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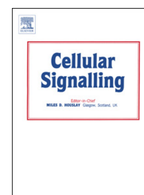

Structural basis of Rad53 kinase activation by dimerization and activation segment exchange ☆

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ABSTRACT

The protein kinase Rad53 is a key regulator of the DNA damage checkpoint in budding yeast. Its human ortholog, CHEK2, is mutated in familial breast cancer and mediates apoptosis in response to genotoxic stress. Autophosphorylation of Rad53 at residue Thr354 located in the kinase activation segment is essential for Rad53 activation. In this study, we assessed the requirement of kinase domain dimerization and the exchange of its activation segment during the Rad53 activation process. We solved the crystal structure of Rad53 in its dimeric form and found that disruption of the observed head-to-tail, face-to-face dimer structure decreased Rad53 autophosphorylation on Thr354 *in vitro* and impaired Rad53 function *in vivo*. Moreover, we provide critical functional evidence that Rad53 trans-autophosphorylation may involve the interkinase domain exchange of helix αEF *via* an invariant salt bridge. These findings suggest a mechanism of autophosphorylation that may be broadly applicable to other protein kinases.

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1. Introduction

The safeguarding of genomic integrity is an essential process whose failure can lead to cell death, gross chromosomal rearrangements or mutations [1]. In vertebrates, genomic stability requires the detection, signaling and repair of DNA damage, and a failure to do so is strongly associated with tumorigenesis [1]. When cells are exposed to DNA damage and in particular to DNA double-strand breaks (DSBs), cells mount an evolutionarily conserved response that coordinates DNA repair and initiates a slow down or arrest of the cell cycle, an event termed the DNA damage checkpoint.

In eukaryotes, the DNA damage checkpoint is initiated when kinases of the ATM/ATR family are activated by DNA lesion sensors [2]. Genetic studies in budding yeast have identified the serine/threonine protein kinase Rad53 and its mammalian ortholog checkpoint kinase 2 (Chk2, product of gene *CHEK2*) as key regulators of the DNA damage checkpoint [3,4]. Loss-of-function mutations of *RAD53* result in loss of viability due to an essential function in maintaining dNTP levels during DNA replication, but hypomorphic *RAD53* mutations result in DNA damage sensitivity and deficits in nearly all checkpoint responses in yeast [5–9]. Similarly, loss-of-function mutations in the mammalian tumor suppressor *CHEK2* also lead to a defective checkpoint response as well as deficiencies in DNA damage-induced apoptosis [10–12].

Rad53 belongs to a subfamily of protein kinases characterized by the presence of one or more phospho-threonine recognition modules known as forkhead-associated (FHA) domains [6,13–15] (Fig. 1A). Rad53 contains two FHA domains, FHA1 and FHA2, which flank a central serine/threonine protein kinase domain [15]. Also, Rad53 contains two serine–glutamine/threonine–glutamine cluster domains (SCD) located N-terminal to FHA1 and immediately C-terminal to the kinase [10,16]. Mammalian Chk2 is similarly organized but notably with only one SCD and one FHA domain N-terminal to the kinase domain [10,11]. Within the SCD of Rad53 and Chk2 are clusters of serine–glutamine and threonine–glutamine (SQ/TQ) motifs. The SQ/TQ motifs are potential target phosphorylation sites of the PI(3) kinase-like protein kinase

Abbreviations: AMPPNP, adenylylimidodiphosphate; AUC, analytical ultracentrifugation; βME, β-mercaptoethanol; Chk2, checkpoint kinase 2; DAPK3, Death-associated protein kinase 3; DTT, dithiothreitol; FHA, forkhead-associated; HU, hydroxyurea; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LOK, lymphocyte-originated kinase; MAPK, mitogen-activated protein kinase; OSR1, oxidative stress-responsive-1; SLK, STE20-like kinase; TEV, tobacco etch virus.

☆ The authors declare no conflict of interest.

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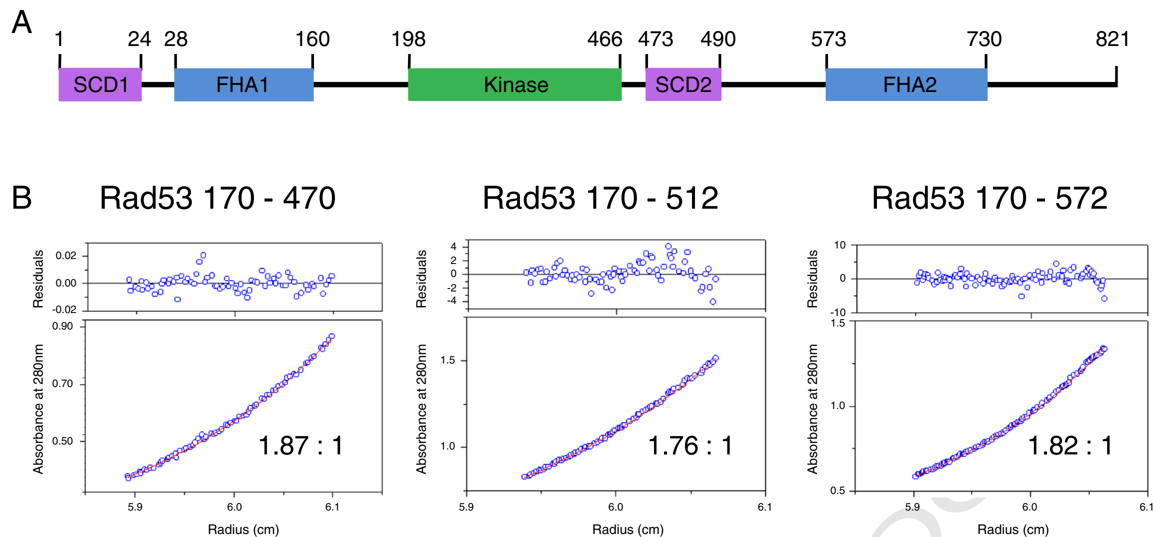

Fig. 1. The Rad53 kinase domain is sufficient for dimerization. (A) The domain structure of Rad53. (B) Data from analytical ultracentrifugation sedimentation equilibrium experiments are shown for Rad53 proteins. The data points depicted were collected at 10,000 rpm and 20 °C for 170–470, 4 °C for 170–512 and 170–572. The red lines in the lower panels correspond to the fit of the data to a monodisperse dimeric model. The residual deviations from the theoretical fits are given in the upper panels.

family members Mec1 and Tel1 in yeast, fungal orthologs of mammalian ATR and ATM respectively, which act upstream of Rad53 and Chk2 as DNA damage sensor proteins [17].

Activation of Rad53 *in vivo* requires its phospho-dependent interaction with the signaling adaptors Rad9 and Mrc1 [15,18,19]. The Rad53–Rad9 interaction is FHA-dependent and occurs following Mec1-dependent phosphorylation of Rad9 at multiple threonine residues [19–21]. Rad9 bound Rad53 proteins are phosphorylated at multiple sites (within the SCD1 by Mec1), which primes Rad53 for activation. Rad53 subsequently undergoes extensive autophosphorylation that is likely facilitated by the clustering of multiple Rad53 molecules on hyperphosphorylated Rad9 [22]. In particular, autophosphorylation of a key regulatory site in the activation segment, Thr354, is required for the catalytic activation of Rad53.

Similar to Rad53, activation of Chk2 kinase requires phosphorylation of a key threonine (Thr383) in the activation segment [23,24]. Notably, dimerization is also necessary for Chk2 activation, possibly by promoting efficient trans-autophosphorylation of the activation segment. Upon DNA damage, Thr68 within the SCD is phosphorylated, which promotes oligomerization of Chk2 *via* the FHA domain and leads to phosphorylation on Thr383 and Thr387 [23–28]. A crystal structure of Chk2 consisting of the FHA and kinase domains revealed a dimer conformation involving intermolecular FHA–kinase and FHA–FHA interactions. In the dimer, the kinase active sites are face-to-face in close proximity and in an arrangement that would facilitate trans-autophosphorylation of their activation loops [29]. A crystal structure of the isolated Chk2 kinase domain revealed a different dimer configuration, in which the kinase domains are positioned tail-to-tail and the activation segments are exchanged between protomers [30]. Intermolecular strand exchange has been observed in the dimeric crystal structures of other protein kinases, including p38α MAP kinase, p70 ribosomal S6 kinase (p70S6K), death-associated protein kinase 3 (DAPK3) and three sterile kinase (STE) family members, namely STE20-like kinase (SLK), lymphocyte-originated kinase (LOK), and oxidative stress-responsive-1 (OSR1) kinase [31–35]. While it has been proposed that activation segment exchange may be a common mechanism for dimerization-driven activation by trans-autophosphorylation, further experimental work is required to fully validate this mechanism of regulation [30,32].

The objective of this study was to understand the mechanisms leading to Rad53 activation by assessing the contribution of dimerization and activation segment exchange in this process. We first found that Rad53 dimerizes in solution and then solved the crystal structure of the Rad53 kinase domain in its dimeric form. The crystal structure reveals a head-to-tail, face-to-face mode of dimerization that is necessary for autophosphorylation of Thr354 on the Rad53 activation loop, as confirmed by *in vitro* and *in vivo* functional analyses. Also, our results provide evidence that Rad53 trans-autophosphorylation may involve the intermolecular exchange of helix αEF *via* an invariant salt bridge, indicating a mechanism of autophosphorylation that may be broadly applicable to other protein kinases.

2. Materials and methods

2.1. Cloning, strains, expression and purification

Overexpression of wild-type (WT) Rad53 from *Saccharomyces cerevisiae* is toxic to bacteria. We serendipitously identified an attenuating mutant Ala225 to serine (A225S) that expressed well in bacteria while remaining catalytically competent for Thr354 autophosphorylation and phosphorylation of a generic myelin basic protein substrate *in vitro*. The A225S form of Rad53 was used as our WT reference and as a template for the generation of mutants for *in vitro* biochemical analyses. *In vivo* studies employed true WT Rad53 as a reference and for the generation of mutants tested *in vivo*. Rad53 constructs were generated by PCR into pPROEx-Hta-TEV and mutants by QuikChange site-directed mutagenesis (Stratagene).

Rad53 constructs were individually transformed into *Escherichia coli* Q2 BL-21 cells and grown in LB media supplemented with 100 μg/ml ampicillin overnight at 15 °C (A_{600} = 0.6, 0.2 mM IPTG induction). Cells were collected by centrifugation, resuspended in buffer A (20 mM HEPES pH 7.5, 200 mM NaCl, 5 mM imidazole) supplemented with 1 mg/ml lysozyme and 5 mM βME, and lysed by sonication on ice. Following centrifugation and filtering to remove cell debris, crude lysate was applied to a HiTrap chelating HP Ni column (Pharmacia). Non-specifically bound proteins were removed by consecutive washes using five column volumes of 5 mM and 30 mM imidazole buffer (20 mM HEPES pH 7.5, 200 mM NaCl) and the 6×HIS-Rad53 proteins eluted with 350 mM imidazole buffer. Removal of the 6×HIS-tag was achieved by addition of 6×HIS-TEV (tobacco etch virus) protease (non-cleavable HIS-tag), 1 mM DTT and 0.5 mM EDTA directly to the final 350 mM imidazole wash fraction (overnight, 4 °C). Following dialysis to remove DTT and imidazole, the TEV-cleaved sample was applied to a Ni column and the Rad53 protein in the flow-through concentrated by ultrafiltration and further purified by gel filtration chromatography (20 mM HEPES

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