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Single-molecule sorting of DNA helicases

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ABSTRACT

DNA helicases participate in virtually all aspects of cellular DNA metabolism by using ATP-fueled directional translocation along the DNA molecule to unwind DNA duplexes, dismantle nucleoprotein complexes, and remove non-canonical DNA structures. Post-translational modifications and helicase interacting partners are often viewed as determining factors in controlling the switch between *bona fide* helicase activity and other functions of the enzyme that do not involve duplex separation. The bottleneck in developing a mechanistic understanding of human helicases and their control by post-translational modifications is obtaining sufficient quantities of the modified helicase for traditional structure-functional analyses and biochemical reconstitutions. This limitation can be overcome by single-molecule analysis, where several hundred surface-tethered molecules are sufficient to obtain a complete kinetic and thermodynamic description of the helicase-mediated substrate binding and rearrangement. Synthetic oligonucleotides site-specifically labeled with Cy3 and Cy5 fluorophores can be used to create a variety of DNA substrates that can be used to characterize DNA binding, as well as helicase translocation and duplex unwinding activities. This chapter describes "single-molecule sorting", a robust experimental approach to simultaneously quantify, and distinguish the activities of helicases carrying their native post-translational modifications. Using this technique, a DNA helicase of interest can be produced and biotinylated in human cells to enable surface-tethering for the single-molecule studies by total internal reflection fluorescence microscopy. The pool of helicases extracted from the cells is expected to contain a mixture of post-translationally modified and unmodified enzymes, and the contributions from either population can be monitored separately, but in the same experiment providing a direct route to evaluating the effect of a given modification.

1. Introduction

Single-molecule fluorescence imaging has revolutionized our ability to study nucleoprotein transactions in real time and in unprecedented detail. The ever-growing repertoire of single-molecule methodologies provides two distinct advantages over traditional ensemble experiments. First, single-molecule assays are designed to consume at least 10^3–10^6 times less material; this can be a limiting factor when the sample is difficult to produce in sufficient quantity for rigorous biochemical analysis. More importantly, examining the activities of individual molecules can reveal characteristics that are otherwise hidden in ensemble experiments due to averaging [1,2]. Stochastic behaviors, for instance, would need to be synchronized and evenly distributed among a population of molecules to be detectable in bulk studies. For these reasons, single-molecule experiments using surface-immobilized proteins are especially useful to study how post-translational modifications (PTMs) affect their biochemical properties since the

Abbreviations: ATP, adenosine triphosphate; BAP, biotin acceptor peptide; bio, biotinylated; BLM, Bloom's syndrome DNA helicase; BRC1, Breast cancer type 1 protein; BRCT, BRCA1 C-terminus; DMEM, Dulbecco's modified eagle medium; DNA, deoxyribonucleic acid; ds, double-stranded; DPBS, Dubelcco's phosphate buffered saline; DPSS, Diode pumped solid state; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMCCD, electon multiplying charge coupled device; FANCJ, Fanconi anemia complementation group J protein; FBH1, F-box containing helicase 1; FBS, fetal bovine serum; FRET, Förster resonance energy transfer; HEK, human embryonic kidney; Hepes, *4-(2-hydroxyethyl)-1-piperazinee thanesulfonic acid*; His, histidine; NHS, N-hydroxysuccinimide esters; -P, phosphorylated; PEG, polyethylene glycol; PEI, polyethylenimine; PMSF, phenylmethylsulfonyl fluoride; PTM, post-translational modification; RAD51, radiation sensitive protein 51; ROS, reactive oxygen species; rpm, revolutions per minute; SiMPull, single-molecule pull down; sm, single-molecule; ss, single-stranded; SUMO, small ubiquitin-like modifier; TE, Tris and EDTA; TIRF, total internal reflection fluorescence; TIRFM, total internal reflection fluorescence microscopy; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TUBE, tandem ubiquitin binding entities; ub, ubiquitylated.

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repeating cycles of activity originating from the same modified or unmodified protein molecule can be monitored.

1.1. Post-translational modifications

PTMs are covalent protein modifications that form either during or after protein synthesis. PTMs can include adding small functional groups such as phosphate, methyl, and acetate, or even protein moieties such as ubiquitin and SUMO, onto the amino acid side chains (or termini) of a target protein [3,4]. Such modifications are widely associated with signal transduction and also provide a mechanism to regulate protein functions. This article will specifically address PTMs of the proteins involved in DNA metabolism, and in particular, a class of proteins known as helicases.

1.2. DNA helicases

DNA helicases are molecular motors that separate double-stranded (ds) DNA in order to form the single-stranded (ss) intermediates needed for DNA replication, recombination, and repair. Helicases can also translocate along ssDNA or dsDNA to remove or remodel protein complexes and unconventional structures [5,6]. Helicase activity is therefore essential during all stages of DNA metabolism. Not surprisingly, mutations in human helicases often contribute to the onset of genetic diseases associated with premature aging and chromosome instability [7]. DNA helicases may act individually or as essential components of multiprotein DNA repair machineries. Many DNA helicases are subject to PTMs that drastically, albeit transiently, alter their biochemical properties and interactions. For example, the BLM helicase (a RecQ-family helicase, mutations in which are associated with Bloom syndrome) can be phosphorylated, ubiquitylated, and SUMOylated; these modifications have an impact on the BLM cellular localization, overall stability, and interactions with protein partners as described in this review [8]. When the FBH1 helicase (F-box containing helicase 1, which switches between pro- [9] and anti-recombinogenic [10–13] activities at the damaged replication forks) is poly-ubiquitylated, it is converted into a pro-recombinase that no longer binds to a RAD51 nucleoprotein filament [14]. Despite their expected importance, the functional consequences for many helicase PTMs are not known. The FANCJ helicase (Fanconi Anemia complementation group J), for example, interacts with the BRCA1 tumor suppressor protein when FANCJ is phosphorylated on serine residue 990 [15–18]. It is not clear whether the FANCJ-BRCA1 complex possesses unique properties distinct to FANCJ alone, or if it combines the DNA structure selective binding properties of the two proteins. Traditional biochemical characterization of this complex is not feasible since FANCJ and BRCA1 are difficult to produce and only a small fraction of FANCJ is phosphorylated on serine 990. As a result, the vast majority of purified FANCJ will not be active in BRCA1-binding. Single-molecule fluorescence imaging provides an elegant solution to both of these experimental constraints.

1.3. Single-molecule sorting overview

This article describes using "single-molecule sorting" to investigate how PTMs influence the activities of the FBH1 and FANCJ helicases. This approach can be adapted to the analyses of a wide variety of systems, with a caveat that the protein of interest must be immobilized on the slide surface for fluorescence imaging and subsequent sorting of the activities; hence, this method is readily applied only to the proteins that either form stable complexes or act as monomers. If the assembly state of the protein is not known, it can be determined empirically by fluorescently labeling the protein site-specifically [19] prior to surface-immobilization and visualizing the number of photobleaching steps in the fluorescence trajectories of the immobilized protein alone. Surface-tethering is commonly achieved by labeling the protein with biotin (described in Section 2) and placing it on a neutravidin-coated slide surface; alternatively, it can also be achieved via a "single-molecule pull down" (SiMPull), an elegant method developed by the Taekjip Ha's group [20,21]. The positions of the individual protein molecules on the microscope slide can then be determined using a fluorescently labeled binding partner (e.g. DNA, antibody, peptide), and protein molecules possessing a particular PTM can be similarly identified with a fluorescently labeled interaction partner that is specific for that epitope. Single-molecule activity assays can be carried out with the entire pool of tethered proteins, either before or after probing for a specific PTM, and their activities can be correlated with the presence or absence of the modification.

2. Methods

2.1. Construction of protein expression vectors

A biotin acceptor peptide (BAP) sequence (GLNDIFEAQKIEWHE) is engineered into the protein expression vector. *Escherichia coli* BirA ligase recognizes and covalently labels the lysine residue within this sequence (Fig. 1A). The BAP can be incorporated into a solvent exposed loop if the three dimensional structure of the protein of interest is known, or at the N- or C-terminus if structural data are not available. Activity assays can be carried out using protein with different anchoring positions as well as with the free protein to test whether surface-immobilization has altered its biochemical properties. Additionally, a FLAG tag (DYKDDDDK) is engineered into the protein expression vector. This tag not only facilitates protein purification via affinity based methods, but also provides an epitope for binding fluorescently labeled antibodies during single-molecule imaging. As an example of "single-molecule sorting" application, this article focuses on the human FBH1 and FANCJ helicases. The open reading frames of these proteins are cloned into a mammalian expression vector (e.g. pcDNA3) for production in human cells so that they can undergo their native PTMs.

2.2. Producing Flag-FBH1-Biotin from HEK293 cells

The following procedure pertains to the expression and purification of the Flag-tagged biotinylated FBH1, but this method can be applied to study any protein of interest. For the initial, prove-of-principle studies we chosen to express human proteins in HEK293 cells (ATCC CRL-1573). HEK293 cells are adherent and display high transfection capacity, thereby allowing transfect the cell with multiple plasmids simultaneously. One, however, needs to exercise judgement with respect to selection of the most appropriate cell lines for each intended study.

2.2.1. HEK293 cell growth

HEK293 cells are seeded in a T75 culture flask using pre-warmed (37°C) Dulbecco's modified eagle medium (DMEM) that is supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, and 1 mM sodium pyruvate. The flask is kept at 37 °C, 5% CO_2 for 3 h, at which time the starting media is exchanged for fresh DMEM to remove any non-adhering cells. Upon reaching 95% surface confluence (usually achieved after 72 h), the media is aspirated out and the cells are washed with Dulbecco's phosphate buffered saline (DPBS). The cells are detached from the culture flask by treating with 1 mL of 0.05% Trypsin-EDTA for 5 min at 37 °C. Trypsin is then inactivated with 9 mL of supplemented DMEM and the cells are further diluted and re-seeded at 1:10 (v/v cells to DMEM) into ten T150 culture flasks.