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Structural and Functional Deviations in Disease-Associated p97 Mutants

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Abstract

Missense mutations that occur at the interface between two functional domains in the AAA protein p97 lead to suboptimal performance in its enzymatic activity and impaired intracellular functions, causing human disorders such as inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD). Much progress has been made in characterizing these mutants at cellular, sub-cellular and molecular levels, gaining a substantial understanding of the involvement of p97 in various cellular pathways. At the tissue level, patient biopsies revealed co-localization of p97 with pathologic proteineous inclusions and rimmed vacuoles, which can be reproduced in various cellular and animal models of IBMPFD. At the subcellular level, alterations in p97's ability to bind various adaptor proteins have been demonstrated for some but not all binding partners. Biochemical and biophysical characterizations of pathogenic p97 revealed altered nucleotide binding properties in the D1-domains compared to the wild type. Structural studies showed that mutant p97 are capable of undergoing a uniform transition in the N-domain from a Down- to an Up-conformation in the presence of ATPγS, while in the wild-type p97, this conformational change can only be demonstrated in solutions but not in crystals. These structural and biochemical analyses of IBMPFD mutants shed new light into the mechanism of p97 function.

Keywords

p97; VCP; IBMPFD; AAA protein; structure and function

1. Linkage between IBMPFD (inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia) and mutations in p97

IBMPFD (OMIM 167320) is an autosomal dominant, progressive, debilitative and ultimately fatal disorder (Kimonis et al., 2000; Watts et al., 2004). It is a late-onset disease with initial symptoms typically appear in adulthood. The disease mainly affects three tissue types: the muscle (myopathy), the bone (Paget's disease of the bone) and the brain (frontotemporal dementia). Among them, myopathy is the most common clinical

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menifestation found in about 85% of IBMPFD patients. Paget's disease of the bone affects about half of the individuals; and frontotemporal dementia occurs in approximately one-third of all cases. Individuals diagonsed with IBMPFD have one or more of these clinical features. However, the clinical phenotypes of the disease do not always correlate with the sites of mutations. In other words, individuals from the same family having the same genetic mutation can exhibit different symptoms. The heterogeneity in clinical presentations makes diagnosis of the disease challenging, thus requiring molecular genetic testing for accurate diagnosis. Since the first description of the disease in 2000, this genetic disorder has been reported worldwide from more than 39 families, but the worldwide prevalence is unknown [for recent reviews, see (Nalbandian et al., 2011; Weihl, 2011)].

Mutations in IBMPFD patients were mapped to the region of chromosome 9p13.3-12 by linkage studies (Kovach et al., 2001). Among various protein candidates encoded in this region, only p97 or VCP (valocin-containing protein) was found affected by these mutations (Watts et al., 2004). Phenotypic characterizations of IBMPFD have been well documented at both the tissue and cellular levels. In patient muscle tissue samples, for instance, rimmed vacuoles were observed and stained positive for both ubiquitin and p97 (Watts et al., 2004). Neuronal nuclear inclusions were also stained positive for ubiquitin and p97 in the brain tissue of a patient harboring the R155C mutation (Schroder et al., 2005). Thus, the presence of ubiquitin and p97 positive inclusions becomes a hallmark feature in IBMPFD patient's tissues. Studies found the inclusions also staining positive for TAR DNA-binding Protein-43 (TDP-43) in patient samples (Weihl et al., 2008), suggesting a defective ubiquitin-proteasome system (UPS). More recently myoblast cells derived from patients showed accumulation of LC3-II, a marker for defective autophagosome maturation (Tresse et al., 2010).

Similar phenotypes can be reproduced in *in vitro* cultured cells transfected with disease-associated p97 mutants (Janiesch et al., 2007; Weihl et al., 2006). Moreover, studies using various animal models further strengthen the linkage between the mutations in p97 and IBMPFD. Transgenic mice bearing a p97 mutation (R155H or A232E) displays dominant-negative phenotypes similar to IBMPFD patients (Custer et al., 2010; Weihl et al., 2007); mutant p97 (R155H) knock-in mice display progressive muscle weakness and other IBMPFD-like symptoms (Badadani et al., 2010).

VCP or p97 is a Type II AAA+ (extended family of ATPases associated with various cellular activities) protein existing in solution as homo-hexamers (Ogura and Wilkinson, 2001). Each monomer consists of three domains: an N-terminal domain (N-domain) followed by two tandem ATPase domains, D1 and D2. Based on the full-length crystal structures of wild-type p97 (DeLaBarre and Brunger, 2003; Huyton et al., 2003), linker regions exist between the major domains. For example, the N-D1 linker is defined for residues in the region between E185 and K211, the D1-D2 linker joins residues L464 and E483, and a C-terminal tail comprises residues after L762 (Fig. 1A). Six monomers assemble into a barrel-like structure, in which N-D1 and D2-domains form two concentric, stacked rings. In the hexameric structures of wild-type p97, the D1-domains are in the ADP-bound state and the N-D1 ring is characteristically larger, compared to the D2 ring, due to the laterally attached N-domains (DeLaBarre and Brunger, 2003; Huyton et al., 2003).

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The functions of p97 have been shown relating to a plethora of cellular pathways including membrane fusion (Kondo et al., 1997; Totsukawa et al., 2011; Uchiyama et al., 2006), DNA repair (Meerang et al., 2011), UPS including endoplasmic reticulum associated degradation (ERAD) (Richly et al., 2005), autophagy (Ju et al., 2009; Krick et al., 2010; Tresse et al., 2010; Vesa et al., 2009), mitochondrial quality control (Tanaka et al., 2010) and endolysosomal sorting (Ritz et al., 2011). The molecular mechanisms that render p97 capable of engaging in these diverse cellular functions remain obscure, and are presumably mediated through binding of different pathway-specific adaptor proteins. Such multi-faceted functionality of p97 is consistent with the embryonic lethality of p97 or its homolog knockout mutants in various organisms (Frohlich et al., 1991; Lamb et al., 2001; Leon and McKearin, 1999; Muller et al., 2007).

So far, 20 missense mutations have been reported through clinical studies of IBMPFD patients (Table 1), involving amino acid changes at 13 different positions in the p97 sequence [reviewed in (Nalbandian et al., 2011)]. In all cases, only a single amino acid substitution was observed. These mutations are mostly found in the N-domain of p97 (I27, R93, R95, P137, R155, G157 & R159), two in the N-D1 linker (R191 and L198) and some in the D1-domain (A232, T262, N387 & A439), but none in the D2-domain (Fig. 1A & Table 1). Among them, the mutation at residue R155 has the highest occurrence (Hubbers et al., 2007; Watts et al., 2004). These IBMPFD mutations can involve changes in charged, polar or apolar residues and can vary from bulky to small; they are not part of the active sites for nucleotide binding or for adaptor protein binding. So far, no clear pattern of any physical or chemical property has emerged from the analysis of these mutations except that they are all located at the interface between the N- and D1-domain (N-D1 interface) in the tertiary structure of the wild-type p97 (Fig. 1B). This observation suggests an important role by residues at the N-D1 interface to the function of p97.

2. Wild-type p97 -- structural and functional properties

The IBMPFD has been characterized as a late-onset disease, suggesting that the effects of mutations in p97 accumulate over a long latency period in affected individuals and that the changes in p97 function must be rather subtle. Thus, it becomes imperative, if the molecular mechanisms underlying the cause of IBMPFD are to be understood, to establish both *in vivo* and *in vitro* assays at the cellular, biochemical, or structural levels that are sensitive to IBMPFD mutations. More importantly, we hope that some of the molecular differences between wild-type and mutant p97, which are detectable by these assays, can be used to correlate with clinical observations, leading to a more accurate prognosis for IBMPFD patients.

2.1. Protein substrate binding and processing activity

Several lines of evidence show that p97 plays an essential role in protein degradation pathways by interacting directly or indirectly with protein substrates. Inhibition of p97 induces ER stress leading to accumulation of various ERAD substrates (Jarosch et al., 2002; Ye et al., 2004). Pathological examinations of IBMPFD patient samples revealed co-localization of p97 with ubiquitinated protein inclusions (Kimonis et al., 2000; Kimonis et al., 2008; Schroder et al., 2005; Watts et al., 2004). *In vitro* experiments showed that p97 is

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able to interact directly with polyubiquitin and polyubiquitinated proteins in the absence of adaptor proteins (Dai and Li, 2001). Therefore, the role of p97 in these pathways has been proposed to unfold, segregate or translocate protein substrates (Braun et al., 2002; Rape et al., 2001; Shcherbik and Haines, 2007), even though a direct demonstration of such activities in an *in vitro* reconstituted system is not available. Intriguingly, introducing two aromatic residues lining the wall of the central channel, together with the removal of the N-domain, rendered p97 a protein unfoldase, effectively unfolding YFP-ssrA substrate (Rothballer et al., 2007).

2.2. Interaction with adaptor proteins

Like other AAA proteins, p97 can interact with a large number of adaptor proteins, consequently being directed to various cellular locations for function. So far, more than twenty adaptor proteins have been identified and the number is still growing [for review see (Buchberger et al., 2001; Madsen et al., 2009)]. The majority of these adaptor proteins such as p47 and FAF1 possess a UBX (ubiquitin regulatory X) domain that binds to the N-domain of p97 (Dreveny et al., 2004; Schuberth and Buchberger, 2008). The UBX domain is an 80-residue module with a β-Grasp fold that highly resembles the structure of ubiquitin (Buchberger et al., 2001). Details of the interaction with UBX were revealed by the crystal structures of isolated p97 fragments in complex with the UBX domain of p47 or FAF1 (Dreveny et al., 2004; Hanzelmann et al., 2011; Kim et al., 2011). In these structures, the UBX domain binds to the cleft between the two subdomains of the p97 N-domain (Fig. 2). Interestingly, although all six sites within a p97 hexamer are available for binding of UBX domains, only three molecules of p47 UBX domains were found in the complex structure (Dreveny et al., 2004). Thus, it should be cautioned that the binding of an isolated UBX domain to p97 may not be physiological, as it was demonstrated by isothermal titration calorimetry (ITC) that interaction between a hexameric p97 N-D1 fragment and the full-length FAF1 is dependent on the presence of the adaptor protein Ufd1-Npl4 (Hanzelmann et al., 2011).

VCP-interacting motif (VIM) represents another group of adaptor proteins that interact with p97. VIM has a linear sequence motif (RX_5AAX_2R) that interacts specifically with the N-domain of p97 (Stapf et al., 2011) and is found in a number of proteins including ubiquitin ligase gp78 (Ballar et al., 2006; Fang et al., 2001), SVIP (small VCP-inhibiting protein) (Ballar et al., 2007) and VIMP (VCP interacting membrane protein) (Ye et al., 2004). Recently, the crystal structure of an isolated N-domain of p97 in complex with the VIM of gp78 was reported, revealing this interaction at atomic detail (Hanzelmann and Schindelin, 2011) (Fig. 2). Unlike the UBX domain that uses a surface loop to interact with the N-domain of p97, VIM forms a α-helix for interaction. However, both UBX and VIM binding sites on p97 overlap at the hydrophobic cleft between the two subdomains of the N-domain (Fig. 2) (Hanzelmann and Schindelin, 2011), which is consistent with the observed competitive binding among different p97 adaptor proteins such as p47 (Bruderer et al., 2004), Ufd1-Npl4 (Meyer et al., 2000), Ubxd1 (Kern et al., 2009), SVIP, FAF1 and gp78 (Hanzelmann and Schindelin, 2011). More importantly, this suggests a regulatory mechanism for p97 function by hierarchical adaptor protein binding (Hanzelmann et al., 2011).

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Another group of p97 adaptor proteins, which interacts with the last 10 residues of p97 at the C-terminus, is the PUB (PNGase/UBA or UBX, or PUG) domain (Allen et al., 2006; Madsen et al., 2008; Zhao et al., 2007) (Fig. 2). Representative of PUB-containing proteins are PNGase (Zhao et al., 2007) and Ubxd1 (Allen et al., 2006; Madsen et al., 2008). The PUB domain is a bundle of four anti-parallel helices (Zhao et al., 2007). The C-terminal peptide of p97 binds to a positively charged groove of the PUB domain, as revealed by the crystal structure. Biophysical studies showed that one hexamer of p97 can only interact with two full-length PNGase monomers with an apparent binding affinity of ~16 μM (Zhao et al., 2007).

2.3. In vitro biochemical characterizations of p97

Isolated p97 displays different binding affinities towards various nucleotides or their analogs for D1- and D2-domain. Most binding measurements for nucleotides were carried out with ITC experiments, giving rise to apparent values of binding affinity and stoichiometry. The D1-domain has a considerably higher affinity (K_d of ~1 μM) for ADP than the D2-domain (K_d of ~80 μM). The affinity for ATP or ATPγS is about the same of ~2 μM for both domains (Briggs et al., 2008). These measurements are consistent with the apparent roles of respective domains. A remarkable observation, though not fully appreciated, is the existence of pre-bound ADP at the D1-domains. Using chemical denaturation experiments, Davies et al first reported that about half of the D1 sites in wild-type p97 hexamers are pre-occupied by ADP (Davies et al., 2005) and it was shown that the pre-bound ADP molecules are difficult to remove in wild-type p97 (Briggs et al., 2008; Tang et al., 2010). The functional significance of the prebound ADP remains to be demonstrated but was speculated to play a role in regulating the movement of the N-domain by the D1-domain (Tang et al., 2010). A practical ramification of this observation is that the presence of pre-bound ADP at the D1-domain deserves a special consideration when interpreting results from various experiments involving ATP binding or hydrolysis.

The ATP hydrolyzing ability of p97 is indispensible for its function (Dalal et al., 2004; DeLaBarre et al., 2006; Kobayashi et al., 2002; Xu et al., 2011; Ye et al., 2003). The two AAA ATPase domains of p97, D1 and D2, are not equivalent, as the D2-domain displays higher ATPase activity than the D1-domain (Song et al., 2003). However, the relationship reported in the literature between the two ATPase rings is not all consistent. By measuring activities of each ring while inhibiting the other, some reported the two ATPase rings operating independently (Song et al., 2003), whereas others showed evidence of inter-dependence (Nishikori et al., 2011; Ye et al., 2003). The ATPase activity of p97 is also influenced by many factors. For example, the overall ATPase activity of p97 can be stimulated by heat or by substrate synaptotagmin (DeLaBarre et al., 2006; Song et al., 2003). Binding of the adaptor protein p47 to the N-domain of p97 has an inhibitory effect on the ATPase activity (Meyer et al., 1998) and restricting the motion of N-domain by cross-linking also inhibits the ATPase activity (Niwa et al., 2012). These observations seem to suggest a communication pathway in p97. More intricate allosteric communication among domains of p97 has also been proposed such as positive (DeLaBarre et al., 2006; Nishikori et al., 2011) and negative cooperativity (Beuron et al., 2003) among domains. These

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interactions are believed to play a role in coordinating movements between domains during the ATP hydrolysis cycle of p97.

2.4. Conformational variability in p97

Unlike many bacterial AAA proteins, assembly of p97 hexamers does not depend on the presence of nucleotide, although it was reported that the binding of ATP accelerates hexamer formation (Wang et al., 2003a). Both the full-length p97 and its N-D1 fragment were purified as stable hexamers; their EM (electron microscopy) and crystal structures are available. Biochemical and structural characterizations showed convincingly that the D1-domains play a major role in oligomerization, whereas D2-domains are more active in hydrolyzing ATP (Song et al., 2003).

Global conformational changes induced by binding of nucleotides in wild-type p97 have been probed for various domains by structural approaches like EM, small-angle X-ray scattering (SAXS) and X-ray crystallography. Specifically, movement in the N-domain for the wild-type p97 has been reported in response to the presence of various nucleotides in solution by EM and SAXS, but not by X-ray crystallography, and the results have not always agreed (Davies et al., 2005; Rouiller et al., 2002). Crystallographic studies of wild-type p97 in the presence of various nucleotides found that the D1-domains are always occupied by ADP, regardless of the type of nucleotide bound in the D2-domains (DeLaBarre and Brunger, 2003; Huyton et al., 2003), whereas the occupants of D2-domains vary depending on the type of nucleotide present in solution. As a result, the conformation of the wild-type N-D1 ring rarely changes in crystals, while the D2 ring undergoes nucleotide-dependent conformational alterations.

EM studies of the complexes between hexameric p97 and adaptor proteins Ufd1-Npl4 and p47 revealed large conformational changes in the N-domain (Bebeacua et al., 2012; Beuron et al., 2006). However, crystallographic studies did not reveal significant conformational changes in p97 when interacting with the UBX domain of p47 (Dreveny et al., 2004). These seemingly conflicting results suggest the possibility of difference in sensitivity among various structural techniques in probing conformational changes in p97. In other words, if a wild-type p97 hexamer acquires some internal asymmetry while undergoing a conformational change in solution, this change is more like to be detected by solution methods such as EM and SAXS than by the X-ray crystallography, which requires crystals to be formed by proteins in a uniform conformation.

3. IBMPFD mutations in p97 – structural and functional alterations

IBMPFD mutations occur at the interface between the N- and D1-domains of p97, which is not part of the nucleotide-binding pocket and remote from any known adaptor protein-binding interfaces. Evidence from different laboratories showed without exception that IBMPFD p97 mutants readily form hexamers in solution just like the wild type (Halawani et al., 2009; Tang et al., 2010; Weihl et al., 2006). Crystallographic study at high resolution further demonstrated that mutations (R155H) induced little structural change in monomeric or hexameric forms of p97 or in the nucleotide-binding pocket when ADP is bound to the D1-domain, as compared to the wild-type protein (Tang et al., 2010). Moreover, IBMPFD

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mutations do not abolish the ATPase activity of p97 (Fernandez-Saiz and Buchberger, 2010; Hubbers et al., 2007; Niwa et al., 2012; Weihl et al., 2006). *In vitro* binding of adaptor proteins p47 or Ufd1-Npl4 to IBMPFD mutants (R95G or R155H) by pull-down assay is comparable to that of the wild type (Fernandez-Saiz and Buchberger, 2010). All these observations indicate that changes, if any, in its structure and function conferred by IBMPFD mutations must be very subtle, which is consistent with the disease being "late onset". Nonetheless, efforts to detect these subtle changes have been made and changes due to mutations have been detected at the protein level by various methods.

3.1. Alteration in cellular functions

IBMPFD mutations have been associated with defects in several intracellular protein degradation pathways including the UPS, autophagy (Tresse et al., 2010), and endosome-lysosome fusion (Ritz et al., 2011). Intracellular protein inclusions that are stained positive for ubiquitin are most often observed in affected tissues (Forman et al., 2006; Hubbers et al., 2007; Schroder et al., 2005). Ubiquitin is a signaling molecule that directs protein substrates to a variety of cellular pathways including protein degradation. Misfolded or unwanted proteins are labeled with polyubiquitin chains for various degradation pathways (Clague and Urbe, 2010). The accumulation of ubiquitinated proteins in cells is therefore indicative of defects in these pathways and can be used, in this case, as a diagnostic indicator of the impaired function of p97 mutants. Indeed, failure to degrade ΔF508-CFTR (Weihl et al., 2006) and Unc-45B (Janiesch et al., 2007), specific substrates for UPS, in cells transfected with IBMPFD mutants (R95G, R155H) validates the utility of the cell-based method.

In addition to ubiquitin, TDP-43 is also found to be co-localized with protein inclusions present in IBMPFD affected tissues or cells (Forman et al., 2006; Hubbers et al., 2007; Ju et al., 2009; Neumann et al., 2007; Ritson et al., 2010; Schroder et al., 2005; Weihl et al., 2008). TDP-43 itself is believed to be a substrate for either proteasome or autophagic degradation (Caccamo et al., 2009; Wang et al., 2010), which provided the first hint pointing to a role of p97 in autophagy, a catabolic degradation process involving the lysosomal machinery. The role of p97 in autophagy has been demonstrated in both mammalian cells and yeast (Ju et al., 2009; Krick et al., 2010; Tresse et al., 2010; Vesa et al., 2009). The protein was found to be essential for autophagosome maturation (Tresse et al., 2010); introduction of IBMPFD mutations (R155H/S) to p97 resulted in accumulation of autophagosome markers p62 and LC3-ll (Ju et al., 2009; Tresse et al., 2010; Vesa et al., 2009). This essential role of p97 has recently been demonstrated also in *Saccharomyces cerevisiae* cdc48, the yeast homolog of p97, which forms a complex with Shp1 needed for autophagosome biogenesis (Krick et al., 2010).

3.2. Alterations in adaptor protein binding

The ability to interact with different adaptor proteins is critical for p97 to perform different cellular functions. Since none of the identified IBMPFD mutations is found at any known adaptor protein-binding interface, the effects of these mutations on protein-protein interaction, if any, should be indirect or subtle. Indeed, inconsistent results have been reported on the observed differences in binding ability between wild-type and mutant p97 to various adaptor proteins, reflecting the complex nature of these interactions. For example, in

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cultured cells transfected with mutant p97 (R95G, R155H/C/P, R191Q, or A232E), an elevated amount of Ufd1-Npl4 was detected by immunoprecipitation associating with mutant p97 compared to wild type (Fernandez-Saiz and Buchberger, 2010; Manno et al., 2010a). Using purified proteins, however, no difference was found in the binding of Ufd1-Npl4 to wild-type or mutant p97 (R93C, R95G, R155H/C) by pull-down assay (Fernandez-Saiz and Buchberger, 2010; Hubbers et al., 2007). Since the conformation of the N-domain is dependent on the nucleotide state at the D1-domain (Tang et al., 2010), it is conceivable that some of the difficulties in detecting changes in adaptor protein binding may be due to uncertainty concerning nucleotide states. For example, structural studies revealed SVIP binding to R155H mutant in the presence of ATPγS is reduced by a factor of 7 (Hanzelmann and Schindelin, 2011). Thus, cautions must be exercised in interpreting results from cofactor binding experiments.

In spite of the challenges in detecting subtle changes, a number of adaptor proteins display altered interactions with mutant p97. Wild-type p97, but not the R155H mutant, forms a ternary complex with ubiquitylated CAV-1 (plasma membrane protein caveolin-1) and Ubxd1 (Ritz et al., 2011). CAV-1 is one of the main constituents of caveolae, small invaginations on the plasma membrane (Parton and Simons, 2007). Degradation of CAV-1 requires mono-ubiquitin modification, which is a signal for endosomal sorting (Haglund et al., 2003). The failure of R155H mutant to form a complex with CAV1-Ubxd1 therefore suggests an altered function of the p97 mutant, affecting the endocytic pathway in cells. Mutant p97 (R95G, R155H) consistently showed reduced binding to the ubiquitin ligase E4B, whereas binding of ataxin 3 is enhanced (Fernandez-Saiz and Buchberger, 2010). Furthermore, competitive binding showed that the presence of high concentration of Ufd1-Npl4 reduced the binding of E4B to IBMPFD mutants (R95G, R155H) but not to wild-type p97 (Fernandez-Saiz and Buchberger, 2010). However, similar pair-wise competition did not show difference between the binding of PNGase and Ufd1-Npl4 (Fernandez-Saiz and Buchberger, 2010; Ritz et al., 2011).

3.3. Alterations in nucleotide binding and the amount of pre-bound nucleotide

Although IBMPFD mutations are mapped to N- and D1-domain of p97, they are not located in the nucleotide binding pocket and do not abolish the ATPase activity of the enzyme. However, these mutations do have a considerable effect on the nucleotide-binding properties, especially on the D1 sites, as detected by titrating the protein with various nucleotides using ITC. As aforementioned, the wild-type p97 has pre-bound ADP in the D1-domain. N-D1 fragments of mutant p97 (R95G, R155H) also showed the presence of pre-bound ADP, but at a much-reduced level, as determined by ITC (Tang et al., 2010). Moreover, these mutants have a 2- to 5-fold reduction in ADP binding affinity (K_d ranging from 1.85 to 4.25 μM) at the D1-domain compared to the wild type, consequently rendering better accessibility to other nucleotides (Tang et al., 2010).

More interestingly, titration of ATPγS into the D1-domain of these mutants (R95G, R155H) consistently yields a biphasic titration exothermal profile from ITC experiments (Fig. 3), which is in contrast to the monophasic profile obtained from titrating wild-type p97 and can be considered as yet another hallmark property of IBMPFD mutants (Tang et al., 2010). The

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biphasic exothermal profile suggests the existence of two types of binding sites for ATPγS in the IBMPFD mutants. Since the D1 sites in IBMPFD mutants have pre-bound ADP and a lowered ADP binding affinity, it was speculated that in mutants, ATPγS can titrate into the D1 sites with pre-bound ADP, whereas in the wild type, it cannot (Fig. 3). In another word, when ATPγS molecules are titrated into IBMPFD mutants, they first take up the empty D1 sites, giving rise to the first phase of the heat profile. As the concentration of ATPγS continues to rise, they displace the pre-bound ADP, resulting in the second phase of the profile. Consequently, at a sufficiently high concentration of ATPγS, all pre-bound ADP in a mutant p97 can be displaced, leading to a uniform nucleotide binding state within the hexamers. Indeed, this speculation is supported by the successful crystallization of the IBMPFD mutants with all D1 sites bound with ATPγS (Tang et al., 2010). By contrast, ATPγS is only able to enter the empty D1 sites of wild-type p97 but unable to dislodge the pre-bound ADP, yielding a monophasic titration profile and a mixed nucleotide binding state at the D1-domains within a hexamer. The presence of a mixed nucleotide binding state at D1 sites in the wild-type p97 is consistent with unsuccessful attempts to crystallize wild-type p97 in the presence of ATP or its homologs at a concentration where mutant p97 form crystals.

There is a disagreement in the literature about the effects of IBMPFD mutations on ATPase activity of p97. Some have reported no significant effect (Fernandez-Saiz and Buchberger, 2010; Hubbers et al., 2007; Weihl et al., 2006), while others have shown higher ATPase activity for some mutants (R93C, R95G, R155C/P/H, R159H, R191Q, L198W, A232E, N387H) (Halawani et al., 2009; Manno et al., 2010b; Niwa et al., 2012). Since IBMPFD mutations alter the binding affinity of ADP at the D1-domain and the majority of ATPase activity comes from the distal D2-domain, it is conceivable that the influence of mutations on activity could also be rather subtle. Given the fact that the ATPase activities of D1 and D2 are not independent of each other (Nishikori et al., 2011; Ye et al., 2003), one would expect the mutations to certainly have an affect on the overall ATPase activity with a magnitude depending upon the site of mutation.

3.4. Conformational changes in mutants

The crystal structure of the IBMPFD mutant R155H with ADP bound at the D1-domain is very similar to the structure of wild-type p97 (Tang et al., 2010). In fact the mutant structure was determined by the molecular replacement method using the wild-type p97 coordinates as a phasing template. Hints of structural differences between the wild-type and IBMPFD mutant p97 were obtained by limited trypsin digestion experiments upon the addition of different nucleotides (Fernandez-Saiz and Buchberger, 2010). In the presence of ATP, not ADP, the N-terminus (residues 1-18) of IBMPFD mutants is significantly less prone to trypsin digestion compared to the wild-type protein. This enhanced protection against trypsin digestion of the N-terminal peptide of IBMPFD mutants in the presence of ATP suggested a structural alternation in the mutant. This observation can well be explained by the crystallographic data on IBMPFD mutants (R95G, R155H) bound with ATPγS at the D1-domain (Tang et al., 2010), but not by the structures of wild-type p97 in the presence of ADP, as in the wild-type structure, the first 24 residues from N-terminus are crystallographically disordered.

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As revealed by their crystal structures with ATPγS bound at the D1-domain, the IBMPFD mutants (R155H, R95G) show a novel N-domain conformation dramatically different from that with ADP bound (Tang et al., 2010). Upon binding of ATPγS to the D1-domain, the N-domains of a p97 hexamer undergo a large rotational and translational movement, moving out of the plane of the D1 ring, which was termed the Up-conformation (Fig. 1C). This is in comparison to the positions of N-domains in plane with the D1-ring in the presence of ADP, the Down-conformation. During this conformational transition, some residues such as Pro178 travel a distance as much as 38 Å. This N-domain movement is accompanied by a transition of the secondary structure of the N-D1 linker from a random coil in the Down-conformation to a two-turn α-helix in the Up-conformation, which is reminiscent of a contracted spring. Furthermore, the presence of ATPγS at the D1-domain stabilizes the conformation of the N-terminal fragment (residues 12-20), rendering it visible in crystallographic electron density maps (Tang et al., 2010) and providing an explanation for the reduced susceptibility of this fragment to trypsin digestion in mutants in the presence of ATP (Fernandez-Saiz and Buchberger, 2010).

The ability to switch between the Up- and Down-conformation is not a unique property to IBMPFD mutants. Similar nucleotide-dependent conformational change was demonstrated for subunits of wild type p97 using SAXS experiments (Tang et al., 2010). Presumably, functional alternations in p97 by IBMPFD mutations are due to the altered regulation in the nucleotide binding state of D1-domain, which controls the N-domain conformation (Tang et al., 2010). Based on the results from structural and biophysical studies of wild-type and IBMPFD mutant p97, a model is proposed to illustrate the subtle change in the regulatory mechanism of the D1-domain caused by IBMPFD mutations and its effect on the N-domain conformation (Fig. 4). In this model, there are four states each representing four different nucleotide-binding states in the D1-domain of a p97 protomer. First, there is an Empty state where no nucleotide is bound at the D1 site; the conformation of the N-domain is unknown (blue sphere). When ATP occupies the D1 site (ATP state), the N-domain adopts an Up-conformation (pink sphere), which has been determined from the crystal structure of IBMPFD mutants (Tang et al., 2010). The hydrolysis of ATP to ADP at the D1-domain will then bring the N-domain to the Down-conformation (green sphere), which is supported by the crystallographic data from both wild-type p97 and its IBMPFD mutants (DeLaBarre and Brunger, 2003; Huyton et al., 2003; Tang et al., 2010; Zhang et al., 2000). Importantly, there are two ADP-bound states existing in equilibrium for a protomer: the ADP-locked and ADP-open states. Both ADP-open and ADP-locked states coexist for different protomers in a p97 hexamer. The ADP-locked state is inspired by the presence of pre-bound ADP at the D1 site in the wild-type p97, which is difficult to remove (Briggs et al., 2008; Davies et al., 2005; Tang et al., 2010). The ADP-open state represents the situation where ADP has a reduced affinity to the D1 site ready to be exchanged.

The major difference between the wild-type and mutant p97 is the regulation or the equilibration between the ADP-open and ADP-locked state. In the wild type, the equilibration favors the ADP-locked state, whereas in the mutant, it prefers the ADP-open state. As supported by the crystal structure of IBMPFD mutant with ATPγS bound at the D1-domain, and inferred from the ITC data (Tang et al., 2010), ATP can only exchange with ADP in the ADP-open state of a D1-domain. This means, in the case of a wild-type p97

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hexamer, that ATP can only get into a subset of D1-domains, driving corresponding N-domains to the Up-conformation. This argument is supported by the SAXS experiment showing that a fraction of subunits in the wild-type p97 hexmer can also undergo nucleotide-driven conformational change in solution (Tang et al., 2010). It is believed that the non-uniform nucleotide binding state in the wild-type p97 in the presence of ATP generates an asymmetry in the N-domain conformation in a p97 hexamer, which could explain the failure in crystallizing the wild-type protein in the presence of ATP or its homologs. The regulation or equilibration between ADP-locked and ADP-open states is shifted toward the latter with the introduction of IBMPFD mutations. Consequently, a uniform nucleotide binding state at the D1-domains and a synchronized N-domain movement can be reached in the presence of a sufficiently high concentration of ATP, forming symmetrical hexamers. More importantly, this implies that the function of p97 requires an asymmetry in the D1 nucleotide binding state in a hexameric ring and interference with this mechanism leads to a defective p97 similar to that found in IBMPFD mutants.

In the proposed model, the nucleotide binding state at the D2-domain was not taken into account simply because there is a lack of data in the literature to support a comprehensive hypothesis on the interaction between D1 and D2. Crystal structures of full-length p97, regardless of the type of nucleotide bound in the D2-domain, reveals high structural similarity in the N-D1 domains, suggesting that the influence of the nucleotide binding state in D2 to that in D1 may be limited (DeLaBarre and Brunger, 2005). Furthermore, biochemical studies have shown that D1-domain is capable of hydrolyzing ATP in the presence of D2-domain (Briggs et al., 2008; Song et al., 2003; Wang et al., 2003b) and the full-length p97 possesses more pronounced pre-bound ADP at the D1-domains (Davies et al., 2005), supporting the idea that a similar nucleotide-dependent N-domain conformational change should takes place in the full-length p97 as well.

4. Concluding remarks

Our effort to understand the underlying molecular mechanism of IBMPFD has clearly been impeded by the multifaceted activities of p97 in various cellular pathways, by the lack of structural knowledge on interactions among subunits and communication between the ATPase domains of p97, and by the shortage of established, reliable and reproducible assays that can be reconstituted *in vitro*. Nevertheless, the association of mutations in the p97 gene with IBMPFD offers a rare opportunity to shed new light on the mechanisms of p97 functions, its roles in associated cellular pathways, and the pathogenesis of its mutations. Already, much progress has been made. A particularly interesting revelation is the mapping of all IBMPFD mutations identified to date to the interface between the N- and D1-domains of p97, suggesting that other mutations found in this region may also be able to cause the disease. Indeed, introducing the mutation R86A to the N-D1 interface of wild-type p97, which is not an IBMPFD mutation, renders the protein IBMPFD-like in biochemical and structural characterizations (Tang et al., 2010).

Studies of IBMPFD have benefited tremendously from cell-based experiments, revealing defects in various cellular pathways (Ritz et al., 2011; Tresse et al., 2010). Understanding at

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molecular level the role of p97 in IBMPFD has been facilitated by biochemical and structural approaches (Fernandez-Saiz and Buchberger, 2010; Niwa et al., 2012; Tang et al., 2010). *In vitro* studies using recombinant, homo-hexameric p97 mutants showed that IBMPFD mutant p97 had altered binding for the adaptor protein SVIP in the presence of ATPγS (Hanzelmann and Schindelin, 2011). ITC measurements have demonstrated the reduced binding of mutants for ADP at the D1-domain and revealed a biphasic exothermal profile when titrated with ATPγS (Tang et al., 2010). Structural studies have shown that in mutants, the N-domains of p97 are capable of undergoing a uniform Up- and Down-conformational change, whereas in wild type, such N-domain conformational change only occurs for a subset of subunits, leading to a conformational asymmetry (Tang et al., 2010). Last but not the least, one should be cautious when attempting to correlate *in vitro* studies with clinical data because not all subunits of a patient's p97 are likely to be mutated. They are more likely to be a mixture of wild-type and mutant subunits, which conceivably would make subtle effects of mutations even more difficult to detect.

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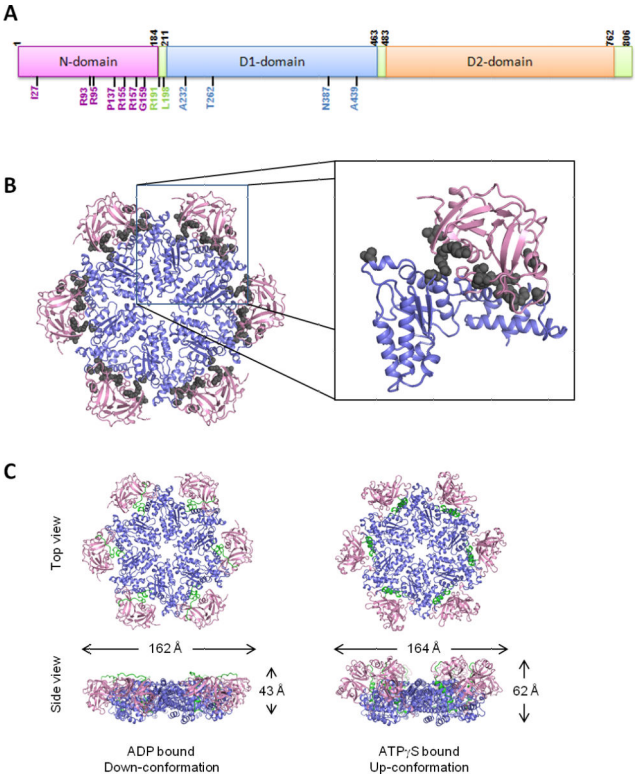

Figure 1. The structure of p97

(A) Domain organization of a full-length p97 subunit with locations of IBMPFD mutations indicated. (B) Ribbon diagram of p97 N-D1 structure in the Down-conformation. IBMPFD mutations are represented as grey spheres. Inset shows a close-up view of a p97 N-D1 monomer. (C) Two conformations of the p97 N-D1 fragment are presented: the Down-conformation with ADP bound on the left and the Up-conformation with ATPγS bound on the right. The ATPγS bound Up-conformation can only be obtained with IBMPFD mutants. The domains are colored based on the color scheme in (A).

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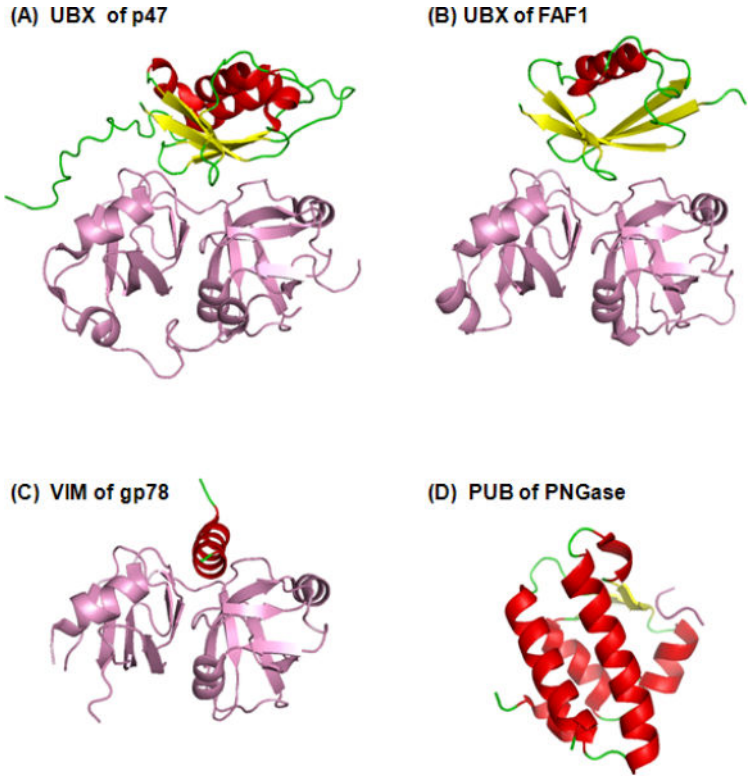

Figure 2. Structures of p97 in complex with different adaptor proteins

isolated p97 N-domain with (A) UBX domain of p47 (PDB: 1S3S), (B) UBX domain of FAF1 (PDB:3QQ8), and (C) VIM motif of gp78 (PDB: 3TIW). (D) The C-terminal peptide of p97 is shown bound to the PUB domain of PNGase (PDB:2HPL). All p97 N-domains are in the same orientation. Models of p97 are in magenta and those for adaptor proteins are shown with helices in red, β-strands in yellow and loops in green.

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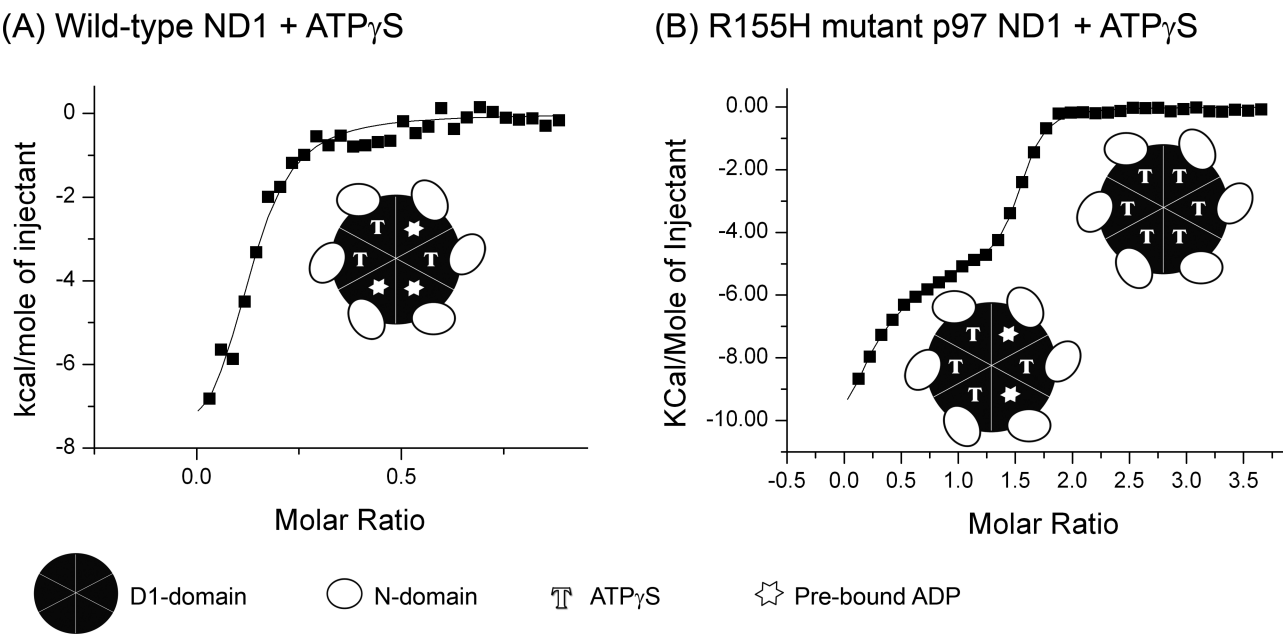

Figure 3. Exothermal titration profiles of the truncated N-D1 wild-type p97 and IBMPFD mutant R155H titrated with ATPγS

The data from the wild-type p97 was fitted by an one-site model and that from the mutant was fitted by a two-site model in Origin® 7 software. The occupants of the D1 sites during different stages of the titration by ATPγS are illustrated by the cartoon inserted underneath the curve. The positions of the N-domains are not considered here.

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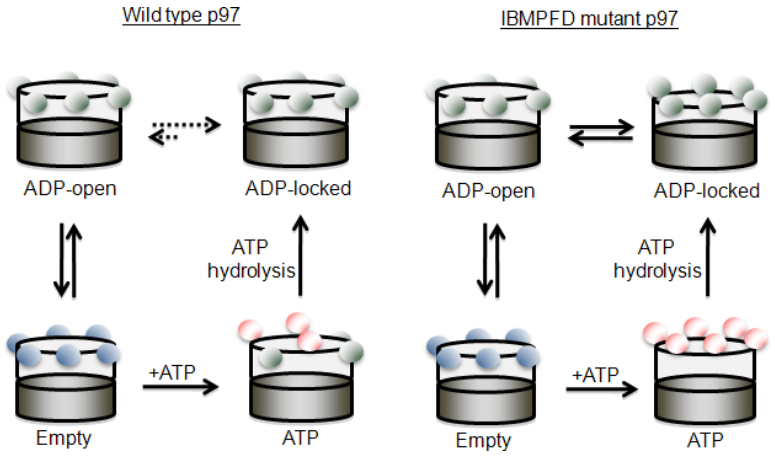

Figure 4. Model proposed for the control of N-domain movement in p97

N-domains are shown as spheres; D1- and D2-domain are represented in light and dark grey cylinders, respectively. There are four nucleotide-binding states in the D1-domain as indicated. In both the ADP-open and ADP-locked states the D1 sites are occupied by ADP and the N-domain is in the Down-conformation. However, the bound ADP can only be displaced by ATP in the ADP-open state. In the case of wild-type p97, there is a tight regulation between the ADP-open and ADP-locked states with a significant portion of D1-domains in the ADP-locked state. The empty state is an intermediate state where no nucleotide is bound to the D1-domain and the conformation of the N-domain is unknown. In the ATP state, ATP binding to the D1-domain triggers the N-domain to adopt the Up-conformation. In IBMPFD mutants, the control over the D1-domain favors the ADP-open state, allowing binding of ATP to all D1 sites and leading to p97 hexamers in a uniform Up-conformation. By contrast, in wild-type p97, only a few protomers are allowed to bind ATP, forming a conformationally asymmetric hexamer. In this simple model, the nucleotide state in D2-domain is not considered.

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Table 1

The 20 missense IBMPFD mutations in p97

N-domain		N-D1 linker region		D1-domain	
Amino acid	**Gene**	**Amino acid**	**Gene**	**Amino acid**	**Gene**
I27V	79 A → G	R191Q	572 G → A	A232E	695 C → A
R93C	277 C → T	L198W	593 T → G	T262A	784 A → G
R95C	283 C → T			N387H	1159 A → C
R95G	283 C → G			A439S	1315 G → T
P137L	410 C → T				
R155C	463 C → T				
R155H	464 G → A				
R155P	464 G → C				
R155S	463 C → A				
R155L	464 G → T				
G157R	469 G → C				
G157R	469 G → A				
R159C	475 C → T				
R159H	476 G → A				