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The S349T mutation of *SQSTM1* links Keap1/Nrf2 signalling to Paget's disease of bone

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ABSTRACT

Mutations affecting the Sequestosome 1 (SQSTM1) gene commonly occur in patients with the skeletal disorder Paget's disease of bone (PDB), a condition characterised by defective osteoclast differentiation and function. Whilst most mutations cluster within the ubiquitin-associated (UBA) domain of the SQSTM1 protein, and are associated with dysregulated NFκB signalling, several non-UBA domain mutations have also been identified. Keap1 is a SQSTM1-interacting protein that regulates the levels and activity of the Nrf2 transcription factor. This in turn controls the expression of numerous cytoprotective genes that contribute to the cell's capacity to defend itself against chemical and oxidative stress, through binding to the antioxidant response element (ARE). The PDB-associated S349T mutation maps to the Keap1-interacting region (KIR) of SQSTM1, however the effects of PDB mutant SQSTM1 on Keap1 function have not been investigated. Here we show that unlike other SQSTM1 mutations, the S349T mutation results in neither impaired ubiquitin-binding function in pull-down assays, nor dysregulated NFκB signalling in luciferase reporter assays. Keap1 is expressed in differentiating osteoclast-like cells and the S349T mutation selectively impairs the SQSTM1–Keap1 interaction in co-immunoprecipitations, which molecular modelling indicates results from effects on critical hydrogen bonds required to stabilise the KIR–Keap1 complex. Further, S349T mutant SQSTM1, but not other PDB-associated mutants, showed reduced ability to activate Nrf2 signalling as assessed by ARE-luciferase reporter assays. Thus, SQSTM1-mediated dysregulation of the Keap1–Nrf2 axis, which could potentially lead to aberrant production of oxidative response genes, may contribute to disease aetiology in a subset of PDB patients.

Introduction

Paget's disease of bone (PDB) is a common disorder characterised by accelerated bone turnover at distinct sites throughout the skeleton [1]. The primary cellular abnormality involves a net increase in the activity of bone-resorbing osteoclasts, with a secondary increase in bone-forming osteoblast activity that increasingly is thought to be important in maintaining the pagetic phenotype [2]. The condition has a strong genetic element and numerous mutations affecting the Sequestosome 1 (SQSTM1) gene, which encodes the SQSTM1 (also known as p62) protein, have been identified in patients with familial and sporadic PDB [3–5]. In different populations *SQSTM1* mutations account for 5–20% of PDB cases [5] and insights into the disease mechanism(s) in patients with these genetic defects are an important first stage in the development of rational drug therapies.

PDB mutations affect the C-terminus of SQSTM1, in and around the ubiquitin-associated (UBA) domain, and we have shown that most [6–8] but not all [9] PDB-associated mutations impair the ability of SQSTM1 to bind to ubiquitin *in vitro*, which can in part be rationalised by structural studies of the UBA domain [10–12]. Despite these insights, there is still limited knowledge about the precise mechanisms by which the mutations manifest their effects at the cellular level. The SQSTM1 protein is multi-functional [13] and within osteoclasts acts as a regulator of the RANK-mediated pathway of NFκB signalling [14]. The altered function of PDB mutant SQSTM1 has been proposed to be linked to an increase in osteoclast activity *via* a mechanism involving gain-of-function with respect to NFκB signalling [8,9,14–17]. In addition, a role for SQSTM1 in mediating the clearance of ubiquitinated proteins, protein aggregates and organelles *via* macroautophagy has been described [18,19], in which SQSTM1 interacts with the LC3 protein to mediate the formation of autophagosomes. Indeed a missense mutation in

Abbreviations: SQSTM1, Sequestosome 1; PDB, Paget's disease of bone; UBA, ubiquitin-associated; ARE, antioxidant response element; KIR, Keap1-interacting region; TPA, 12-O-tetradecanolylphorbol 12-myristae 13-acetate; tBHQ, tert-butylhydroquinone.

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the UBA domain of SQSTM1, equivalent to the most common PDB-associated P392L mutation, is sufficient to cause a PDB-like disorder in mice with evidence of dysregulated autophagy in osteoclasts [20].

Further insights into the normal function of SQSTM1 which are likely to be altered upon its mutation can be derived from the identification of its protein interaction partners. We [21] and others [22–25] identified Keap1 as a SQSTM1-interacting protein and found that SQSTM1 over-expression leads to reduced levels of Keap1, indicating that SQSTM1 can regulate Keap1 degradation [21]. Keap1 is a Cul3 adaptor which regulates the levels and activity of the Nrf2 transcription factor, which in turn binds to the antioxidant response element (ARE) to regulate the expression of a battery of detoxification enzymes (e.g. HO-1 and NQO1), antioxidant proteins (e.g. glutathione synthesis enzymes), xenobiotic transporters (e.g. multidrug resistance protein 1) and other stress response proteins that serve to protect against chemical and oxidative insults that have the capacity to provoke cellular harm [26]. Thus, SQSTM1 over-expression can lead to reduced Keap1 levels with a concomitant increase in Nrf2 activity, which is dependent on direct interaction of SQSTM1 with Keap1. Replacement of six critical residues within the Keap1-interacting region (KIR) of SQSTM1 (347-DPSTGE-353 in the human sequence) with alanines abrogates SQSTM1–Keap1 binding and prevents SQSTM1-mediated activation of Nrf2 signalling, as determined by NQO1 ARE-luciferase reporter assays [22]. At least in liver, SQSTM1-mediated stabilisation of Nrf2 leads to deleteriously high chronic production of oxidative response genes [23].

Although several studies have addressed the functional interaction between wild type SQSTM1 and Keap1, the effects of PDB-associated SQSTM1 mutations on Keap1/Nrf2 signalling have not been determined. Notably, although many PDB mutations affect the UBA domain of SQSTM1, several mutations outside of this ubiquitin-binding region have been identified [8,9] including a S349T missense mutation in a single patient [27], which maps to the KIR. Here we show that S349T-mutant SQSTM1, but not other PDB-associated UBA domain mutants, shows impaired interaction with Keap1 and reduced ability to activate Nrf2 signalling.

Materials and methods

Plasmids

Plasmids (pGEX-4T-1; GE Healthcare) for expression in *E. coli* of the full-length wild type human SQSTM1 protein (440 residues) as well as the G425R and E396X PDB mutants, as GST fusions, were described earlier [6]. The S349T mutant was generated from the wild type SQSTM1 construct by site-directed mutagenesis (QuikChange kit, Stratagene) and verified by DNA sequencing. Mammalian expression vectors (pcDNA3.1; engineered to produce an N-terminal His_6-FLAG-tagged fusion) for wild type, G425R and E396X mutant SQSTM1 were described earlier and the S349T mutant was again generated by site-directed mutagenesis. Plasmids for the NFκB and Nrf2 reporter assays are detailed below (see NFκB reporter assays and Nrf2 reporter assays).

In vitro ubiquitin pull-down assays

Pull-down assays of the GST–SQSTM1 fusion proteins were carried out as described previously [6]. Briefly, proteins were expressed using 10 ml cultures of *E. coli* (XL10-Gold; Stratagene) and the harvested cells were lysed by sonication in 1 ml of 10 mM Tris, 150 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.5 (TBS-T). Supernatants containing the recombinant proteins, produced by centrifugation (13,000 g for 10 minutes) of cell lysates, were incubated for 30 minutes with an excess of glutathione-Sepharose (GE Healthcare), ubiquitin-Sepharose (10 mg/ml bovine ubiquitin covalently immobilised on CNBr-activated thiol-Sepharose 4B) or control-Sepharose (without the addition of ubiquitin). All reagents were maintained at 37 °C throughout the binding and washing stages. After extensive washing with TBS-T, bound proteins were eluted from the beads with gel-loading buffer and detected by western blotting with anti-SQSTM1 antibody, as described below (Western blotting). Three independent replicate experiments were performed.

NFκB reporter assays

HEK293T cells were cultured in 24-well plates and co-transfected with 100 ng per well of NFκB Firefly luciferase reporter construct, 0.2 ng per well of control *Renilla* luciferase plasmid [28] and 700 ng per well of wild type or PDB mutant His-FLAG-SQSTM1 pcDNA3.1 construct (empty pcDNA3.1 in the control) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The NFκB reporter contained the −415/−93 fragment of the human IL8 promoter cloned into the *Kpn*I and *Hind*III restriction sites of the pGL2 plasmid (Promega) [8]. Cells were washed with sterile phosphate-buffered saline (PBS) 30 hours after transfection, lysed in 100 μl passive lysis buffer (Promega), and then Firefly and *Renilla* luciferase activities were measured according to the manufacturer's instructions using the Dual-Glo Luciferase Assay System (Promega) with a GloMax-96 Microplate Luminometer (Promega). Firefly luciferase activity was normalised to *Renilla* luciferase activity within the same transfection and relative luciferase activity was expressed with the value for wild type SQSTM1 set at 1.0. Experiments were performed in quadruplicate and assays repeated on four independent occasions. For each experiment the normalised values of the quadruplicates were averaged, and data are presented as the mean of the average values from the four separate experiments ± SD. Statistical analyses were performed using a one-way ANOVA (Prism5) and a Dunnett's test to determine the level of significance of differences between values for wild type SQSTM1 and PDB mutants, with significance set at $p<0.05$. Western blotting analysis (anti-SQSTM1) was used to determine expression levels of the different SQSTM1 constructs under the assay conditions (see Western blotting).

Differentiation of U-937 cells

U-937 cells were maintained in RPMI-1640 medium in suspension. For differentiation into osteoclast-like cells [29–31], the cells were first pelleted and re-suspended in fresh RPMI-1640 culture medium containing TPA (12-O-tetradecanolylphorbol 12-myristae 13-acetate) at a concentration of 0.1 μg/ml and cultivated in 6-well plates at a density of $0.8–1.0\times10^6$ cells/well in 3 ml medium. Cells were incubated for two days (day −2 to day 0 of differentiation). On day 0, the TPA-containing medium was removed and attached cells rinsed with fresh medium. RPMI-1640 medium containing TNFα at a concentration of 0.3 ng/ml was added to each well and incubated for up to 16 days with the medium changed every two days. Differentiation of the U-937 cells was evaluated during the time course between day 0 and day 16 by western blotting for TRAP; in this case cells were solubilised in RIPA buffer (Sigma) and protein concentrations determined using the BCA assay (Pierce BCA protein assay kit, Thermo Scientific). Blots were also probed for SQSTM1, β-actin and Keap1 (see Western blotting).

Immunoprecipitations

HEK293T cells were seeded in 6-well plates at 1.25×10^6 cells/well. Cells were transfected using Lipofectamine 2000 (Invitrogen) with 4 μg of the wild type or PDB mutant His-FLAG-SQSTM1 pcDNA3.1 construct (empty pcDNA3.1 in the control) overnight (14–16 hours). Transfected cells were washed twice with sterile PBS and cells were pelleted at 800 g for 5 minutes. The cells were then lysed in 200 μl RIPA buffer (Sigma). The insoluble debris was removed by centrifugation and an aliquot of the cleared lysate from each sample was kept at −80 °C for subsequent analysis by western blot (anti-SQSTM1 and anti-Keap1; Western blotting). Protein concentrations were measured