-3'

5'-GCAGGATCCGGTCTCAACCTCCAATCTGTTCGCGGTG-3'.

(The reannealing temperature was 55 °C for all PCRs.) These primers introduced *Nco*I and *Bam*HI sites at the 5' and 3' ends, respectively, allowing for insertion of this gene into the multiple cloning site of pET24d(+) following digestion with these two enzymes. In addition, a unique *Bsa*I site was introduced at the 3' end of the *Smt3* coding sequence, which allows direct fusion of the downstream protein coding sequence to the Gly-Gly dipeptide at the C terminus of SUMO. This plasmid, pET24-6H-SUMO, was subjected to DNA sequencing (MWG Biotech) to confirm the final construct.

The poliovirus (Mahoney strain) 3D^{pol} gene segment was PCR amplified from the previously described pET26b-Ub-3D-GSSG-6H plasmid [34] using synthetic primers incorporating a *BsaI* site at the 5' end and a *BamHI* site at the 3' end to facilitate cloning into the pET24-6H-SUMO vector:

5'-GCAGGTCTCAAGGTGGTGAAATCCAGTGGATGAG-3'

5'-GCAGGATCCCTAGTGGTGGTGGTG-3'.

The structure of the final construct, pET24-6H-SUMO-3D^{pol}, was verified by DNA sequencing.

Expression and purification of SUMO-3D^{pol}

The SUMO-3D^{pol} plasmid was transformed into BL21 (DE3). BL21 (DE3) containing the pSUMO-3D plasmid (LifeSensors) was grown overnight in 100 ml of NZCYM supplemented with 25 µg/ml kanamycin (K25) and 0.4% glucose at 30 °C. The overnight culture was then used to inoculate 1 L of K25-supplemented media to OD₆₀₀ = 0.05. The cells were grown at 37 °C to OD₆₀₀ = 0.6–0.8 and then cooled to 25 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 500 µM and was grown for an additional 4 h. The cells were harvested by centrifugation in a Sorvall SLC-6000 rotor at 6000 rpm for 15 min, washed once in 200 ml of T₁₀E₁ (10 mM Tris [pH 8.0], 1 mM EDTA), and centrifuged again before the cell paste was weighed. Typical yields were 7–9 g of cell paste per liter of culture. The cells were then frozen and stored at –70 °C until used.

Frozen cells were thawed and suspended in lysis buffer (100 mM potassium phosphate [pH 8.0], 0.5 mM EDTA, 20% glycerol, 5 mM of 2-mercaptoethanol, 1.4 µg/ml pepstatin A, and 1.0 µg/ml leupeptin) and 5 ml/g of cell paste. The suspended cells were lysed by passing through a French press, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM, and NP-40 was added to 0.1%. The extract was then centrifuged in a Beckman JA-30.50 rotor for 30 min at 30,000 rpm (117,000g) at 4 °C. The supernatant was decanted and loaded onto a 5-ml Ni-NTA column at a flow rate of 1 ml/min (~1 ml bed volume/20 mg total protein). The column was washed twice with buffer A (50 mM Tris [pH 8.0], 5 mM of 2-mercaptoethanol, 20% glycerol, 0.1% NP-40, and 500 mM NaCl) containing 5 and 50 mM imidazole. The protein was eluted with buffer A containing 500 mM imidazole. Then 1-ml fractions were collected and assayed for purity by SDS-PAGE. The pooled fractions were dialyzed overnight against buffer A containing 50 mM NaCl at 4 °C. The dialyzed sample was loaded onto a 5-ml phosphocellulose column (1 ml bed volume/20 mg protein) at 1 ml/min. The column was loaded in buffer A containing 50 mM NaCl, washed, and eluted with a linear gradient (6 column volumes) from 50 to 350 mM NaCl in buffer A. Fractions were pooled based on purity. The fractions were pooled and diluted to 50 mM NaCl based on conductivity. The diluted sample was loaded onto a 1-ml Q Sepharose column and concentrated by stepping the protein off in 2 column volumes of buffer B (50 mM Hepes [pH 7.5], 500 mM NaCl, 20% glycerol, 5 mM of 2-mercaptoethanol, and 0.1% NP-40). The protein concentration was determined by measuring the absorbance at 280 nm in 6 M guanidine-HCl (pH 7.0) and using a calculated molar extinction coefficient of 73,350 M⁻¹ cm⁻¹. The sample was aliquoted and frozen at –70 °C. When frozen in glycerol-containing buffer at –70 °C, the enzyme was stable indefinitely and lost less than 10% activity after a single cycle of freezing and thawing.

Authentic poliovirus 3D^{pol}, used as a control in some of the experiments, was purified as described in the literature [34].

Cleavage using SUMO protease

C-terminal, His-tagged, SUMO protease 1 [Ulp1 (403–621)p] [9,39] was expressed from pET24d in Rosetta-(DE3) pLysS (Novagen). The recombinant protein (apparent M by SDS-

PAGE ~31 kDa) was purified using Ni-NTA resin and buffer-exchanged into 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 5 mM of 2-mercaptoethanol using a PD-10 column (Amersham Biosciences). Purification from 125 ml cell culture ($OD_{600} = 1.2$) yielded sufficient SUMO protease that was 99% pure. The preparation, when adjusted to 50% glycerol, yielded 10 ml of SUMO protease stock at specific activity = 425 U/ml. A unit of SUMO protease is defined as the amount of enzyme that cleaves 15 mg of SUMO-M-green fluorescent protein (GFP) fusion substrate at 25 °C in 60 min in buffer containing 20 mM Tris-HCl (pH 8.0) and 5 mM of 2-mercaptoethanol. Concentrated stocks of SUMO protease were kept in storage buffer (50% glycerol, 7 mM Tris [pH 8.0], 0.5 mM dithiothreitol [DTT], 1 mM EDTA). Hydrolysis of fusions was performed in 50 mM sodium phosphate for a pH range of 6.0–8.0. Reactions were run in 100- μ l volumes with 15 μ g of SUMO-fusion protein substrate. Hydrolysis was initiated by the addition of 1 U of SUMO protease and incubation at 25 °C for an appropriate time, dictated by the experimental protocol, after which the reaction was stopped by the addition of 21 μ l of stop solution (15 μ l of 25 mM Tris-HCl [pH 8.0], 150 mM NaCl + 6 μ l of 6 $\times$ concentrated SDS-PAGE buffer). After the termination of the reaction, samples were boiled for 5 min and analyzed by SDS-PAGE. Activity was assessed by quantification of scanned gels using Scion Image software.

Purification, 5'- 32 P labeling, and annealing of sym/sub

RNA oligonucleotides were purified, labeled, and annealed as described previously; sym/sub is a short, symmetrical, heteropolymeric RNA primer template [40].

Assays for 3D^{pol} activity

The polymerase activities of 3D^{pol} and its fusion derivative were assayed by either radiolabeled nucleotide incorporation or primer extension. The radionucleotide incorporation assays contained 50 mM Hepes (pH 7.5), 10 mM β -mercaptoethanol, 5 mM MgCl₂, 60 μ M ZnCl₂, 500 μ M UTP, 0.4 μ Ci/ μ l [α - 32 P]UTP, 1.8 μ M dT15/0.15 μ M poly(rA)₄₀₀ primer/template, and 3D^{pol}. Reactions were performed in a total volume of 25 μ l using 250 ng of purified 3D^{pol} at 30 °C for 5 min. Reactions were quenched by the addition of 0.5 M EDTA to a final concentration of 83 mM. Then 10 μ l of the quenched reaction was spotted onto DE81 filter paper discs and dried completely. The disks were washed three times for 10 min in 250 ml of 5% dibasic sodium phosphate and rinsed in absolute ethanol. Bound radioactivity was quantified by liquid scintillation spectrometry in 5 ml of scintillation fluid.

The primer extension reactions contained 50 mM Hepes (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM MgCl₂, 500 μ M ATP, 1 μ M end-labeled sym/sub, and 1 μ M 3D^{pol}. Reactions were quenched by the addition of EDTA to a final concentration of 50 mM. An equal volume of loading buffer, 10 μ l (90% formamide, 0.025% bromophenol blue, and 0.025% xylene cyanol), was added to 10 μ l of the quenched reaction mixtures and heated to 70 °C for 2–5 min prior to loading 5 μ l on a denaturing polyacrylamide gel of the appropriate percentage containing 1 $\times$ TBE and 7 M urea. Highly cross-linked gels contained 1.7% bisacrylamide. Electrophoresis was performed in 1 $\times$ TBE at 75 W. Gels were visualized by using a PhosphorImager and quantified using ImageQuant software (Molecular Dynamics).

Results

Design of SUMO-3D^{pol} fusion gene

We employed a cloning strategy that makes use of *Bsa*I, a type II restriction enzyme, allowing us to designate the N terminus at the junction between SUMO and the 3D^{pol} protein while preserving the C-terminal Gly-Gly dipeptide structure found in SUMO and other UBLs. During poliovirus infection, 3D^{pol} is initially synthesized as part of a large polyprotein, which then undergoes posttranslational processing to release the mature active polymerase. We have

designed the fusion protein junction such that the native 3D^{pol} amino terminus, NH₂-Gly-Glu-Ile ..., is generated on cleavage by SUMO protease (Fig. 2) (LifeSensors).

Expression of SUMO-3D^{pol} in *E. coli*

IPTG induction of mid-log cultures of *E. coli* harboring the pET24-6H-SUMO-3D^{pol} expression plasmid resulted in the accumulation of the predicted 65.9-kDa SUMO-3D^{pol} fusion protein (Fig. 3). Cell fractionation revealed that although much of the fusion protein was contained within the insoluble inclusion body fraction, enough fusion protein remained soluble to allow further purification. The fraction of SUMO-3D^{pol} protein that remained soluble could be altered by varying the growth and lysis conditions.

Cleavage of SUMO-3D^{pol} and activation of enzyme

Purification is summarized in Fig. 4 (fusion) and Fig. 5 (cleaved → pure 3D^{pol}). The SUMO-polymerase fusion from the various purification fractions migrated in SDS-polyacrylamide gels at the position of approximately 66 kDa (Fig. 4). A comparison of lane 4/5 (lysate and clarified lysate, representing the soluble protein fraction) with lane 6 (pellet or insoluble fraction) indicates that, although not all of the polymerase was expressed in soluble form under the conditions used, a significant and workable percentage of the enzyme was soluble. Thus, further purification focused on the soluble fraction, obviating the need to refold the expressed protein, thereby eliminating the possibility of severely diminished 3D^{pol} activity owing to incorrect refolding. After three column steps—Ni-NTA, phosphocellulose, and Q Sepharose (Fig. 4, lanes 7, 8, and 9, respectively)—highly pure SUMO-3D^{pol} fusion was obtained, as judged by Coomassie staining. Cleavage of this fusion by SUMO protease was essentially complete (Fig. 5, lanes 2 and 3). Pure polymerase was recovered from the hydrolysis reaction mixture by subtractive chromatography (see Materials and methods).

3D^{pol}-polymerase assays

The N-terminal dependence of the activity of 3D^{pol} was tested. Ribonucleotide incorporation by uncleaved and cleaved fusion protein (SUMO-3D^{pol}) was assayed over a 30-min period (Fig. 6). The data from simple incorporation assays showed that the activity of the polymerase fused at the N terminus with SUMO was absolutely dependent on cleavage precisely at the N-terminal fusion junction. The signal/noise ratio on the linear portion of the curve, using radioactivity to measure RNA polymerase activity, was greater than 20:1, indicating that configuration for high-throughput screening should be feasible. In a high-throughput mode, fluorescent nucleotides would likely be used in place of radioactivity, which would be expected to increase the signal/noise ratio as well as to give acceptable precision. SUMO protease, shown to be a robust enzyme capable of acting under broad temperature, pH, and ionic strength ranges [38], apparently can release active polymerase from the fusion quite rapidly given that similar kinetics of incorporation was seen in the cases of precleaved 3D^{pol} and fused 3D^{pol} incubated simultaneously with SUMO protease and components of the polymerase reaction. Moreover, incorporation kinetics using 3D^{pol} purified in the traditional fashion [34] was similar to that of the 3D^{pol}-SUMO fusion cleaved with SUMO protease (Fig. 6). It will be of interest to examine more closely the time courses of cleavage and appearance of polymerase activity as well as to compare the specific activity and inhibitor effects of the polymerase cleaved in vitro with that of the conventionally prepared enzyme.

In primer extension experiments employing cleaved and uncleaved SUMO-3D^{pol}, similar results were found; SUMO-3D^{pol} fusions had no activity except when treated with SUMO protease (Fig. 7). Under the conditions of the experiment shown, conventionally purified 3D^{pol} appeared to have a faster initial rate than the polymerase liberated from the SUMO fusion, likely reflecting the dependence of polymerase activity on liberation of the active enzyme from the fusion.

Discussion

The list of enzymes known to be able to cleave ubiquitin or UBLs from their target proteins or linear fusions has been growing as a variety of genomes have been sequenced. A facile assay that is applicable to all, or at least most, of these enzymes would be very helpful in mechanism studies as well as in drug discovery and development. The assay described here measures activity at a linear peptide bond (involving the α -amino group of lysine), whereas many DUBs hydrolyze isopeptide bonds (involving the ϵ -amino group of lysine). Designing isopeptide-linked substrates for ubiquitin or UBLs that will provide a convenient way in which to assay isopeptidase activities has been problematic. The linear assay described here, however, is appropriate for both types of peptide bond, for two reasons. First, isopeptidases can cleave linear fusions as well as isopeptide bonds [41]. Therefore, the assay is biochemically appropriate. Second, both linear and isopeptide fusions have physiological relevance. Ubiquitination at the amino termini of substrate proteins can occur in vivo and lead to target protein degradation [42–44]. In addition, DUBs are responsible for the normal processing and maturation of the genome-encoded ubiquitin precursors, all of which are linear fusion proteins. Structural studies and X-ray crystallographic analysis of UBPs and UCHs have demonstrated that substrate recognition by these enzymes relies on extensive contacts with structural features located on the surface of the ubiquitin/UBL molecules themselves [39,45,46]. This type of interaction takes place regardless of the nature of the linkage between the C terminus of ubiquitin/SUMO and the target protein.

Assays that have been available heretofore are less than ideal for high-throughput screening, for reasons ranging from poor adaptability to 96- or 384-well plates to prohibitive cost of reagents. The assay described here does not suffer from any of these disadvantages; rather, the results suggest that a reasonable DUB/isopeptidase assay platform can be constructed by identifying enzymes that are inactive in the ubiquitin and SUMO-fused configuration (with bound N termini) but that, on cleavage by ubiquitin or SUMO protease, become activated by the liberation of their N termini. The N terminus could be part of the catalytic site or otherwise essential to the catalytic mechanism, and a range of reporter fusions could ensure that most (if not all) DUBs can be assayed in this way. Fusion of ubiquitin or UBLs to the N termini of such enzymes inactivates them, and the removal of ubiquitin or SUMO rapidly restores activity. Thus, the generation of the active enzyme is a direct function of the respective protease. The 3D^{pol} fusion is the first validated substrate in such a platform, as demonstrated here for the case of SUMO and as demonstrated previously for ubiquitin fusion [34]. A series of protease-activated fused reporters should constitute a valuable assay platform for deubiquitinases. Viral RNA-dependent RNA polymerases constitute an important class of these fusions.

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Fig. 1.

Biochemical functions of deubiquitinating enzymes. Ubiquitin (Ub) is synthesized as a fusion protein and must be processed by ubiquitin-specific proteases (USPs). Removal of conjugated Ub by USPs serves an editing function. At the proteasome, the poly-Ub chain must be removed from the docked conjugates and disassembled to replenish the Ub pool and prevent accumulation of free poly-Ub chains. “Salvage” is the removal of cellular thiols, amines, and other small adducts that inactivate free Ub (catalyzed by the ubiquitin terminal hydrolase [UCH] enzymes).

Fig. 2. Schematic map of the pET24-6H-SUMO-3D^{pol} expression vector. The vector contains the T7 promoter driving the expression of the His-tagged SUMO-3D^{pol} fusion protein. DNA and protein sequence at the junction between the two proteins is shown on the right, with the SUMO protease cleavage site indicated.

Fig. 3. Expression of SUMO-3D^{pol}. Coomassie-stained SDS-polyacrylamide (10%) gel of bacterial cell lysates before IPTG induction and after 4 h of induction at 37 °C. The induced SUMO-3D^{pol} migrates at the 66-kDa marker. Lane 1, molecular weight markers; lane 2, uninduced; lane 3, induced.

Fig. 4. Purification of SUMO-3D^{pol}. Coomassie-stained SDS-polyacrylamide (10%) gel of the samples from each purification step. Lane 1, molecular weight markers; lane 2, uninduced; lane 3, induced; lane 4, lysate; lane 5, clarified lysate; lane 6, pellet; lane 7, Ni-NTA elution; lane 8, phospho-cellulose elution; lane 9, Q Sepharose elution. Approximately, 1.0–2.5 μ g of total protein was loaded in lanes 7–9.

Fig. 5. Cleavage of SUMO-3D^{pol} by SUMO protease: Coomassie-stained SDS-polyacrylamide (10%) gel of purified SUMO-3D^{pol} before and after proteolytic cleavage by SUMO protease. SUMO-3D^{pol} (5 μ g) was incubated with 2 U of SUMO protease on ice for 30 min prior to loading samples on the gel.

Fig. 6.

Poly(rU) polymerase activity assay of SUMO-3D^{pol}. Reactions contained 0.05 μM SUMO-3D^{pol} and were performed as described in Materials and methods. The figure shows kinetics of nucleotide incorporation by 3D^{pol} where SUMO-3D^{pol} was added to the reaction in the absence of SUMO protease (filled circles), where SUMO-3D^{pol} was added to a reaction containing SUMO protease (filled squares), and where SUMO-3D^{pol} was incubated with SUMO protease on ice for 30 min prior to initiating the reaction (open circles). Conventionally purified 3D^{pol} is shown as a control (open squares).

Fig. 7.

Primer extension. Reactions contained 1 μ M sym/sub (0.5 μ M duplex), 1 μ M enzyme, and 500 μ M ATP. Reactions were initiated by the addition of enzyme and were quenched at the indicated times by EDTA. SUMO-3D^{pol} was preincubated with SUMO protease on ice for 30 min prior to initiating the reaction.