Molecular and Cellular Neuroscience 76 (2016) 52–58

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

ALS-FTLD associated mutations of *SQSTM1* impact on Keap1-Nrf2 signalling

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ARTICLE INFO

Article history:
Received 12 May 2016
Revised 3 August 2016
Accepted 18 August 2016
Available online 20 August 2016

Keywords:
ALS-FTLD
SQSTM1/p62
Keap1-Nrf2
Oxidative stress

ABSTRACT

The transcription factor Nrf2 and its repressor protein Keap1 play key roles in the regulation of antioxidant stress responses and both Keap1-Nrf2 signalling and oxidative stress have been implicated in the pathogenesis of the ALS-FTLD spectrum of neurodegenerative disorders. The Keap1-binding partner and autophagy receptor SQSTM1/p62 has also recently been linked genetically to ALS-FTLD, with some missense mutations identified in patients mapping within or close to its Keap1-interacting region (KIR, residues 347–352). Here we report the effects on protein function of four different disease associated mutations of SQSTM1/p62 which affect the KIR region. Only mutations mapping precisely to the KIR (P348L and G351A) were associated with a loss of Keap1 binding in co-immunoprecipitations comparable to wild-type SQSTM1/p62. These selective effects on Keap1 recognition were entirely rational based on protein structural models. Consistent with impaired Keap1 binding, the P348L and G351A KIR mutants showed reduced ability to activate Nrf2 signalling compared to wild-type SQSTM1/p62 in antioxidant response element (ARE)-luciferase reporter assays. The results suggest that *SQSTM1* mutations within the KIR of SQSTM1/p62 contribute to aetiology of some cases of ALS-FTLD through a mechanism involving aberrant expression or regulation of oxidative response genes.

1. Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are devastating neurological diseases that lie within the same clinicopathological spectrum, with approximately 5–10% of cases being familial. ALS is a debilitating disease characterised by loss of both upper and lower motor neurones whilst FTLD is recognised as the second most common early onset dementia. Variants in numerous genes are associated with susceptibility to ALS-FTLD including *SQSTM1* (Rubino et al., 2012), which was previously found to carry mutations in patients with the skeletal disorder Paget's disease of bone (PDB) (Rea et al., 2014). Coexistence of PDB and ALS-FTLD is apparently rare and precisely how different *SQSTM1* mutations (some of which are common to both disorders) can lead to either neurodegeneration or PDB is currently unknown, although in some cases co-occurrence of an additional mutation such as a pathogenic *C9orf72* expansion may account for the neurodegenerative phenotype (Almeida et al., 2015).

SQSTM1 encodes the SQSTM1/p62 protein and the overwhelming majority of mutations associated with PDB cluster within and around the C-terminal ubiquitin-associated (UBA) domain, whereas mutations associated with ALS-FTLD occur more widely throughout the protein sequence (Rea et al., 2014; Majcher et al., 2015). In addition to the UBA domain, the multi-domain SQSTM1/p62 protein consists of an N-terminal Phox1 and Bem1p (PB1) domain, zinc finger domain (ZZ), TRAF6-binding domain, LC3-interacting region (LIR), Keap1-interacting region (KIR) and two PEST sequences. The KIR sequence allows SQSTM1/p62 to physically link to the oxidative stress-related Keap1/Nrf2 pathway (Copple et al., 2010; Lau et al., 2010), whereas the LIR allows interaction with Atg8/LC3 proteins that facilitates SQSTM1/p62's function as a receptor for ubiquitin-dependent autophagy (Pankiv et al., 2007). Due to the close proximity of the LIR and KIR, SQSTM1/p62 cannot interact with LC3 and Keap1 protein simultaneously (Jain et al., 2010). Very recently we showed that an ALS-associated L341V mutant of SQSTM1/

Abbreviations: ALS, Amyotrophic lateral sclerosis; ARE, Antioxidant response element; FTLD, Frontotemporal lobar degeneration; IKKβ, Inhibitor of nuclear factor kappa-B kinase subunit beta; Keap1, Kelch-like ECH-associated protein 1; KIR, Keap1-interacting region; LIR, LC3-interacting region; mTORC1, Mammalian target of rapamycin complex 1; *NFE2L2*, Nuclear factor erythroid 2-like 2; NQO1, NAD(P)H dehydrogenase, Quinone 1; Nrf2, Nuclear erythroid 2-related factor 2; PDB, Paget's disease of bone; RBM45, RNA binding motif protein 45; SALS, Sporadic ALS; SOD1, Superoxide dismutase 1; SQSTM1/p62, Sequestosome 1/p62 protein; TDP-43, TAR DNA-binding protein 43; UBA, Ubiquitin-associated (domain).

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http://dx.doi.org/10.1016/j.mcn.2016.08.004

p62, which maps directly to the LIR sequence, is defective in recognition of LC3B and associated with autophagy defects in motor neurone-like cells (Goode et al., 2016).

Previous studies have indicated that the Keap1-Nrf2 pathway, involving the transcription factor Nrf2 and its repressor protein Keap1, is altered in animal models of ALS (Mimoto et al., 2012) and post mortem tissues from patients (Sarlette et al., 2008). In addition to the prominent role of SOD1 in familial ALS, several components of the cellular oxidative stress response have been genetically linked to ALS-FTLD including variants in *NFE2L2* and *KEAP1*, encoding Nrf2 and Keap1 proteins respectively (Bergstrom et al., 2014). Furthermore Keap1 immunoreactivity has also been detected in skein-like inclusions in the spinal cords of ALS patients (Tanji et al., 2013) but the precise role of this pathway in ALS-FTLD motor neurodegeneration remains to be fully elucidated. The Keap1-Nrf2 pathway is crucial in the cellular defence response against oxidative and chemical stress (Komatsu et al., 2010). Under normal conditions monomeric Nrf2 is bound to a homodimer of Keap1 in the cytoplasm via a tight interaction with the Nrf2-ETGE motif and a weaker interaction with the Nrf2-DLGex ('ex' denoting 'extended' from the DLG tripeptide) motif (McMahon et al., 2006; Tong et al., 2006). The N-terminus of Keap1 also associates with Cullin-3 to form an E3 ubiquitin ligase complex which ubiquitinates Nrf2, signalling it to be constitutively degraded by the ubiquitin-proteasome system. Under conditions of oxidative stress Keap1 undergoes modification of cysteine residues changing its structural conformation, allowing Nrf2 to dissociate and translocate to the nucleus, where it binds antioxidant response elements (ARE) in promoter regions of many cytoprotective antioxidative genes (Kobayashi et al., 2006). Liberation of Nrf2 regulates *SQSTM1* gene expression in a positive feedback loop (Jain et al., 2010) and Nrf2 expression is also induced when SQSTM1/p62 binds to Keap1, via its KIR (residues 347–352), (Komatsu et al., 2010; Lau et al., 2010). Indeed regulation of this interaction further indicates the interdependence of Keap1-Nrf2 signalling and autophagy; SQSTM1/p62 bound to ubiquitin-modified autophagic targets is phosphorylated by mTORC1 at residue S349 within the KIR, to promote interaction with Keap1 and displacement of Nrf2 (Ichimura et al., 2013; Tanji et al., 2014). Keap1 also has a role in the autophagic degradation of IKKβ (Kim et al., 2010) which may impact NF-κB-dependent regulation of autophagy, additionally SQSTM1/p62-dependent autophagic degradation of Keap1 has been reported (Bae et al., 2013).

A previous study by Rubino and co-workers determining the frequency of *SQSTM1* mutations in ALS-FTLD patients attending clinics in Italy identified four novel *SQSTM1* variants, including the missense mutations K344E and P348L (Rubino et al., 2012). The K344E mutation affects a region of the protein between the LIR and KIR whereas the P348L mutation is located directly within the KIR sequence (see Fig. 1). K344E was found in 1/170 patients affected with FTLD and P348L in 1/124 patients affected with ALS (neither were present in controls). Both mutated residues are highly conserved in evolution but only P348L is predicted to be pathogenic (Rubino et al., 2012). The patient with the P348L mutation was 53 years old at age of onset but died at 55 years with a rapidly worsening condition; the FTLD patient with the K344E mutation was 69 years at age of onset and suffered from dementia. An additional disease associated KIR missense mutation, G351A, was identified in a subject from a large cohort of FTLD patients from the North West of England (Miller et al., 2015) in an individual who also had a repeat expansion in *C9orf72*, with approximately 2500 repeats (absent in controls). The patient suffered from behavioural variant FTD, with delusions as an atypical feature. She progressed slowly and died 18 years after onset of symptoms, however the authors of the study were unsure of the pathogenicity of the G351A mutation due to the co-occurrence of the *C9orf72* repeat expansion. Interestingly the G351A mutation was previously studied as an 'artificial' mutation to probe SQSTM1/p62-Keap1 interactions, prior to any disease association. In that context the G351A mutation was shown to reduce Keap1 binding in MBP pull-down assays and was associated with reduced Nrf2 activity in NQO1-ARE luciferase reporter assays (Jain et al., 2010). Similarly we previously found that a PDB-associated S349T KIR mutation of SQSTM1/p62, which unusually for PDB affects a region of the protein outside the UBA domain, impacts interaction with Keap1 and Nrf2 activity in reporter assays (Wright et al., 2013).

Here we verify that the disease associated G351A KIR mutation of SQSTM1/p62 impairs recognition of Keap1 and show that this mechanistic defect extends to an additional KIR mutation, P348L. In contrast, mutations located close to but not precisely mapping within the KIR (L341V, K344E) bind Keap1 normally. Consistent with this Keap1 interaction code, only the mutants associated with loss of Keap1 binding show reduced ability to activate Nrf2 signalling. Thus aberrant production of oxidative response genes may be a feature of a subset of cases of ALS-FTLD with *SQSTM1* mutations.

2. Materials and methods

2.1. Plasmids

The plasmids for expression of full-length human wild-type and L341V mutant SQSTM1/p62 protein (residues 1–440) as GST fusion proteins (pGEX-4T-1, GE Healthcare) in *E. coli* were described previously (Goode et al., 2016). The K344E, P348L and G351A mutants were created from the wild-type plasmid by site-directed mutagenesis (QuikChange kit; Stratagene) and subsequently verified by DNA sequencing. The L341V, K344E, P348L and G351A mutations were also introduced into the wild-type pcDNA3.1 His-FLAG-SQSTM1/p62 construct (Goode et al., 2014) via site-directed mutagenesis and verified by DNA sequencing. The cDNA of human LC3B was cloned into the pGEX-4T-3 plasmid (GE Healthcare) between the *Eco*RI and *Sal*I cloning sites allowing expression as a GST fusion.

2.2. Co-immunoprecipitations

Co-immunoprecipitations of transfected His-FLAG-SQSTM1/p62 and endogenous Keap1 have been described previously (Wright et al., 2013). Briefly, HEK293T cells were seeded in 6-well plates at 1.0×10^6 cells/well. 24 h after cells were transfected with 4 μg of the wild-type or mutant His-FLAG-SQSTM1/p62 pcDNA3.1 construct (empty pcDNA3.1 in the control) for 24 h. Transfected cells were harvested and lysed in 200 μL RIPA buffer (150 mM NaCl, 1% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, including

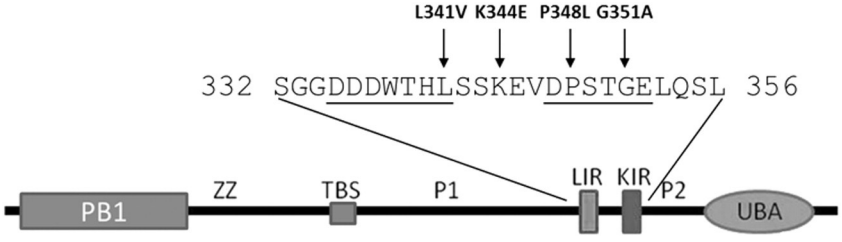

Fig. 1. Schematic of the SQSTM1/p62 protein highlighting the amino acid sequence containing the Keap1-interacting region (KIR) and LC3-interacting region (LIR). Selected residues mutated in ALS-FTLD are indicated.