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Development of a homogeneous AlphaLISA ubiquitination assay using ubiquitin binding matrices as universal components for the detection of ubiquitinated proteins☆

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ABSTRACT

The Ubiquitin Proteasome Pathway (UPP) has become a target rich pathway for therapeutic intervention as its role in pathogenic disease is better understood. In particular many E3 ligases within this pathway have been implicated in cancer, inflammation and metabolic diseases. It has been of great interest to develop biochemical assays to identify inhibitors of catalytic E3 ubiquitination activity as a means of abrogating the disease mechanism. Here we describe a homogeneous biochemical assay that utilizes native ubiquitin and Tandem-repeated Ubiquitin-Binding Entities (TUBEs) as a detection technology for ubiquitination activity. We developed a TUBEs based proximity AlphaLisa® assay for Mdm2, which is an E3 ligase that negatively regulates p53 tumor suppressor protein. We further demonstrate that this assay strategy or design can also be applied to the development of assays to detect autoubiquitination of other E3 ligases that are also of interest for therapeutic intervention. This article is part of a Special Issue entitled: Ubiquitin Drug Discovery and Diagnostics.

1. Introduction

The covalent postranslational modification of protein substrates with ubiquitin is of growing interest to the pharmaceutical industry as the process regulates proteins involved in many cellular functions, to include cell cycle, cell motility, transcriptional regulation, receptor internalization and protein trafficking [1–3]. The process of ubiquitin ligation consists of a three enzyme cascade that begins with the activation of ubiquitin by a ubiquitin activating enzyme, E1, which forms a high energy thioester bond with ubiquitin. The activated ubiquitin is then passed to a ubiquitin conjugating enzyme, E2, and then the E2-ubiquitin conjugate is brought together with an E3 ubiquitin ligase and it is the E3 in complex with the E2 and substrate that facilitates the transfer of ubiquitin to the substrate protein (Fig. 1). The protein components within the pathway can potentially be modulated by small molecules, and given the fact that there are >600 ubiquitin ligases that exist [4], this process is being examined for therapeutic intervention. Many ubiquitin ligases possess an intrinsic autoubiquitination activity which we have used as a read out for the catalytic activity of the ligase complex.

Indeed this approach has been utilized to screen for, and identify, small molecule inhibitors of Mdm2 ubiquitination activity [5]. Two biologically alluring ubiquitin ligases that might provide therapeutic value are Mdm2 and Traf6. Mdm2 is the ligase dedicated to regulating the tumor suppressor p53 [6,7], and as such, inhibition of Mdm2 catalytic activity would provide increased amounts of p53 to the cell. In support of this, evidence exists that transformed cells are more sensitive to p53 induced apoptosis and therefore increases in p53 may sensitize tumor cells to increased cell death [8,9]. Traf6 is an E3 ligase that plays a role in canonical NFκB activation. Upon TNFR, IL-1R and TLR stimulation, Traf6 is activated, leading to a cascade which

Abbreviations: UPP, ubiquitin proteasome pathway; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; ECL, electrochemiluminescence; HECT, Homologous to E6AP Carboxy Terminus; RING, Really Interesting New Gene; Smurf1, SMad Ubiquitin Regulatory Factor 1; TGF-β, transforming growth factor beta; DTT, Dithiothreitol; BSA, bovine serum albumin; GST, glutathione-s-transferase; UBA, ubiquitin association domain; IL-1, Interleukine1; TLR, Toll Like Receptor; TNFR, tumor necrosis factor receptor; NFκB, Nuclear factor κB; TR-FRET, Time-Resolved Fluorescence Resonance Energy Transfer; TUBE, Tandem-repeated Ubiquitin-Binding Entities; HTS, High Throughput Screening; MS, Mass Spectrometry; DMSO, Dimethylsulfoxide

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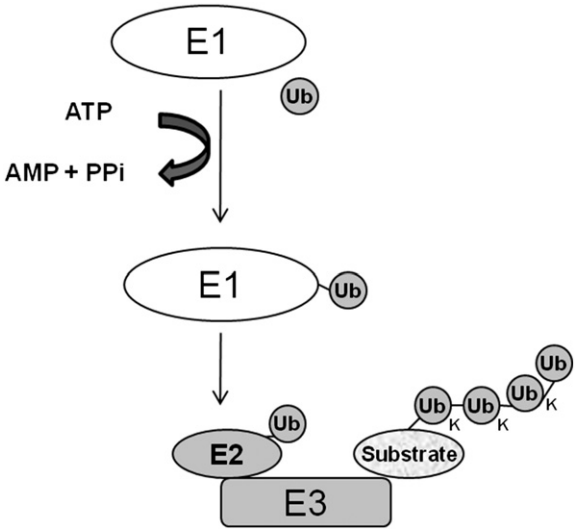

Fig. 1. Ubiquitination pathway. The ubiquitination pathway is a cascading reaction involving several proteins. The first step in the reaction is enzyme E1 activation in the presence of ATP and ubiquitin by forming a high energy thioester bond between ubiquitin and E1. The E2 is then charged by the transfer of ubiquitin from an activated E1 to the E2, also by a thioester bond. Finally, a substrate is ubiquitinated when complexed with the charged E2 and a substrate specific E3 that facilitates the transfer of ubiquitin from E2 to substrate. In the absence of a substrate many E3s are known to be themselves the substrate via an autoubiquitination reaction.

results in NFκB activation and translocation to the nucleus [10]. Inhibition of this cascade with small molecules, and specifically the activity of Traf6, would result in the inhibition of NFκB activation and the inflammatory responses it regulates.

A number of assay technologies have been developed to identify small molecule inhibitors of ubiquitin ligases. These technologies include plate based assays such as electrochemiluminescence (ECL) [11,12] and homogeneous assays such as Time Resolved Fluorescence Resonance Energy Transfer (TR-FRET) [13]. Here we describe the development of a homogeneous autoubiquitination assay utilizing the PerkinElmer AlphaLISA® technology and ubiquitin binding matrices (TUBEs) [14], as a universal component for the detection of ubiquitinated proteins. This approach provides the sensitivity of the AlphaLISA® technology, relative simplicity as a homogeneous assay and is advantageous since native ubiquitin is used, eliminating the need to optimize assay conditions and ratios of tagged ubiquitin molecules to native ubiquitin, which other homogeneous assay formats require. Additionally, the utilization of native ubiquitin eliminates the possibility of a tagged ubiquitin interfering in the kinetics of the ubiquitination event.

2. Materials and methods

2.1. Purification of recombinant proteins

Human Ube1 gene encoding amino acids 2–1058 (Genbank accession CAA40296) was PCR amplified from a liver cDNA library (Amgen) and cloned into pFastBac1 H6T vector through an enzyme-free cloning method as described previously [15]. UBE1 (2–1058) was then expressed in sf9 insect cells as an N-terminal His6 fusion protein using Bac-to-Bac Baculovirus Expression Systems (Invitrogen, Carlsbad, CA). The preparation of BacMid and production of recombinant baculovirus were carried out according to the manual of Bac-to-Bac system (Invitrogen). The full length Ube1 protein was expressed by infecting mid-log phasesf9cells (2.5×10^6 cells/ml) with a high-titer baculovirus stock at a MOI of 5–10. Insect cells were then cultured in sfx medium (Hyclone) at 27°C for 48 h. Recombinant His-tagged UBE1 expressed from Sf9 insect cells is sequentially purified through three steps: cell lysis, Nickel (Sigma) affinity chromatography and gel filtration (Superdex 200, Amersham Biosciences). The cell paste was resuspended in lysis buffer, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, supplemented with complete protease inhibitor mixture (Sigma), and disrupted by microfluidizer. Cell debris was removed by centrifugation (45,000 *g*, 1.5 h, 4), and then, the supernatant was incubated for overnight at 4°C with Nickel resin (Sigma). The resin was washed with 20 CV of lysis buffer and lysis buffer containing 10 mM imidazole first, then eluted target protein with lysis buffer containing 100 mM imidazole. The eluted protein solution was concentrated to a small volume, and was loaded on Gel-Filtration column (Superdex 200, XK16) which was equilibrated in 20 mM Tris, pH8, 150 mM NaCl, 1 mM β-mercaptoethanol. The final protein purity was about 95% based on SDS-PAGE. A clone of huSmurf1 was obtained from Origene (Rockville, MD). The gene was PCR amplified using the following oligo pair:

5′-AAC AAC AAC GGT CTC GGA TCC ATG TCG AAC CCC GGG ACA CGC AG-3′//5′-CCG CCG CCG GAA TTC CTA TTA CTC CAC AGC AAA CCC GCA GGT CTC-3′. The product was purified with a QIAquick PCR purification kit (Qiagen), digested with BamHI and EcoRI and cloned into pGEX2T (GE Healthcare). The insert was sequence confirmed. Expression was done at 16 °C in strain BL21-Codon Plus-RIL (Stratagene) in TB media supplemented with 100 μg/ml carbenicillin and 30 μg/ml chloramphenicol. The culture was induced with 0.2 mM IPTG at about 1 OD and induction went overnight. Cell pellets were resuspended in 25 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 0.1% CHAPS, 5 mM DTT, 10% glycerol and Complete "Free" protease inhibitor cocktail (Roche) and lysed by a microfluidizer. Lysate was cleared by centrifugation at 14 K rpm for 2 h and filtered through a 0.45 μm cellulose acetate membrane before application to Glutathione Sepharose 4B beads (GE Biosciences) for one hour batch-incubation at 4 °C. The beads were washed extensively with same buffer and protein was eluted with the buffer containing 10 mM reduced glutathione. The elution was loaded on Superdex 200 XK 26/60 column (GE Biosciences) equilibrated with 25 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 5% glycerol, 0.1% CHAPS and 5 mM DTT.

The protein identity was confirmed by LC–MS and concentrations were determined using Bradford assay, and snap frozen in liquid nitrogen and stored at −80 °C.

A DNA fragment encoding TRAF6 (1–181) was amplified from the full length TRAF6 DNA template using PCR primers: GACTACTAGTGA-GAACCTCTACTTCCAGGGAAGTCTGCTAAACTGTGAAAACAGC (F) and GCG AAT TCA ATC CTT CAG AAT GTG AAT ATT AAT ATG (R). The bacterial expression plasmid pET42 GST-Tev-TRAF6 (1–181) was constructed by inserting the SpeI and EcoRI (New England Biolabs) double digested PCR fragment into the pre-digested pET42 vector (Novagen). To express recombinant TRAF6 (1–181) protein, BL21 (DE3) cells carrying pET42 GST-Tev-TRAF6 (1–181) plasmid were grown in TB at 37°C, and induced by 1 mM IPTG at 18°C overnight. The protein was first purified by GST affinity chromatography and sequentially by size-exclusion chromatography (Pharmacia S200) in a buffer containing 20 mM Tris pH8, 5% glycerol, 100 mM NaCl, 5 mM βME. Uev1A gene was amplified a cDNA library (Amgen) using PCR primers: GAC TAC CGG TGA GAA CCT CTA CTT CCA GGG ACC AGG AGA GGT TCA AGC GTC (F) and GAC TAC CGG TGA GAA CCT CTA CTT CCA GGG ACC AGG AGA GGT TCA AGC GTC (R). The PCR product was then digested by AgeI and BamHI (NEB) and cloned into pET45 (Novagen) between AgeI and BamHI sites. The recombinant Uev1A protein with a His6 tag followed by a Tev cleavage site at its N-terminus was expressed in BL21 (DE3) cells under the same conditions as TRAF6 (1–181). The frozen cell paste was resuspended in lysis buffer (35 mM Tris pH8, 5% glycerol, 150 mM NaCl, 5 mM BME, cocktail protease inhibitors) and lysed by microfluidizer. The lysate was centrifuged, filtered and mixed with Nickel affinity resin, then, slowly rocked at 4°C overnight. After low imidazole washing, target protein was eluted with 150 mM imidazole buffer. The eluted target protein was