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The R1441C mutation alters the folding properties of the ROC domain of LRRK2

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ABSTRACT

LRRK2 is a 250 kDa multidomain protein, mutations in which cause familial Parkinson's disease. Previously, we have demonstrated that the R1441C mutation in the ROC domain decreases GTPase activity. Here we show that the R1441C alters the folding properties of the ROC domain, lowering its thermodynamic stability. Similar to small GTPases, binding of different guanosine nucleotides alters the stability of the ROC domain, suggesting that there is an alteration in conformation dependent on GDP or GTP occupying the active site. GTP/GDP bound state also alters the self-interaction of the ROC domain, accentuating the impact of the R1441C mutation on this property. These data suggest a mechanism whereby the R1441C mutation can reduce the GTPase activity of LRRK2, and highlights the possibility of targeting the stability of the ROC domain as a therapeutic avenue in LRRK2 disease.

1. Introduction

Leucine rich repeat kinase 2 (LRRK2) is a member of the ROCO family of proteins, defined by the presence of a ROC (Ras of complex proteins) domain followed by a domain of unknown function termed C-terminal of ROC, or COR [1]. In LRRK2, these domains are flanked towards the C- and N-termini respectively by leucine rich repeat (LRR) and kinase domains that give this protein its name, along with a number of protein/protein interaction domains [2]. The function of LRRK2 is unknown, but several mutations in this protein have been shown to cause autosomal dominant Parkinson's disease (PD) [3,4]. Overall, mutations in LRRK2 are the most common genetic cause of PD [5,6]. Due to their recent description and large size, very little is known about the biochemistry and function of the ROCO proteins. Studies focusing on the enzymatic activities of LRRK1 and LRRK2 have suggested that the GTPase activity of these proteins regulates their kinase activity, in a manner analogous to the interaction of small GTPases and associated kinases such as Ras and Raf [7-9]. Studies of mutations linked to Parkinson's disease have shown that the kinase activity of LRRK2 is required for cytotoxicity associated with mutations [10-14]. However, while mutations in the kinase domain have a modest activating effect on kinase activity, mutations in the ROC domain of LRRK2 have not consistently been shown to affect kinase activity [15]. These same ROC domain mutations consistently disrupt the GTPase activity of the protein, which might lead to altered regulation of kinase activity, although the mechanistic basis for this is unclear [16-19]. These studies highlight the role of the enzymatic activities of LRRK2 in the disease process, but the exact functional and spatial relationship between these two domains is unknown.

We have previously demonstrated that the R1441C mutation decreases the GTPase activity of LRRK2 in the context of the full-length protein [16]. The crystal structure of LRRK2 ROC domain showed that the R1441 residue is distal to the active site of the protein and revealed that the R1441 residue sits at the interface between two constituent monomers in a dimeric structure [19]. This observation suggests that mutations at this residue could destabilize the interaction between the monomers, which might be the mechanism of enzymatic dysfunction. Consistent with this observation, we demonstrated that the ROC domain containing the R1441C mutation has a decreased ability to precipitate the full-length protein from cell lysates. However, the recent report of the crystal structure of a fragment of a ROCO protein from the prokaryote *Chlorobium Tepidium* consisting of the ROC and COR domains (the *C. Tepidium* ROCO protein does not contain a kinase

Abbreviations: DSF, differential scanning fluorimetry; CD, circular dichroism; GDP, guanosine diphosphate; GTP, guanosine triphosphate; LRRK2, leucine rich repeat kinase 2; ROC, Ras of complex proteins; PD, Parkinson's disease

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domain) suggested that the role of R1441 is mainly inter-domain association. This alternative model implied that mutations at LRRK2 R1441 should not perturb the structure of the ROC GTPase domain itself, and instead acts to disrupt its interaction with the COR domain [20]. To further investigate the mechanism whereby the R1441C mutation disrupts ROC/ROC interactions and how it decreases the GTPase activity of LRRK2, we have used a variety of biophysical approaches including circular dichroism spectropolarimetry and differential scanning fluorimetry to assess the folding characteristics of the ROC domain and to test whether the R1441C mutation alters these characteristics.

2. Materials and methods

2.1. Protein production and purification

The ROC domain of LRRK2 was expressed and purified as previously reported [21]. Where only the wild type protein was used, the 6× polyhistidine tag was removed as described [21]. The R1441C mutant protein was expressed and purified as a 6xHis tagged fusion and was used to compare with the 6xHis tagged WT ROC protein. Attempts to remove the 6xhis tag from the mutant protein failed due to the drastically decreased stability of the protein (data not shown). Attempts to purify a GTP binding dead (K1347A) mutant form of the protein in a soluble form were also unsuccessful (data not shown).

2.2. Circular dichroism

Spectra were recorded with a Jasco J715 spectropolarimeter and measured at a protein concentration of 1 mg ml^{-1} with a 0.01 cm path length. The spectra are an average of 50 scans at 25 °C, with background molecular ellipticity due to the buffer subtracted, in the presence of 4 mM GDP. Analysis of protein stability was carried out with a protein concentration of 0.1 mg ml^{-1} and a 5 mm path length (measurements were taken over a 10 nm bandwidth centered around 220 nm). Unfolding was carried out by melting over a temperature gradient from 25 °C to 85 °C, ramping by 1° every minute. The ellipticity signal (h) was converted to proportion of molecules in the native state α_N, according to the relationship $\alpha_N = (\theta - \theta_U)/(\theta_N - \theta_U)$, where θ_U and θ_N are the ellipticity signals for the unfolded and native states, respectively. Data were fitted to the Van't Hoff function and the mid-point of thermal denaturation (T_m) defined as DH/DS.

2.3. Differential scanning fluorimetry

Thermal denaturation curves were also obtained using a Bio-Rad iCycler Single Wavelength Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and SYPRO Orange fluorescence dye (Invitrogen, Carlsbad, CA) with an excitation and emission wavelength of 490 nm and 520 nm, respectively. The 6xHis tagged ROC domain proteins (WT and R1441C) were subjected to a thermal gradient of 0.5 °C increments from 23 °C to 75 °C. The 50 µl reaction consisted of 0.25 mg/ml protein in 100 mM HEPES, 150 mM NaCl, 20 mM $MgCl_2$, pH 7.5 and a 2× SYPRO Orange final concentration diluted from concentrated stock. Guanine nucleotide ligands (GDP, GTP and GppNP) were added at a final concentration of 2 mM accordingly. All experiments were performed with five replicates. The T_m for each sample was calculated using the first derivative of the normalized fluorescence.

2.4. Pulldown assay

Purified WT and R1441C 6xhis tagged ROC proteins were mixed with excess amounts of purified WT v5-tagged ROC. The protein mixtures were then incubated with Ni-NTA superflow resin for 1 hour at 4 °C, in buffer A containing 20 mM Tris, 500 mM NaCl, 20 mM Imidazole, 20 mM $MgCl_2$, 10% Glycerol, pH 8.0. Different guanine nucleotide ligands (GDP, GTP and GppNP) were added to a final concentration of 2 mM accordingly. After extensive wash with buffer A, proteins were eluted from Ni-NTA. Co-purified proteins were analyzed by SDS-PAGE and western blotting with biotinylated anti-V5 antibody (1:10000). Streptavidin conjugated HRP (1:2000) were used for developing the membrane. The SDS gels were scanned by CanonPI CS-U 3.8.1×scanner and the western blot was developed and scanned on Alpha Innotech FluoChem Instrument. The intensities of the bands were integrated and recorded using the

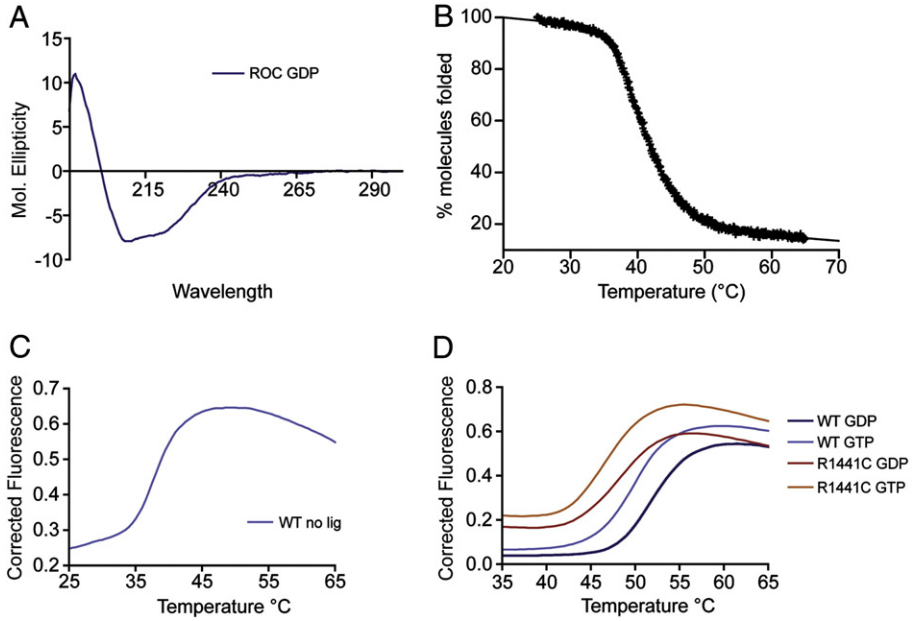

Fig. 1. (A) CD analysis of the wild type ROC domain, displaying absorption spectra typical of a predominantly α-helical fold B, (C) Thermal melt data from the ROC domain in the absence of ligand using CD (B) and DSF (C) to follow loss of structure (D) DSF measurement of protein stability in the presence of GDP and GTP for wild type and R1441C ROC domain.