Information Processing and Molecular Signalling

p62 mutations, ubiquitin recognition and Paget's disease of bone

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Abstract
Functional analyses of PDB (Paget's disease of bone)-associated mutants of the p62 [also known as SQSTM1 (sequestosome 1)] signalling adaptor protein represent an interesting paradigm for understanding not only the disease mechanism in this skeletal disorder, but also the critical determinants of ubiquitin recognition by an ubiquitin-binding protein. The 11 separate PDB mutations identified to date all affect the C-terminal region of p62 containing the UBA domain (ubiquitin-associated domain), a ubiquitin-binding element. All of these mutations have deleterious effects on ubiquitin binding by p62 *in vitro*, and there is evidence of an inverse relationship between ubiquitin-binding function and disease severity. The effects on ubiquitin-binding function of most of the mutations can be attributed to either reduced UBA domain stability, and/or the mutations affecting the presumed ubiquitin-binding interface of the UBA domain. However, a subset of the mutations are more difficult to rationalize; several of these affect sequences of p62 outside of the minimal ubiquitin-binding region, providing insights into non-UBA domain sequences within the host protein which mediate ubiquitin-binding affinity. The p62 mutations are presumed to result in activation of (osteoclast) NF-κB (nuclear factor κB) signalling. Understanding how loss of ubiquitin-binding function of p62 impacts on signal transduction events in osteoclasts will undoubtedly further our understanding of the disease mechanism in PDB at the molecular level.

Introduction

PDB (Paget's disease of bone), a disorder characterized by focal increases in bone turnover, affects up to 3% of individuals over 55 years of age in the U.K. and other Caucasian populations [1,2]. In up to a third of cases, symptoms can include bone pain, deformity and susceptibility to pathological fractures, and therefore PDB represents a serious clinical problem. There is a clear genetic predisposition for PDB, and mutations in the gene (known as *SQSTM1*) that encodes the p62 protein are commonly found in patients with familial and sporadic PDB [3,4]. p62 is a multifunctional protein that, in the context of bone-resorbing osteoclasts (the cells principally affected in PDB), acts an adaptor or scaffolding protein in the RANK [receptor activator of NF-κB (nuclear factor κB)]–TRAF-6 [TNF (tumour necrosis factor)-receptor-associated factor 6]–NF-κB signal transduction pathway, an important mediator of (induced) osteoclastogenesis [5]. The 11 separate p62 mutations identified to date all affect the C-terminal region of the protein [6–9], which contains the ubiquitin-binding element, the UBA domain (ubiquitin-associated domain).

Effects of p62 mutations on ubiquitin-binding function

We have demonstrated that all of the p62 mutations found in PDB patients have deleterious effects on ubiquitin binding by the p62 protein *in vitro*, although notably some of these deficits are only uncovered at physiological temperature, i.e. at 37°C [10,11]. These findings led us to suggest that loss of ubiquitin binding may be a unifying mechanism by which mutations of p62 manifest their effects [11]. Interestingly, the rare progressive disorder, inclusion body myopathy associated with PDB and frontotemporal dementia (IBMPFD), a condition in which PDB is a feature, is caused by mutations clustering in a ubiquitin-binding region of the VCP (valosin-containing protein; or p97; [12]), further indicating that abberant ubiquitin recognition may be a critical event in PDB and related conditions.

Correlating p62 ubiquitin-binding function with phenotype in PDB

Although there is some debate as to whether a causal relationship between p62 mutations and PDB exists, several observations suggest that the magnitude of loss of ubiquitin-binding activity of the p62 protein may indeed be related to PDB severity. First, there is clear evidence that the missense mutations of p62 are clinically less severe than truncating mutations [9]. The latter delete most or all of the p62 UBA domain and exert the greatest negative effects on ubiquitin-binding. Secondly, there are indications that clinically the P387L missense mutation may result in a relatively mild phenotype [6] and we found that P387L was the only missense mutant that retained partial function in ubiquitin-binding assays [11]. Finally, a recent study indicates the possible segregation of an M404V missense mutation of p62, which we find to be one of the most detrimental to p62–ubiquitin binding *in vitro* [10,11], with forms of PDB in which an increased number of bones are affected (i.e. polyostotic forms) in an Italian family [13]. Taken together, and combined with the fact that the mutations are only found in PDB patients and not in controls, these observations are supportive of a causal role of p62 mutations in PDB.

Insights into ubiquitin recognition by p62

While providing insights into the disease mechanism in PDB, functional analyses of disease-associated mutants of p62 also represent an interesting paradigm for understanding the critical determinants of ubiquitin recognition by an UBA domain protein.

The effects on ubiquitin-binding function of the four separate p62 truncating mutations [390X, 394X (two different mutations) and E396X] are simple to rationalize, since we have refined the polyubiquitin-binding region of p62 to residues 392–436 of the 440-amino-acid protein [11]; clearly each of the truncating mutations removes most or all of this ubiquitin-binding region.

The effects on ubiquitin-binding function of the majority of the seven p62 missense mutations (P387L, P392L, S399P,

M404V, M404T, G411S and G425R) can be attributed to either reduced UBA domain thermodynamic stability and/or direct effects on the presumed ubiquitin-binding surface of the UBA domain (J. Long, T. Gallagher, R. Layfield and M. Searle, unpublished work), the structure of which we previously determined by NMR spectroscopy [14]. For example, the S399P missense mutation is located within the first helix of the structured region of the UBA domain (which is a three-helix bundle) and mutation at this site decreases the thermodynamic stability of the UBA domain [11]. The G425R missense mutation introduces a highly polar arginine side chain into the hydrophobic patch on the surface of the p62 UBA domain, which is equivalent to the surface implicated in ubiquitin binding by UBA domains from other proteins and seems likely to be involved in the p62–ubiquitin interaction. Precise mapping of the p62 UBA domain interaction surface to determine whether UBA domains all utilize the same binding epitope is in progress.

However, the effects of a subset of the p62 missense mutations are more difficult to rationalize. For example, the P387L mutation affects p62 outside of the minimal polyubiquitin-binding region, has no effects on thermodynamic stability of the UBA domain and, based on current structural models, P387L is unlikely to form part of the binding interface with ubiquitin. Yet this mutation is still able to exert subtle detrimental effects on p62–ubiquitin binding *in vitro* [11]. These findings support the conjecture that non-UBA domain sequences within the host protein can (and presumably often do) mediate ubiquitin-binding specificity and affinity (discussed in more detail in [11]). This is an interesting extension to the notion that ubiquitin-binding properties and/or specificity of UBA domain proteins can be regulated by inter-molecular interactions. The p47–p97 complex, in which p47 is only able to bind ubiquitin (via an UBA domain) in the presence of its p97 binding partner [15], is evidence for the regulatory effects of an inter-molecular interaction. Notably, both the P387L and the most common P392L mutation found in PDB patients affect the 'flexible' N-terminus of the p62 UBA domain, with the latter (which affects the P392 'capping residue') having subtle effects on the UBA domain structure, extending the N-terminus of helix 1 by four residues [14]. P387 is located within a predicted β-turn conformation just prior to helix 1 [11], and substitution with a leucine residue at this site is predicted to perturb this conformation. Whether these mutations affect 'conformational flexibility' at the N-terminus of the UBA domain, which is required for efficient p62-binding function, or alternatively whether these residues become important following structural rearrangements of the UBA domain upon ubiquitin binding (note that the existing NMR structure of the p62 UBA domain is of the uncomplexed polypeptide [14]) remains to be clarified.

In summary, our studies of PDB-associated mutants indicate that key determinants of ubiquitin recognition by the p62 protein are the presence of an UBA domain that is thermally stable with an intact hydrophobic interaction surface, as well as a non-UBA domain sequence(s) capable of modulating

UBA-binding affinity and specificity. This reliance on non-UBA domain components may explain the apparent lack of polyubiquitin chain-binding specificity of the isolated p62 UBA [16], which contrasts with evidence that p62 is functionally involved (at least in some contexts) with the regulation of processes reliant on Lys^{63}-linked polyubiquitination (see, e.g., [17]).

Disease mechanism

The p62 protein is multifunctional and has been implicated in several diverse cellular processes, including that which is likely to be most relevant in PDB, regulation of NF-κB signalling pathways [18]. In this case, p62 functions in concert with TRAF-6 as a scaffolding/adaptor protein in pathways downstream of the IL-1 (interleukin 1), TNF-α, NGF (nerve growth factor) and RANK receptors, which ultimately lead to activation of the NF-κB transcription factor following receptor stimulation. RANK-mediated activation of NF-κB signalling is an important regulator of osteoclastogenesis, and accordingly mutations affecting the RANK–NF-κB signalling axis can result in human skeletal disorders, including several PDB-like conditions (reviewed in [19]). Additionally, skeletal phenotyping of mice with targeted deletion of p62 showed no obvious abnormality in osteoclast activity, but when these animals were challenged with a bone resorbing factor (parathyroid hormone-related protein) there was a clear impairment of osteoclastogenesis. Also, these workers reported that the overexpression of the P392L mutant of p62 activated NF-κB signalling in reporter assays in HEK-293 cells (human embryonic kidney cells) more efficiently than wild-type p62 [5]. Such observations are consistent with the proposal that Pagetic osteoclasts may be abnormally sensitive to inflammatory cytokines and 1,25-dihydroxyvitamin D$_3$ [19].

The mechanistic links between ubiquitin conjugation and the regulation of signal transduction are complex, with ubiquitination of signalling proteins and their subsequent recognition by ubiquitin-binding proteins representing critical events at several different steps within NF-κB signalling pathways. For example, ubiquitination can serve as a receptor internalization, endocytic sorting and degradation signal; as a scaffold to establish signal-induced protein–protein interactions during signalling complex assembly; and to target proteasomal degradation of the IκB (inhibitor of NF-κB) protein [20]. The precise role of the ubiquitin-binding activity of p62 within such pathways is unclear, although there is some evidence that (at least in response to NGF) p62 may regulate the Lys^{63}-linked ubiquitination of TRAF-6 in a UBA domain-dependant manner [17]. However, since TRAF-6 ubiquitination positively regulates NF-κB signalling by promoting the binding of TAK1 (transforming growth factor-β-activated kinase 1) adaptor proteins (TABs; [21]), the apparent activation of an NF-κB reporter [5] by the non-ubiquitin-binding P392L mutant of p62 [10] seems somewhat contradictory. This discrepancy may in part reflect the different *in vitro* assay systems used. Regardless, key questions that await resolution include: what is the physiological significance of ubiquitin binding by p62 in osteoclasts and, moreover, does loss of ubiquitin binding by p62 lead to disordered NF-κB signalling in osteoclasts? Answers to either or both of these questions will provide insights that are likely to underpin advances in therapeutic strategies in PDB, as well as other disorders of abnormal osteoclast activity.

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