Ubiquitin binding domains – from structure to application

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Abstract: Ubiquitin is one of the most important signal molecules in biological processes and ubiquitination plays essential roles in many signal transduction pathways. In most cases, they function as a post-transcription modification on target proteins as a monomer or a chain, which could recruit other proteins with ubiquitin binding domains (UBDs) to enable signal transduction. The UBDs are variant on structure and recognition site on ubiquitin. For the variant function the UBDs gives, UBDs are good tools pool for material design such as ubiquitin pathway inhibitor, ubiquitin enrichment material and manufactural specific ubiquitin chain antibody. In this review, we summarize some recent work on UBDs characterization and application.

1. UBIQUITIN AND UBIQUITINATION
Ubiquitin consists of 76-amino-acid residues, which is highly conserved from yeast to man. Reversible PTM on proteins play important role in signal sensing and intracellular signal transduction. The ubiquitination of target proteins is a widespread and conserved regulatory post-translational modification in eukaryotes during protein degradation, signal transduction and DNA repair pathways. Conjugation of ubiquitin to a target protein as a PTM or to itself resulting in polyubiquitin chains is regulated by activity of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes. The addition of single ubiquitin or ubiquitin chains can alter protein activity and its localization such as target to 26 S proteasome. So far, many cellular progresses are known to involve ubiquitination, including DNA repair, protein degradation, endocytosis, vesicular trafficking and gene silencing. In these processes, ubiquitin acts as a reversible signaling tag that can trigger biological events in cells by recruiting ubiquitin-binding domains (UBDs) in cellular proteins.

So far, most UBDs characterized well interact with ubiquitin through three surfaces on ubiquitin including hydrophobic patch including and surrounding isoleucine 44 (Ile44), acidic hydrophilic Asp58-centred face (Asp58) and flexible glycine tail (Gly76) as figure 1 shows. Helical UBDs share a common attraction to the same binding surface on the ubiquitin moiety, formed by the hydrophobic patch including and surrounding isoleucine 44 (Ile44). In contrast, some of ubiquitin binding zinc fingers (ZNFs), such as the A20-ZnF and the ZnF-UBP, prefer binding to acidic hydrophilic Asp58-centred face. In this review, we look into molecular basis of ubiquitin recognition by UBDs mainly through the structure properties of different UBDs and comprehend how different types of UBDs recognize corresponding ubiquitin or polyubiquitin chains as well as their roles in cellular signaling transduction.
2. UBDs interact with ubiquitin hydrophobic patch including Ile44

The interaction of UBDs and ubiquitin hydrophobic patch is based on hydrophobic interaction. The hydrophobic α-helical regions of UBDs enable their interaction with ubiquitin hydrophobic face. The UBDs fold into α-helical based structures constituting the largest class of ubiquitin-binding domains including UBA \(^{10}\) (ubiquitin associated), UIM (ubiquitin-interacting motif), MIU (motifs interacting with ubiquitin), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum). All of them above known to interact with the single hydrophobic region on ubiquitin including Ile44. Both UBA and CUE domains contain a common three-helical bundle architecture thus they bind to the Ile44 patch in similar pattern. The UIM and MIU domain is also helical and interact with hydrophobic patch.

2.1 UBA

The UBA domain was the first reported and well-characterized ubiquitin-binding domain from Rad23 and R23A, Dsk2\(^{11}\) and NBR1 which are key protein in proteasome targeting, kinase regulation and autophagy process\(^{12}\). UBA domains were discovered as a homology region in many proteins involved in ubiquitination cascades\(^{13}\). UBA domains consist of three compact helix bundles. Two of the helical, α1 and α3 helices, form a conserved hydrophobic patch which is able to bind hydrophobic Ile44 patch on mono-ubiquitin\(^{14}\) as figure 2 shows. UBA domains are able to bind mono-ubiquitin in vitro with affinity in mM magnitude. What’s more, they have been found to play a role in a variety of other protein–protein interactions. Through some orientation variation of the UBA domain relative to ubiquitin exist in some reports, but the identity of the binding sites on both allows of no dispute.
Besides, the UBA domains is essential in polyubiquitin recognition because its ability to interact with two ubiquitin hydrophobic Ile44 faces simultaneously through two opposite hydrophobic faces formed by three bundle helix. The UBA domains of hHR23A is a good example to illustrate how two surface patches on the UBD allow it to be sandwiched into the hydrophobic binding cleft formed by Lys48-diubiquitin using the Ile44 faces of both proximal and distal ubiquitin moieties. NMR studies of the hHR23A UBA domain bound to Lys48-linked di-ubiquitin in the centre of a ‘sandwich’ between the two mono-ubiquitin moieties. More interesting, some UBA domains show preference to the ubiquitin chain in specific linkage form. Taking hHR23A, A human Rad23 orthologue, for example, hHR23A effectively bind to chains containing up to eight ubiquitin molecules with its C-terminal UBA domain sandwiching between the ubiquitin moieties of Lys48-linked diubiquitin, contacting closely with the linker peptide bonds connecting two moieties. These facts give reason to the ability of R23A to inhibit substrate deubiquitylation thus influence binding and activation of Uch37 by Rpn13. They probably make Rad23 proteins better shuttling factors for passing substrates to the proteasome because substrates are more likely to remain ubiquitylated during transport. Cecile Pickart group pioneered studies on interaction between 30 different UBA domains and polyubiquitin chains in different types of linkage and concludes that the UBA domains can be sorted into four major specificity classes. The class 1 selectively binds to Lys48-linked polyubiquitin including two known members, hHR23A UBA and Mud1 UBA while class 2 prefers Lys63-linked polyubiquitin. In contrast, class 3 and class 4 show no selectivity on polyubiquitin linkages. However, with the fact that this classification does not show patterns of sequence conservation and most of the discrimination mechanistic basis remains to be elicited, this result must be considered provisional.

![Figure 2. Structure of UBA-mono ubiquitin complex](image)

### 2.2 UIM
The UIM is found in S5a, Vps27, STAM, epsins and RAP80, most of which are trafficking progress related proteins. The UIM contains a single α-helix, centred around a conserved alanine residue and binds to mono-ubiquitin with relatively low affinity in mM magnitude. They mediate the recognition and direction of some protein complex to corresponding ubiquitinated cargo. For example, s5a, the first characterized ubiquitin-binding site in a proteasome subunit having only one ubiquitin interacting surface, is necessary for proteasome to recognize their substrates. The NMR structures of ubiquitins and S5a UIMs complex show that the UIM helix interact with a shallow hydrophobic groove on ubiquitin. Another UIM containing protein, receptor associated protein 80 (RAP80), targets BRCA1 through its double UIMs which binds Lys63-linked ubiquitin chains.

### 2.3 MIU
The MIU contains a single helix and seems to be unique to one protein, the Rab5 exchange factor Rabex-5, so far. The Rabex-5 is necessary in endocytosis and kinase regulation. The MIU in Rabex-5 is in tandem with A20 ZnF (zinc finger) domain and attach to the helical C-terminus of the...
A20 ZnF domain. The MIU consists a functionally essential alanine residue, which contacts Ile44 hydrophobic face of ubiquitin in similar mechanism with UIM. However, the MIU helix lies on the same hydrophobic groove that binds UIM but in the opposite orientation, which gives MIU another name, invert UIM. The N-terminus part of the UIM show similarity with MIU C-terminus and vice versa. Different from UIM, the MIU contains one more turn of helix that contacts with ubiquitin thus its affinity for ubiquitin is relatively higher to µM magnitude.

2.4 CUE domain
The CUE domain was digged by bioinformatics analysis of proteins related to Endocytosis and kinase regulation pathways including Vps9, TAB2 and TAB3. CUE domains share structurally similarity to the UBA domains as both of them consists of three-helix bundles and bind ubiquitin via conserved hydrophobic residues at the C-terminus of the α1 helix. With screens for mono-ubiquitin interactors in yeast, the CUE function in ubiquitin binding was uncovered. Ubiquitin binding ability seems to be the universal property of CUE domains as all the domains in CUE family show binding affinity to mono ubiquitin. Interestingly, one member of CUE domains, CUE domains in Vps9, shows special property as it bind to mono-ubiquitin with much higher affinity than the other CUE domain. The researcher find that the Vps9 CUE domain forms a domain-swapped dimer which is not observed in other CUE domains. In the dimeric structure, one of the α-helices in each domain is swapped, which forms a structure that is kinked to allow the two equivalent CUE domain surfaces to contact different portions of the same ubiquitin molecule. This structural contortion expands the CUE domain-ubiquitin interface from 550 Å² to 870 Å², such that it includes residues near the C terminus of ubiquitin. This type of binding interaction probably explains the relatively high binding affinity of this particular CUE-domain-containing protein for ubiquitin.

3. UBDs interact with ubiquitin acidic hydrophilic Asp58-centred face
ZnFs are the second biggest family of ubiquitin binding domains including NZF domains, A20 ZnF domains and ZNF UBP domains. The ZnF ubiquitin-binding domains offer much more diversity in recognition and binding affinity than the helical domains and both NZF fomains and A20 ZnF domains recognize Asp58-centred polar face. Like other UBDs, they were also discovered initially through domain dissections of known ubiquitin-binding proteins. In studies on Ufd1 Npl4, a ubiquitin binding adaptor in endoplasmic reticulum-associated degradation pathway, the NZF (Npl4 Zn-F) domain was established. subsequently, NZF domains in Vps36, TAB2 and TAB3 were discovered. The A20 ZnF domain was found in the NF-κB signalling cascade.

3.1. NZF domains
NZF domains contain approximate 30-residue and built around a single zinc-binding site. For the NZF domains tested to bind ubiquitin including Npl4, TAB2, TAB3 and Vps36, their affinities are about 100 µM or weaker. Two residures, Thr and Phe, lie in the first ‘zinc knuckle’ and a hydrophobic residue in the second knuckle, the TF fingerprint, appear to be the most important position for ubiquitin binding.

3.2. A20 ZnF domains
A20 ZnF domains were first discovered in ubiquitin binding protein, A20 protein, which function as a Lys63-linked polyubiquitin chain remover from its substrate, RIP (receptor-interacting protein), using another domain, OTU family de-ubiquitination domain and subsequently attaches Lys48-linked polyubiquitin chains to the substrate protein via its A20 ZnF domains. Another protein, Rabex-5, which also contains a A20 ZnF domain is a homologues of yeast Vps9 vin mammals. Unlike Vps9, Rabex-5 binds to ubiquitin with a N-terminal A20 ZnF domain fused to a MIU instead of CUE domain in Vps9 (Figure 3). The Rabex-5 A20 ZnF domain binds to ubiquitin with Kd about 15 µM. The A20 ZnF domains use a pair of aromatic residues and several polar residues to bind to a predominantly polar patch on ubiquitin centred on Asp58. The A20 ZnF binds to ubiquitin surface epitope which does
not overlap with the Ile44 patch.

Figure 3. Structure of A20_ZNF-mono ubiquitin complex

3.3. ZnF UBP
The ZnF UBP (ubiquitin-specific processing protease) ZnF domain was discovered originally in Isopeptidase T (USP5) via an exploration of the ubiquitin-binding sites of histone deactylases. The ZnF UBP domain is wildly distributed in different sorts of deubiquitinating enzymes including the ubiquitin ligase IMP/BRAP2 and the microtubule deacetylase HDAC6. The ZnF UBP has also been referred to as the PAZ (polyubiquitin-associated zinc binding) domain, even though it has no relation with the widely studied RNA binding PAZ domain. The ZnF UBP domain is approx. 130 residues in length, much larger than other ubiquitin-binding ZnFs. The domain is built around a single zinc-binding site in its Nterminal half which is fused to an α/β fold. The ZnF UBP is one of the ubiquitin binders with highest affinity among known binding domains: Kd = 3 µM for the human isopeptidase T ZnF UBP. The free C-terminal glycine residue of ubiquitin is required for ZnF UBP binding. The structure of the isopeptidase T ZnF UBP domain–ubiquitin complex shows that the extended C-terminus of ubiquitin insert deep into a tube-shaped cavity in the ZnF UBP domain, which tightly binds and enclose the free C-terminal ubiquitin tail (the sequence: RGG) through an aromatic pocket.

4. Application of UBDs

4.1. Tandem UBDs as enrichment materials or inhibitor
When the research on ubiquitin has accumulated\textsuperscript{27-31}, some are inspired by the series arrangement of UBD in nature, trying to construct multiple repetitive UBD modules in the same protein chain to enhance their binding ability\textsuperscript{32}. This idea does not require much thought, it is very easy to think of, because nature in the process of evolution is doing the same. However, this constructed element, or tandem ubiquitin binding element (TUBEs), has achieved quite good results.\textsuperscript{33} According to reports, most of these elements contain more than five UBD repeats, separated by flexible amino acid linkers, and contain commonly used protein tags in the sequences so that they can be immobilized.

This kind of component has been put into use and has shown quite good performance. For example, TUBEs of UBA1 domain based on hHR23A and UQ1-UBA domain based on ubiquilin-1 exhibit high affinity for tetramer ubiquitin chains linked by Lys48 and Lys63, which is 100-1000 times higher than that of single UBA domain\textsuperscript{34}. These two non-specific domains are most widely used.
4.2. Isolation free mono ubiquitin and ubiquitin chains
This form of UBD is bound to the carrier, and its affinity effect depends on its concentration on the carrier. These UBDs contain Znf-UBP domain and A20 Znf domain of ZNF216 protein etc. Their binding sites include targets from C terminal, Asp34 to Ile44. Among them, the Znf domain is used to capture the polyubiquitin chains that do not bind to proteins in the samples.

In summary, although these types of UBDs are unconventional and relatively few, they are still used in many special situations and may be extended in the future.

4.3. UBD as endo-cellular monitor or indicator
In this case, UBD with fluorescent labels, such as GFP, RFP and so on, can be recombined to obtain UBD with fluorescent effect. These UBD tools can bind to target proteins like fluorescent antibodies. When observed by fluorescent microscopy, they can indicate their location with fluorescent signals, this method can be used to monitor the ubiquitination of proteins, as well as the level and process of ubiquitination of various parts of cells.

There is also a large family of enzymes in cells, called deubiquitinating enzyme (DUBs), which hydrolyze ubiquitin molecules from ubiquitin-linked proteins or precursor proteins by hydrolyzing ester, peptide or isopeptide bonds at the carboxyl end of ubiquitin. The human genome encodes nearly 100 deubiquitinating enzymes, making them the largest family of ubiquitin system enzymes. DUBs genes in humans can be divided into two categories: cysteine protease family and metalloproteinase family. Ubiquitin can be produced in areas where DUB is active in cells, so UBD can be used as indicator the activity of DUBs. However, fusion proteins are only a fairly basic method. In order to improve its specificity, some people have designed fluorescence-quenching mechanism or fluorescence recovery mechanism. Both of them are targeted at specific ubiquitin junction types and use multiple junctions to improve their specificity.

The reason is very simple. If a lock core has only one identifying position with the key, the lock will also be easily cracked, and even may be unlocked by the key that does not match the lock core by accident. This will be a disaster. If a lock requires more sophisticated key, and unlocking them "by chance" becomes difficult, or even, impossible.

Facing problem how to ensure that only when all matches will phenomena occur, FRET come into people's sight. Two UBDs targeting the adjacent ubiquitin junctions are recombined with fluorescent and quenching atom groups respectively. Only by observing the phenomenon of fluorescence appearing and quenching, can the existence of a specific ubiquitin junction structure be explained, otherwise, there will be false positive possibilities. Or two kinds of UBD can be recombined to easily stimulated substances and fluorescent substances that are difficult to stimulate respectively, so that the existence of specific ubiquitin junction structure can be judged by observing whether there is "no fluorescence at first, then fluorescence gradually appears". Examples of this are Venus proteins. It is expected that more of these kits will be available in the future, and this approach may be used to monitor ubiquitinated proteins in the body.

4.4. Inhibitors of living activities
UBD can bind to ubiquitinated proteins to act as an indicator protein, but from another point of view, when proteins in a metabolic or signaling pathway are combined with engineered UBD, it will not perform its normal physiological function, at least will be more or less affected. So UBD can also be directly used to bind to a protein, it can be a signal molecule, it can be a metabolic precursor. UBD can inhibit ubiquitin-dependent pathways in cells by binding these proteins.

In addition, the ability of UBD as an inhibitor goes far beyond that. UBD plays a recognition role by binding with ubiquitin chains. DUBs also rely on its ubiquitin-binding domain to recognize and decompose ubiquitin chains. UBD and DUBs normally compete with each other for polyubiquitin chains. In this way, UBD with high affinity can be designed to protect ubiquitinated proteins from being deubiquitinated by DUBs or from being decomposed by proteases. Such a mechanism has been widely observed in organisms such as yeast.
Based on this mechanism, we can design some inhibitors to regulate the process of life activities, or to protect certain target proteins from decomposition, which can play a role in controlling the symptoms of some incurable genetic diseases.

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