Structure of the Ubiquitin-associated Domain of p62 (SQSTM1) and Implications for Mutations That Cause Paget’s Disease of Bone*

Barbara Cianfi, Robert Layfield§, James R. Cavey§, Paul W. Sheppard, and Mark S. Searle‡***

From the §School of Chemistry, University Park, Nottingham NG7 2RD, §School of Biomedical Sciences, University of Nottingham Medical School, Nottingham NG7 2UH, and Affiniti Research Products Ltd., Palantine House, Matford Court, Exeter EX2 8NL, United Kingdom

The p62 protein (also known as SQSTM1) mediates diverse cellular functions including control of NF-κB signaling and transcriptional activation. p62 binds non-covalently to ubiquitin and co-localizes with ubiquitylated inclusions in a number of human protein aggregation diseases. Mutations in the gene encoding p62 cause Paget’s disease of bone (PDB), a common disorder of the elderly characterized by excessive bone resorption and formation. All of the p62 PDB mutations identified to date cluster within the C-terminal region of the protein, which shows low sequence identity to previously characterized ubiquitin-associated (UBA) domains. We report the first NMR structure of a recombinant polypeptide that contains the C-terminal UBA domain of the human p62 protein (residues 387–436). This sequence, which confers multiubiquitin chain binding, forms a compact three-helix bundle with a structure analogous to the UBA domains of HHR23A but with differences in the loop regions connecting helices that may be involved in binding accessory proteins. We show that the Pro392 → Leu PDB substitution mutation modifies the structure of the UBA domain by extending the N terminus of helix 1. In contrast to the p62 PDB deletion mutations that remove the UBA domain and ablate multiubiquitin chain binding, the Pro392 → Leu substitution does not affect interaction of the UBA domain with multiubiquitin chains. Thus, phenotypically identical substitution and deletion mutations do not appear to predispose to PDB through a mechanism dependent on a common loss of ubiquitin chain binding by p62.

The ubiquitin-binding protein p62 (SQSTM1) functions as a scaffold in a range of signaling pathways associated with cell stress, survival, and inflammation and also controls transcriptional activation and protein recruitment to endosomes (1). p62 binds ubiquitin-dependent proteolysis (8). Consistent with the ability of other UBA domains to bind non-covalently to ubiquitin and multiubiquitin chains (9–12), the C-terminal region of the p62 protein is also reported to have these properties (13, 14). We report the first structure of a recombinant polypeptide containing the UBA domain of the human p62 protein (residues 387–436; Fig. 1b) and NMR investigations of the Pro392 → Leu mutant (the most common p62 PDB mutation). This structural model allows us to rationalize the differential effects on ubiquitin binding of p62 PDB deletion and substitution mutations detected in parallel in vitro ubiquitin chain binding assays.

EXPERIMENTAL PROCEDURES

Expression and Isolation of p62 UBA and Mutants—The cDNA containing the UBA domain of human p62 was amplified from IMAGE clone 2906284 by PCR and cloned into the BamHI and XhoI sites of plasmid pGEX-4T-1 (Amersham Biosciences). The Pro392 → Leu PDB mutation was introduced by site-directed mutagenesis (Quick-Change Mutagenesis kit, Stratagene), and constructs were verified by sequencing. Glutathione S-transferase fusion proteins were expressed in XL-10 Gold Escherichia coli (Stratagene). Protein purification involved growing transformed cells at 37 °C until the absorbance at 600 nm reached 0.6 to 0.7, and induction of overexpression was achieved by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 200 μM. Cleavage of purified glutathione S-transferase fusion proteins retained on glutathione-Sepharose beads with thrombin released polyepitides equivalent to residues 387–436 of human p62 with an additional Gly-Ser dipeptide at the N terminus. The cleaved proteins were subjected to a final purification step with a Superdex-200 gel filtration column and dialyzed to remove excess salt.

NMR Spectroscopy and Structure Calculation—NMR data were collected on 2-mm samples of the wild-type p62-UBA domain and the Pro392 → Leu mutant at pH 5.8. Two-dimensional TOCSY, DQF-COSY, and 3D-, 50-, 100-, 250-, and 300-ms two-dimensional NOESY experiments were performed at 25 and 35 °C on a Bruker Avance-600 instrument equipped with a triple-resonance probe with solvent suppression achieved using the WATERGATE sequence (15). Data were processed using Bruker XWINNMR (version 2.5) and ANSIG software (16). NOE restraints from NOESY spectra at 25 and 35 °C were classified as weak.

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†To whom correspondence may be addressed. Tel.: 44 115 9249924 35722; Fax: 44 115 9249924. E-mail: robert.layfield@nottingham.ac.uk.

§To whom correspondence may be addressed. Tel.: 44 115 951 3567; Fax: 44 115 951 3564; E-mail: mark.searle@nottingham.ac.uk.

** The abbreviations used are: PDB, Paget’s disease of bone; UBA, ubiquitin-associated; NOE, nuclear Overhauser effect; NOEys, nuclear Overhauser effect spectroscopy; RMSD, root mean square deviation.
Structure of the UBA Domain of p62

RESULTS

The one-dimensional $^1$H NMR spectrum of the p62-UBA recombinant polypeptide (residues 387–436) has a widely dispersed amide and methyl region consistent with a compact folded structure which CD spectra indicate has a high helical content (~55%). NMR resonance assignments and secondary structure were determined from homonuclear $^1$H NMR TOCSY and NOESY experiments following well established procedures (20). The pattern and intensity of sequential NOEs and TOCSY cross-peak intensities establish that the p62-UBA domain is helix-rich. Structure calculations based on 735 NOE and 56 dihedral angle restraints show that the structure is well defined between residues Pro$^{392}$ and Ile$^{431}$ (see Table I); however, the short N- and C-terminal regions are much more dynamic and show no signs of stable secondary structure (Fig. 2).

The polypeptide forms a compact three-helix bundle (Fig. 3a), with a helical alignment analogous to that found for the UBA domains of HHR23A (21, 22) (Fig. 3b), a highly conserved protein that functions in nucleotide excision repair. The RMSD between the $\alpha$-helical backbone C$\alpha$ atoms of the UBA (2) domain of HHR23A and that of p62 is 1.7 Å indicating a common helical alignment. Although the residues forming the hydrophobic core are mostly conserved, the major differences between the p62-UBA and HHR23A UBA (2) domain are in the loops (L1 and L2) connecting the helices (Fig. 3b). In the p62-UBA, Glu$^{409}$ and Gly$^{415}$ extend loop 1 by 2 residues forming a turn partially stabilized by interactions between the side chains of Asp$^{408}$/Glu$^{409}$ and residues in helix 2, as clearly evident from the NOE data. The conserved Gly-Phe-Xaa se-

![Fig. 2. Stereoview showing the family of 27 lowest energy NMR structures of p62 UBA (residues 387–436) showing a well structured three helix bundle motif between residues 392–431 (RMSD to the mean 0.51 Å), with more flexible N and C termini.](image)
We find that in contrast to the loss of multiubiquitin chain binding that results from PDB deletion mutations, modification of secondary structure by the Pro392 → Leu mutation does not affect either multiubiquitin chain binding in the isolated UBA (Fig. 4) or p62 holoprotein (not shown), a similar effect is predicted for the 390Stop mutation. To determine whether the Pro392 → Leu mutation similarly causes loss of multiubiquitin chain binding, we assessed the functional consequences of this substitution in the p62-UBA domain. In contrast to the PDB deletion mutations, the Pro392 → Leu substitution did not affect either multiubiquitin chain binding in the isolated UBA (Fig. 4) or p62 holoprotein (not shown), suggesting that neither Pro392 nor residues immediately preceding are critical for multiubiquitin chain binding per se.

Consistent with the above findings, analysis of our structural model shows that Pro392 does not form part of the hydrophobic surface patch previously implicated in ubiquitin binding in other UBA domains (21). Additionally, residues that are involved in the modification of secondary structure induced by the Pro392 → Leu substitution (Glu389, Ala390, and Asp391) are not included in this proposed ubiquitin-interacting surface.

DISCUSSION

We have determined the first NMR structure of the p62-UBA domain and shown that the Pro392 → Leu substitution mutation, which causes PDB, modifies secondary structure by extending the N terminus of helix 1. The functional significance of the UBA domain in the p62 protein is currently not known; however, it is able to bind unanchored Lys48-linked multiubiquitin chains (13), and as evident in Fig. 4b, which when attached to target proteins, are proteasomal degradation signals (24). Additionally, p62 binds to multiubiquitinated proteins that accumulate when proteasome degradative function is impaired (13, 25) further suggesting a role for p62 in the regulation of ubiquitin-dependent proteolysis as is the case for other UBA domain proteins (26). We find that in contrast to the loss of multiubiquitin chain binding that results from PDB deletion mutations, modification of secondary structure by the Pro392 → Leu substitution does not affect interaction of the UBA domain with Lys48-linked multiubiquitin chains. The observation that deletion and substitution mutations are phenotypically identi-
interaction with such proteins leading to altered osteoclast activity. Finally, the lack of well defined secondary structure in the N-terminal sequence preceding helix 1 (residues 387–391; Fig. 1b) might suggest that this portion of the polypeptide chain constitutes a flexible linker between the PEST and UBA domains in p62. In this context, it is possible that PDB mutations affect the half-life of p62, for example by exposing PEST sequences that render the protein more susceptible to ubiquitin-dependent proteolysis (28).

Regardless of the precise molecular mechanism by which p62-UBA domain mutations contribute to PDB pathogenesis, it is tempting to speculate that functionally these mutations result in activation of NFκB signaling. Such a proposal is based on the observation that signal peptide mutations in receptor activator of NFκB, which lead to an increase in NFκB signaling, cause the PDB-like condition familial expansile osteolysis (29). Establishing the precise in vivo function of the p62 protein in bone metabolism, and in particular the identification of ubiquitylated and non-ubiquitylated UBA domain-interacting proteins, should provide further insights into the pathogenesis of both familial and sporadic forms of PDB.

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