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Regulation of tankyrase activity by a catalytic domain dimer interface

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ABSTRACT

Tankyrases (TNKS and TNKS2) are enzymes that catalyze poly-ADP-ribosylation (PARsylation) of their target proteins. Tankyrase-mediated PARsylation plays critical regulatory roles in cell signaling, particularly in the Wnt/β-catenin pathway. The sterile alpha motif (SAM) domain in tankyrases mediates their oligomerization, which is essential for tankyrase function. The oligomerization regulates the catalytic activity of tankyrases, but the underlying mechanism is unclear. Our analyses of crystal structures of the tankyrase catalytic domain suggest that formation of a head-to-head dimer regulates the catalytic activity. Our activity assays show that residues in the catalytic domain dimer interface are important for the PARsylation activity of tankyrases both in solution and cells. The dimer is weak and may only form in the context of the SAM domain-mediated oligomers of tankyrases, consistent with the dependence of the tankyrase activity on the SAM domain.

1. Introduction

Tankyrases 1 and 2 (TNKS and TNKS2) are two highly homologous members of the poly-ADP-ribosylation polymerase (PARP) family. PARPs catalyze the transfer of the ADP-ribose group from NAD^+ to their target proteins for PARsylation, a post-translational modification that is involved in regulating many cellular processes, including telomere maintenance, DNA repair and Wnt/β-catenin signaling [1]. Dys-regulation of TNKS and TNKS2 by altered expression levels or mutations is associated with malignant tumors [2]. Inhibitors of TNKS and TNKS2 are currently being developed for targeting colorectal cancer and other cancer types [3].

PARsylation of the target proteins often leads to their degradation through the ubiquitin-proteosomal system [4,5]. One of the PARsylation substrates of tankyrases are the Axin proteins (Axin1 and 2), negative regulators of the Wnt/β-catenin pathway [6]. Axin is a core component of a multi-protein complex referred to as the destruction complex, which also contains adenomatous polyposis coli (APC) and glycogen synthase kinase 3α/β (GSK3 α/β) [7,8]. In the absence of Wnt, the destruction complex constitutively drives the degradation of β-catenin, keeping the downstream pathways off. Tankyrases promote Wnt/β-catenin signaling by PARsylation of the Axin proteins thereby inducing degradation of Axin proteins and lifting the suppressive action of the destruction complex on signaling [6]. Consequently, levels of β-catenin are increased, which enters the nucleus to promote gene transcription. Tankyrases also undergo auto-PARsylation, which promotes their own destruction through the ubiquitin-proteasome pathway [6,9].

Tankyrases are distinct members in the PARP family in that they contain several unique regulatory domains in addition to the conserved catalytic domain. There are five N-terminal ankyrin repeat clusters (ARCs), which are responsible for substrate recruitment by recognizing "RXXXDG" (single-letter amino acid code) motifs in substrate proteins [10,11]. Another unique domain in tankyrases is the Sterile Alpha Motif (SAM) domain located between the ARCs and the catalytic domain [12,13]. The SAM domain mediates tankyrase oligomerization by forming a left-handed spiral with six subunits per turn [14–16]. The positive regulation of Wnt signaling by tankyrases is dependent on the SAM domain-mediated oligomerization, which has been shown to both control tankyrase subcellular localization and promote substrate recruitment.

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Disruption of the SAM domain-mediated oligomerization has also been shown to cause diminished auto-PARsylation of tankyrases, suggesting that the oligomerization is required for the catalytic activity ([12,14,15]). The mechanism by which the oligomerization regulates the catalytic activity remains unclear. From the analyses of crystal structures of the catalytic domains of tankyrases in the database, we found a reoccurring dimer that is correlated with the open conformation of the substrate-binding site in the catalytic domain. Our mutational analyses support the notion that the dimerization of the catalytic domain in the context of the SAM mediated-oligomers promotes the catalytic activity of tankyrases.

2. Materials and methods

2.1. Protein expression and purification

The catalytic domain of human TNKS (residues 1105–1313) cloned into a modified pET28 vector (Novagen) was expressed and purified as described previously [17]. The SAM-catalytic domains (residues 1017–1315) were cloned into the same vector and expressed in a similar manner. The SAM-catalytic domains were purified using a similar procedure as for the catalytic domain and stored at −80 °C before use. Mutations were introduced by site-directed mutagenesis.

2.2. In vitro auto-PARsylation assay and histone PARsylation assay

Two methods were used to detect the auto-PARsylation of TNKS SAM-catalytic domains. In the first purified proteins were run on SDS-PAGE and transferred to PVDF membrane (Millipore). Ponceau staining was used to check loading of proteins. Auto-PARsylation of proteins that has occurred during expression in E. Coli was detected by Western blot with an anti-PAR polyclonal antibody (Trevigen, #4336-APC-050). In the second protocol, purified proteins, both WT and mutants, were mixed with 6-biotin-17-NAD (TREVIGEN) in the reaction buffer (50 mM Hepes pH7.5, 150 mM NaCl, 2 mM $MgCl_2$) at 30 °C for 90 min. Reaction products were resolved on SDS-PAGE and detected by Western blot using HRP conjugated anti-biotin antibody (Cell Signaling Technology, #D5A7). To check loading of protein, the same membrane was stripped and probed with anti-His antibody (Takara, #631212). Histone PARsylation assay was conducted as described in HT Universal Color PARP Assay Kit w/Histone Coated Strip Wells (Trevigen, #4677-096-K) [17].

2.3. Analytical ultracentrifugation

Various concentrations of wild-type TNKS catalytic domain (140 μM, 70 μM, 37 μM, 11.6 μM and 3.7 μM) were loaded into the "sample" side of dual-sectored charcoal-filled Epon centerpieces, while the "reference" sectors were loaded with the centrifugation buffer (10 mM Tris, pH8.0, 150 mM NaCl and 1 mM DTT) of the same volume. Filled cells were loaded into an An50Ti rotor and equilibrated for 2 h under vacuum in a Beckman Coulter Optima XL-I ultracentrifuge at 4 °C prior to centrifugation. Data were acquired at 50,000 rpm at 4 °C via absorbance at 305 nm and interference optics. Data were analyzed using the c(s) methodology in the program SEDFIT [18]. The figure featuring c(s) distributions were rendered with the program GUSSI [19].

2.4. Thermodenaturation assay

The TNKS catalytic domain in a buffer containing 10 mM Tris, pH8.0, 150 mM NaCl and 1 mM DTT was mixed with the protein thermal shift dye from Applied Biosystems (#4461141) to reach the final protein concentration of 30 μM. The samples (20 μl) in 96-well plate were subjected to thermodenaturation by using a CFX96 Real-time PCR machine (Bio-Rad). Fluorescence signal was recorded during temperature increase from 4 °C to 85 °C.

2.5. Structural and sequence analyses

The PyMOL software package (http://www.pymol.org) was used for structure analyses and figure rendering. The sequence alignment was rendered using ESPript [20].

2.6. Immunoprecipitation and western blot

The entire coding human TNKS cDNA sequence was fused to the IgG Fc domain by ligating into the pcDNA3 EK FC vector using flanking restriction sites introduced by PCR. Human TNKS-FLAG DNA constructs harboring various mutations were engineered using site-directed mutagenesis. HEK293 or COS1 cells were transfected with the corresponding cDNA construct using Effectene (Qiagen) according to the manufacturers protocol. To chemically inhibit TNKS, cells were treated with the corresponding inhibitor for 24 h. For immunoblotting, cells were lysed in 1X protein sample loading buffer diluted from Biorad 4X Laemmli protein sample buffer (#1610747), and proteins were separated on SDS-PAGE. For immunoprecipitation or IgG pull-down studies, cleared lysates were mixed with Protein A agarose beads in the presence or absence of 2 μg of desired antibody and rotated for 4 h at 4 °C. Beads were then washed three times with lysis buffer (1% NP40 in PBS). Bound proteins were eluted using 2x protein sample loading buffer and separated on SDS-PAGE. Antibodies were acquired from the following sources: anti-FLAG (Sigma, #A2220), anti-TNKS1/2 (Santa Cruz Biotechnology, #sc-8337), anti-NPT (Millipore, #06–747), and anti-ACTIN (Sigma, #A1978).

3. Results

3.1. Catalytic activity of tankyrases is dependent on the SAM domain-mediated oligomerization

Previous studies have shown that loss of the SAM domain-mediated oligomerization reduces self-PARsylation of tankyrases from mammalian cells [14,15]. We found that TNKS SAM-catalytic domains purified from E. Coli is auto-PARsylated (Fig. 1). We made two oligomerization-impaired SAM-catalytic mutants, V1056G and Y1073A, based on the crystal structures of the SAM domain tankyrases [14,15]. Consistent the previous studies, the mutants showed much lower levels of PARsylation than the wild-type protein (Fig. 1B). These observations suggest that the SAM domain-mediated oligomerization promotes the catalytic activity of tankyrases. Alternatively, the diminished self-PARsylation of the mutants may be caused by impaired PARsylation *in trans* of tankyrase molecules that cannot oligomerize, rather than reduced catalytic activity *per se*. To distinguish these two possibilities, we examined the PARsylation activity of the SAM-catalytic domains of TNKS by using histone as a substrate. The results show that wild-type SAM-catalytic domains PARsylated histone much more efficiently than the V1056G and Y1073A mutants (Fig. 1C). The isolated catalytic domain was even less active (Fig. 1C). These results support the notion that the catalytic activity of tankyrases is dependent on the SAM domain-mediated oligomerization.

3.2. A catalytic domain dimer in crystal structures of TNKS and TNKS2

To understand how the oligomerization regulates the

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