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Review

Ubiquitin fragments: their known biological activities and putative roles

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Abstract

Ubiquitin (Ub) is involved in many key processes of cell biology. Identification of compounds that could interfere in the ubiquitination process is of importance. It could be expected that peptides derived from the Ub-binding regions might be able to interact with Ub receptors themselves and modify an ability of the Ub receptors interactions. This review summarizes current knowledge about known Ub-derived peptides and discusses putative activity of unexplored Ub fragments. Among identified biologically active Ub-derived peptides, its decapeptide fragment of the LEDGRTLSDY sequence was found to exhibit strong immunosuppressive effects on the cellular and humoral immune responses, comparable to that of cyclosporine. Some of the Ub fragments possess strong antibacterial and antifungal potency. In the search for new peptides that could interfere in the interaction of Ub with other proteins, we investigated the pentapeptide Ub sequences present in non-ubiquitin proteins. Based on examination of the Swiss-Prot database, we postulated that sequences of some Ub fragments often exist in other protein molecules. However, some of those motives are represented more frequently than others and could be involved in regulation of cellular processes related to Ub.

Keywords: cryptides; peptic fragments; ubiquitin; ubiquitin-binding domain.

Introduction

Ubiquitin (Ub) is a 76-amino acid polypeptide present in all eukaryotic cells. It has been highly conserved throughout evolution, with human and yeast Ub differing only by three residues. The remarkable degree of sequence conservation highlights the important physiological role of Ub. Ub is known as a post-translational addition to proteins that targets them for degradation by the proteasome. Therefore, Ub plays an important role in protein turnover as well as in removing the damaged or misfolded proteins (1, 2). In addition to protein degradation, Ub is known to activate cell signals in several pathways: tolerance to DNA damage, inflammatory response, protein trafficking, and ribosomal protein synthesis (3).

The conjugation of Ub to a target protein is regulated by the sequential activity of Ub-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3) enzymes. The E3 ligase usually determines substrate specificity, although the E2-conjugating enzyme can also play a role in substrate selection. A Ub moiety can be attached either to the ε-amino group of a lysine residue or to the amino terminus of a target protein. There are seven lysine residues in Ub (K6, K11, K27, K29, K33, K48, and K63), which allow formation of seven possible homotypic linkage types and multiple possible heterotypic chains. Further Ub moieties can then be attached to the first Ub forming a polyubiquitin chain with various topologies and functions (4). The covalent attachment of Ub is a versatile signaling event with a wide range of possible consequences. Modification of a protein with Ub chains (polyubiquitination), in which Ub is linked via K48, targets the substrate to the proteasomal pathway, whereas attachment of a single Ub moiety (monoubiquitination) and/or oligomeric K63-linked Ub chains, i.e., during the process of endocytosis, marks substrate proteins for degradation in lysosomes (5). By contrast, the attachment of a single Ub or Ub chains linked through K6 serves a non-proteolytic role (6).

The Ub-dependent proteolysis plays a critical role in the regulation of many cellular processes and therefore the malfunction of the Ub system is involved in various human diseases including cancer, viral infection, neurodegenerative disorders, muscle wasting, diabetes, and inflammation (7).

Ub has been discovered as a normal component of human blood and seminal plasma (8). An increased serum concentration of this protein was observed in several diseases (9, 10). It was suggested that Ub might be involved in the modulation of the immune response (11, 12). Ub was shown to exert inhibitory effects on circulating leukocytes (13), regulate the local inflammatory process, and enhance the Th2-type cytokine response (14).

The involvement of Ub in inflammation suggests its therapeutic potential in transplantation (15, 16). Earle et al. reported that Ub inhibited alloreactivity and prolonged skin allograft survival in fully mismatched mouse strain combinations (17). It was also proven that Ub does not cause acute toxicity or local side effects after intravenous administration in animals (14). Ub can act as an immunophilin by binding to some important immunosuppressive drugs, such as tacroli-

mus and sirolimus, which can alter the tagging for destruction of specific proteins responsible for T cell activation (18). Such a mechanism of action suggests a possibility of synergistic effects of co-administration of Ub and immunosuppressive drugs. Therefore, Ub-combined treatment could be a novel strategy to improve immunosuppressive therapy in transplantation.

Because of the intra- and extracellular occurrence of Ub in all organisms and cells, it is impossible to generate high-affinity antibodies against Ub (19). Therefore, there is a need for new and specific Ub ligands both for molecular studies and potential therapeutic applications.

Ub is the most studied member of the family of Ub-related proteins, consisting of the ubiquitin-like proteins (UbLs) and the ubiquitin-domain proteins (20). A crystal structure as well as high-resolution nuclear magnetic resonance data are available for Ub (21). Although Ub is a small protein (76 residues in length), which does not contain disulfide bonds, its structure is relatively rigid. The protein was found to have a tightly packed globular structure in which three strands of mixed parallel–anti-parallel β-sheet pack against an α-helix to form the hydrophobic core. This creates an extensive hydrophobic core contributing to a remarkable stability of Ub (from pH 1.2 to 8.5; T_m ~90°C at pH 4.0) (22, 23). Such a structure, called a β-grasp fold, is widespread and many proteins and protein families are found to adopt this topology.

It has been observed that the N-terminal 35-residue fragment of Ub forms a native-like structure, whereas the remaining fragment forms a non-native structure (24). The shorter Ub peptide comprising the N-terminal 17 residues exists in a native-like β-hairpin structure both in aqueous methanol and water (25). Interestingly, the N-terminal 51-residue fragment of Ub forms a folded symmetrical dimer in solution with orientations similar to the intact Ub (26). These facts correlate with the high stability of the N-terminal region as well as an independent folding manner of Ub.

Ub molecule comprises two functional domains: a non-structured carboxyl terminus responsible for Ub activation and a globular region responsible for interactions with diverse downstream effectors. A synthetic C-terminal fragment of Ub was found to stimulate the pyrophosphate–ATP exchange, the first step during Ub activation by E1 enzyme (27). Madden et al. reported that the C-terminal Ub fragment with RLRGG sequence can serve as Ub surrogate for the ubiquitination pathway (28).

Any intrusion in the ubiquitination process affects cell physiology. One of the viral strategies against host defense is based on the interference with the Ub system which emerges as a central theme around virus replication. Many viruses encode proteins that can modify Ub and Ub-like machinery to interfere with class I major histocompatibility complex (MHC)-restricted antigen presentation and thus escape from T cell recognition, facilitate the assembly of virus particles, and also cause degradation of host surface receptors [for reviews see Ref. (29)]. It can be concluded from such evolutionary examples that the identification of any compound interfering in the ubiquitination process is of great importance.

Ub interactions with Ub-binding domains (UBDs)

Ub has been reported to interact with numerous proteins. These interactions regulate various processes such as NF-κB-mediated transcription, DNA repair, endocytosis, and vesicular trafficking (30, 31). Recognition and transmission of Ub signals is mediated by binding to different Ub-binding domains (UBDs) of Ub receptors. So far, 20 classes of such motifs have been identified including: UIM, IUIM, DUIM, UBM, UBAN, UBA, GAT, CUE, VHS, UBZ, NZF, ZnF A20, Znf UBP, PRU, GLUE, UEV, UBC, SH3, PFU, and Jab1/MPN [reviewed by Dikic et al. (32)]. Sloper-Mould et al. reported that Ub carries a limited number of essential surface residues with defined functions in proteasome-mediated degradation or endocytosis. All essential residues are in, or adjacent to, two distinct hydrophobic surface patches (Figure 1). One of these surfaces (residues surrounding I44) is multifunctional and is involved in proteasome degradation and receptor endocytosis. The second, smallest hydrophobic surface (residues surrounding F4) is required for endocytosis but not for conjugation or proteasomal degradation (33).

It has been found that most of the receptors use α-helical regions to bind to Ub, except for the receptor Rpn13 where the loops rather than other secondary structural elements are used to capture Ub (34). The interactions of UBDs – Ubs are rather weak; however, the affinity is usually increased by Ub polymerization, as well as by the presence of several UBD motifs in one receptor or multimerization of the Ub receptor (35).

Studies investigating an understanding of how different forms of polyubiquitin are recognized by receptors and

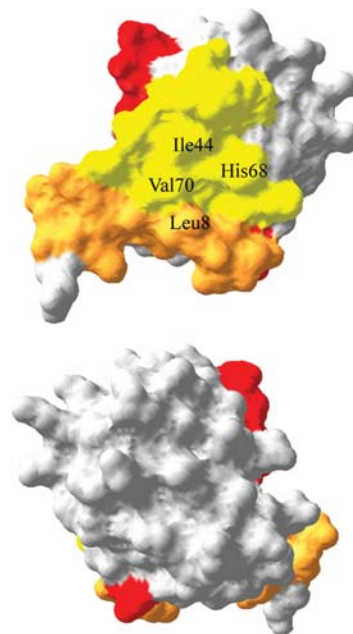

Figure 1 Visualization of the regions responsible for most of the Ub protein–protein interactions (yellow), the fragments most frequently found in other proteins (red) and the overlap of both (orange) over Ub molecular surface (both faces of Ub are given).

UBDs focused on the monoubiquitin derivatives and K48- or K63-linked polyubiquitins. However, many proteins contain more than one copy of the UBD. Usually each of them binds Ub independently (36–38). In some cases only one of UBDs presented in a molecule actually binds Ub (39). Proteins with tandem repeats of UBDs can also interact with K48- or K63-linked Ub chains (40, 41). UBDs with two Ub-binding interfaces were also found (42, 43). These domains can dimerize which allows both surfaces to form contact with a single Ub molecule (44), or they can interact with two Ub molecules simultaneously (45). Moreover, many proteins contain more than one type of the UBDs. An example of the protein having two different UBDs is Rabex5. It contains MIU (motif interacting with Ub) and RUZ (Rabex5 Ub-binding zinc finger) domains. Within each molecule, the RUZ and MIU domains contact two different Ub molecules. Conversely, each Ub molecule contacts two Rabex5 molecules using two disjoint surfaces (46, 47).

The structures of different UBDs in complexes with Ub reveal intermolecular interactions involving the hydrophobic patch on the Ub surface, formed by L8, I44, and V70, although there are known exceptions, such as the aforementioned RUZ domain in Rabex5, which binds to a surface of Ub centered on D58 (46, 47). It can be concluded that the hydrophobic interactions between the UBDs and Ub are strengthened by hydrogen bonds and/or salt bridges. In many complexes of Ub with UBDs, the side chains of K48 and K63, two major sites for polyubiquitin formation, are far away from the contacting surface (48). The known examples of complexes of the Ub with UBD-containing proteins are presented in Table 1.

The detailed information about the interaction surface of the Ub with other proteins can be helpful in designing new modulators of these interactions (49–51). The modulator can exhibit a broad range of biological activities, as the Ub is involved in many biological processes.

Recently, Roth et al. (52) examined the sequences and crystal structures of proteins that interact with Ub to select the interacting motifs and Ub-binding sites. The authors identified several small peptides as potential Ub ligands. One of the discovered peptides with DPDELRFNAIAL-NH_2 sequence turned out to be a specific Ub-interacting ligand. This peptide could serve as a small affinity tag for the detection of Ub and ubiquitinated proteins. Therefore, the fragments of other known proteins involved in interactions with Ub (including those presented in Table 1) can also specifically bind to the Ub molecule.

It could also be expected that some Ub fragments can interact with Ub-binding proteins and act as selective probes for detection of unknown Ub-binding proteins involved, for example, in regulation of the ubiquitination process, or even in some previously unknown Ub activities.

Bioactive Ub-derived peptides

Immunomodulatory peptides

The regulatory functions of ubiquitination in the immune system were reviewed by Ben-Neriah (53). It has been reported that the proteasomal degradation of the polyubiquitinated protein targets the peptide fragments to MHC class I molecules, which is crucial for cellular immune response (54). Our investigations revealed that a nonapeptide fragment located in the β164–172 loop of MHC class II molecules (HLA-DQ) suppressed the humoral and cellular immune responses and inhibited interaction with certain integrins (55). The fragment contained the RGD sequence, characteristic for several proteins involved in mediating cell adhesion. The shortest biologically active fragment (56) is located in the HLA-DQ loop exposed towards the microenvironment and, therefore, could be involved in the interactions with other proteins. The corresponding fragments of other MHC class II molecules (HLA-DP and HLA-DR) showed immunological properties similar to the HLA-DQ fragment (57). There are some topological correspondences between the β164–172 loop of HLA-DQ and the external 50–59 loop of Ub (58), suggesting that their biological roles could be similar, although the Ub fragment contains the retro-RGD sequence. We found that a decapeptide fragment 50–59 of the Ub with LEDGRTLSDY sequence exhibited strong immunosuppressive effects on the cellular and humoral immune responses, comparable to that of cyclosporine (59). The peptide was much less toxic than cyclosporine, particularly at higher doses. The DGRTL pentapeptide was the shortest, effective immunosuppressive fragment of Ub, although its potency was significantly weaker than that of LEDGRTLSDY. We also synthesized a cyclic peptide, cyclo(Glt-QLEDGRTLSDK)-NH_2 (Glt is glutaryl) obtained by reacting the C-terminal lysine side chain with the glutarylated N-terminus. The peptide was designed to mimic the immunosuppressory Ub loop. The cyclization product strongly suppressed the immune response, indicating that Ub and its LEDGRTLSDY fragment can interact with the same hypothetical receptors.

The strong suppressive effects of Ub on a mixed leukocyte reaction and allogenic skin graft survival, described by Earle et al. (17) suggest that T cells are the cellular target. Therefore, it could be expected that Ub receptors exist on the T cell surface. The hypothetic receptor can also bind Ub fragments corresponding to the Ub-binding site. The immunosuppressory potency of LEDGRTLSDY and its cyclic analog, designed to mimic the Ub loop, suggests that the loop and the decapeptide fragment can interact with the same hypothetical receptors (59).

Although the mechanisms of immunosuppressive activity of Ub or its fragments are not fully understood, a possibility of synergistic effects, resulting from co-administration of Ub with tacrolimus or sirolimus was recently suggested (17). That finding could initiate a new therapeutic approach by application of the peptides as drugs diminishing side effects of the routinely used immunosuppressants.

Interestingly, the mixture of peptides, derived from the proteolytic digestion of Ub exhibit higher immunomodulatory potency than that of Ub and its 50–59 (LEDGRTLSDY) fragment (60). The unusually high immunosuppressive potency of the mixture could suggest the existence of a very potent fragment, exhibiting high activity regardless of its rel-

Table 1 Ubiquitin fragments and the respective ubiquitin-binding domains.

Ubiquitin fragments interacting with domain[a]	Ubiquitin-binding domains				PDB code[c]	References
	Protein	Domain	Protein size[b]	Domain location		
8 (L); 42–47 (RLIFAG); 68–73 (HLVLRL)	Vps27	UIM1	622	258–277	1q0w	(37)
6–8 (KTL); 42–49 (RLIFAGKQ); 68–73 (HLVLRL)	Hrs	DUIM	777	258–277	2d3g	(45)
$Ub_{proximal}$: 8 (L); 42–49 (RLIFAGKQ); 68–73 (HLVLRL) Ub_{distal}: 8 (L); 42–49 (RLIFAGKQ); 68–73 (HLVLRL)	Rap80	UIM1-UIM2	727	UIM1: 79–96 UIM2: 104–124	3a1q	(40)
$Ub_{proximal}$: 6–9 (KTLT); 42–49 (RLIFAGKQ); Ub_{distal}: 68–76 (HLVLRLRGG)	S5a	UIM1-UIM2	377	UIM1: 211–230 UIM2: 282–301	2kde	(41)
8 (L); 42–47 (RLIFAG); 51–58 (EDGRTLSD); 66–73 (TLHLVLRL)	Rabex5	MIU-ZnF A20	492	ZnF: 13–47 MIU: 47–73	2c7m	(46, 47)
$Ub_{proximal}$: 1–6 (MQIFVK); 10–16 (GKTITLE); 64–66 (EST) Ub_{distal}: 8 (L); 42–47 (RLIFAG); 68–74 (HLVLRLR)	NEMO	UBAN	412	289–320	2zvn	(94)
8–10 (LTG); 42–48 (RLIFAGK); 68–73 (HLVLRL)	TOM1	GAT	492	215–303	1wrd	(42)
8–9 (LT); 42–49 (RLIFAGKQ); 68–70 (HLV)	GGA3	C-GAT	723	171–298	1wr6	(43)
6–8 (KTL); 42–47 (RLIFAG); 68–70 (HLV)	GGA3	GAT	723	171–298	1yd8	(95)
6–8 (KTL); 42–49 (RLIFAGKQ); 68–70 (HLV)	Cue2	CUE1	443	8–51	1otr	(36)
8–9 (LT); 34–36 (EGI); 42–49 (RLIFAGKQ); 68–73 (HLVLRL)	Vps9p	CUE	451	408–451	1p3q	(44)
8 (L); 42–47 (RLIFAG); 60–70 (NIQKESTLHLV)	TSG101	UEV	390	1–133	1s1q	(96)
8 (L); 44–47 (IFAG); 60–72 (NIQKESTLHLVLR)	Vps23	UEV	385	87–164	1uzx	(97)
8 (L); 42–49 (RLIFAGKQ); 68–73 (HLVLRL)	Npl4	NZF	608	580–608	1q5w	(98)
8 (L); 44–49 (IFAGKQ); 68–70 (HLV)	Dsk2	UBA	373	327–371	1wr1	(99)
6–8 (KTL); 44–47 (IFAG); 68–73 (HLVLRL)	Ede1	UBA	1381	1338–1380	2g3q	(100)
8 (L); 42–49 (RLIFAGKQ); 68–71 (HLVL)	EDD	UBA	2799	178–230	2gho	(101)
8 (L); 44–49 (IFAGKQ); 68–70 (HLV)	BMSC-UbP	UBA	380	333–377	2den	(102)
7–8 (TL); 44–49 (IFAGKQ); 68–70 (HLV);	UQ1	UBA	589	546–586	2jy6	(103)
8 (L); 42–49 (RLIFAGKQ); 68–73 (HLVLRL)	Eap45	GLUE	386	1–138	2dx5	(104)
7–8 (TL); 42–49 (RLIFAGKQ); 68–73 (HLVLRL)	Sla1	SH3 3	1244	353–415	2jt4	(39)
8 (L); 42–49 (RLIFAGKQ); 68–72 (HLVLR)	Rpn13	PRU	156	22–130	2z5g	(34)
8 (L); 36 (I); 71–76 (LRLRGG)	IsoT	ZnF UBP	858	197–269	2g45	(105)
8–11 (LTGK); 71–74 (LRLR)	UCH-L3	UCH-L3	169	1–169	2xd3	(106)
8 (L); 40–49 (QQRLIFAGKQ); 70–75 (VLRLRG)	OTU1	OTU	301	109–229	3by4	(107)
$Ub_{proximal}$: 4 (F); 62–64 (QKE) Ub_{distal}: 8 (L); 44–49 (IFAGKQ), 68–76 (HLVLRLRGG)	AMSH-LP	DUB	436	264–436	2znv	(108)

[a]Sequences are given in parentheses; underlined residues make direct contact with domain.
[b]Number of amino acid residues.
[c]Complex structure deposited in the Protein Data Bank (48).

atively low concentration. In the search for immunomodulatory fragments of Ub, we identified sequences of the peptic peptides using electrospray mass spectrometry (ESI-MS). The following sequences were found: 5–15 (VKTLTGKTITL), 16–21 (EVEPSD), 25–40 (NVKAKIQDKEGIPPDQ), 41–45 (QRLIF), and 59–67 (YNIQKESTL). A major peptide component containing the retro-RGD sequence: 46–58 (AGKQLEDGRTLSD) was detected; the other four DGR containing sequences: 46–59 (AGKQLEDGRTLSDY), 46–55 (AGKQLEDGRT), 50–58 (LEDGRTLSD), and 52–58 (DGRTLSD) were also present, although their abundances were low. We presume that these peptides yield the highest contribution to the suppressive activity of the Ub digest; however, the effect of other peptides and/or their synergistic or accumulative effects cannot be excluded. Interestingly, some shorter analogs of the suppressory fragment of Ub (e.g., DGRT) strongly stimulated the humoral immune response *in vivo* (59). This finding could suggest a presence of self-regulatory properties of Ub fragments, depending on the peptide chain length, with the longer peptides possessing strong immunosuppressory potencies, and their degradation products exhibiting opposite activity.

The reported synergistic effects of different immunosuppressory peptides suggested separate mechanisms of action (61, 62). Assuming that specific Ub fragments can interact with distinct receptors responsible for the immunosuppressory effect, a possibility of their synergistic or additive effect is likely. To reveal such possible phenomena, further studies on synthetic Ub fragments as well as their mixtures should be performed, using selected immunological assays.

Although the digestion products of Ub in the presence of various proteases in extracellular environment can vary from the peptic fragments, it is possible that some active immunosuppressive fragments of Ub also appear in circulation. Their half-life can be short, which makes the identification of these fragments difficult.

It has been found that the decapeptide LEDGRTLSDY has an ordered conformation in methanol solution, although its conformation differs from the structure of the 50–59 loop in human Ub. Assuming that Ub and its LEDGRTLSDY fragment interact with the same hypothetical receptor binding site, the interaction of the decapeptide with a receptor is thermodynamically less favorable. This could explain a higher immunosuppressive potency of previously cited cyclic peptide, designed to mimic the Ub 50–59 loop structure (59).

Antimicrobial peptides

Alonso et al. imply that Ub-derived peptides cause lysosomal killing of *Mycobacterium* (63). The authors found that the induction of autophagy in infected macrophages enhanced the delivery of Ub conjugates to the lysosome and increased the bactericidal capacity of the lysosomal soluble fraction. Strong antibacterial properties of Ub-derived peptides, as compared with the relatively inert full-length Ub, suggest that the Ub-derived peptides contribute to the bactericidal capacity of the lysosomal milieu. The lysosomal compartment contains a complex mixture of hydrolytic enzymes, including proteases. Therefore, it is likely that Ub-derived peptides in the lysosomal lumen would act synergistically with these other compounds to promote bacterial killing. Using nano-liquid chromatography/tandem mass spectrometry, the authors found Ub fragments: 12–27 (TITLEVEPSDTIENVK), 55–63 (TLSDYNIQK), and 64–72 (ESTLHLVLR) in the soluble fraction of lysosomes isolated from bone marrow-derived macrophages.

Kieffer et al. (64) discovered relatively strong antifungal effect of N- and C-terminal fragments of Ub (residues 1–34 and 65–76, respectively). Interestingly, the C-terminal peptide is able to cross the cell wall and the plasma membrane and to accumulate in fungi, whereas the N-terminal peptide is stopped at the fungal cell wall level. However, these two peptides act synergistically to kill filamentous fungi.

Search for novel bioactive peptides

Several bioactive peptides hidden within larger precursors, which are liberated by the action of proteases (cryptides), have been established recently (65). The search for such sequences is based on several strategies. The proteolytic approach reflects the natural origin of short peptides in a living organism (66). The alternative method utilizes synthetic peptide fragments to identify protein epitopes, including spatially addressable positional scanning libraries or mixture-oriented peptide libraries (67, 68).

Also, a bioinformatic procedure could be devised to perform similarity searches. Previously, we used a search for tuftsin- and tymopentin-like sequences in bioactive proteins to discover several immunomodulatory peptide sequences (69–71).

The application of the similarity search to a immunosuppressory hexapeptide VTKFYF sequence from Interleukin-1 receptor antagonist led us to the observation that an analogous fragment VTRFYF appears in a putative C10L protein of Vaccinia virus, which corresponds to the known viral strategy of ‘borrowing’ parts of the immune system of the host to suppress the reaction to viral invasion (72).

Therefore, the analysis of a protein sequence aimed at establishing short, potentially bioactive fragments should combine information about the protein (structure, metabolism, and interactions), the data on the activity of known fragments and the bioinformatic approach (similarity searches, binding motifs, etc.) (67, 73, 74).

In our opinion, Ub could be considered as a source of biologically active sequences. The exposed Ub fragments can serve as functional epitopes for intermolecular binding; therefore, peptides that correspond to those fragments can interfere in interactions of the Ub molecule with other proteins. There are only few examples of known biologically active Ub fragments, which have been mentioned in this review. However, we believe that there are many other, yet uninvestigated, Ub-derived peptides, which also possess biological activities and can be appealing from a pharmacological point of view.

Occurrence of Ub fragments

The interactions between proteins play an important role in many biochemical processes. Although they usually involve large interfaces with many intermolecular contacts, the peptides that mimic the small binding epitopes can block these interactions. Therefore, several research groups focus their attention on the potential functional epitopes that could be targeted for the design of new inhibitors of the protein interactions.

In the search for new peptides that could interfere with the interaction of Ub with other proteins, we investigated the similarity between Ub and sequences of synthetic peptides [deposited in the PepBank database (75)], the presence of small functional motifs [using Minimotif Miner (76) and Eukaryotic Linear Motif ELM (77)], and the occurrence of pentapeptide Ub sequences in the non-ubiquitin proteins [Swiss-Prot (78)].

Ub fragments in PepBank

The results of PepBank searches gave only a few positive hits on synthetic peptides containing Ub-derived pentapeptide sequences (Table 2) and only one of these, a nonadecapeptide fragment of horse fibrinogen, turned out to have a non-ubiquitin origin. Furthermore, according to the data mined from PepBank, only one Ub fragment 50–59 was studied previously for its biological activity (59). The small number of positive query results seems to come from the limited number of references on synthetic peptides (PepBank database contains over 20 000 records, whereas the protein Swiss-Prot database contains more than 500 000 entries).

Furthermore, some experimental data concerning the antimicrobial Ub-derived synthetic peptides were not found in PepBank. Although these results are not sufficient to investigate the structural or functional relationship between Ub cryptides and known synthetic peptides, it is interesting to point out that the region proved to possess immunosuppressive activity (59) overlaps with the sequence overrepresented among animal, including human, and bacterial proteomes (described in section 'Ub fragments in other proteins').

Short linear motifs in Ub

The naturally occurring protein regions frequently involved in protein–protein interactions, described as short linear motifs, were collected in several databases (79). We investigated the presence of such motifs in the Ub sequence. The application of Minimotif Miner (76) and ELM (77) searches resulted in over 40 hits (Table 3). Among these were potential phosphorylation/dephosphorylation sites, proteolytic enzyme substrate motifs and binding sites. In the context of diverse activity of Ub, not related to proteasome pathway, these regions could be regarded as potential functional sites, involved in interactions with other proteins, and could be used in design of novel bioeffectors.

Ub fragments in other proteins

All of the possible Ub-derived penta- and hexapeptides are listed and their frequency of occurrence in a whole Swiss-Prot database and selected taxonomic groups were examined. The total number of direct hits was used to calculate the simple moving average (SMA) according to formula given in the experimental section. In our procedure, the SMA serves to relate the penta- and hexapeptide hits to a single amino acid; it could also be interpreted as a frequency of occurrence of a certain residue in a specific amino acid neighborhood. We used pentapeptide sequences as the optimal size search frames, taking into account the results obtained by Otaki et al. (80) and Tuller et al. (81).

In the Swiss-Prot database all protein sequences encoded by a same gene are merged into a single UniProtKB entry to have minimal redundancy and to improve sequence reliability (82).

To neutralize a possible bias caused by proteins (gene transcripts) from various genomics projects, the search was conducted using the general Swiss-Prot database, and separate queries were performed for specified taxonomic kingdoms

Table 2 The list of synthetic peptides studied to date containing Ub-derived pentapeptide sequences.

Synthetic peptide sequences	Overlapping ubiquitin sequence	Peptide description	References
MQIFVKTLTGKTITLEV MQIFVKS(L)PGKTITLKV MQIFVKS(D)PGKTITLKV	1–17 (MQIFVKTLTGKTITLEV) 1–17 (MQIFVK-GKTITL-V) 1–17 (MQIFVK-GKTITL-V)	Model peptides for β-hairpin folding dynamics studies	(109)
QLEDGRTLSDK LEDGRTLSDY	49–58 (QLEDGRTLSD) 50–59 (LEDGRTLSDY)	Immunosuppressive fragments of human ubiquitin	(59)
LDYDHEEEDGRTKVTFDAR	51–55 (EDGRT)	Fragment of horse fibrinogen (fibrinopeptide B)	(110)
LRLRGG RLRGG	71–76 (LRLRGG) 72–76 (RLRGG)	Conjugating C-terminal motif characteristic for UbLs; substrates for ubiquitin activating enzyme; modulators of isopeptidase T activity; probes for ubiquitination	(27), (28), (111), (112), (113)

Result obtained through PepBank search (75).

Table 3 Short functional peptide motifs identified in ubiquitin sequence by Minimotif Miner (76) and ELM (77).

Motif pattern	Ub positions	Function of consensus motif	Modification required[a]
Minimotif Miner			
RGG	74–76	In FMDV virus binds integrin	No
(RK)(LVI)xxxxx(HQ)(LA)	42–50	Mevalonate kinase binds sequence and is trafficked to peroxisomes	No
(LV)x(1,5)Yx(1,5)(RK)	56–63	Binds cholesterol	No
YxxQ	59–62	In Stap2 is phosphorylated by Stat3; phosphorylated on Y	No
(DE)xxxL(LI)	39–44	In LIMP_II binds the trunk domain of AP1, AP2, AP3 beta subunits	No
TxxD	55–58	Binds the #1 FHA domain of Rad53	T^P
(E/D)Y	58–59	In peptide is phosphorylated by EGFR; Y residue is phosphorylated	No
DxxG	32–35	Binds the G protein domain of phosphate	No
(EDY)Y	58–59	In peptide is dephosphorylated by TC-PTP; Y is dephosphorylated	Y^P
ExT	64–66	In p27(Kip1) binds Skp2	T^P
Txx(IL)	12–15, 66–69	Binds the #2 FHA domain of Rad53	T^P
(RK)xL	6–8, 48–50, 54–56	In cyclin A binds Cdk2	No
PxxxR	38–42	Binds the #2 SH3 domain of Grb2	No
(KR)xx(ST)	6–9, 11–14, 54–57, 63–66	In Rsk is phosphorylated by RSK; phosphorylation of S/T	No
(AG)(KR)	10–11, 28–29, 47–48, 53–54	In hepatocyte growth factor, urokinase is proteolyzed by matripase; cleaves after basic residue	No
(TS)xxxx(VI)	12–17, 65–70	In SLAM binds the SH2 domain of SH2D1A	No
(FILVW)xxxxxx(FILV) xxxxx(FILVW)	4–17, 23–36, 43–56	Binds the #1 calmodulin domain of calmodulin	No
Motif expression	**Occurring positions**	**Function of consensus motif**	
ELM			
(RK)x(AILMFV)(LTKF)x	6–10, 11–15, 42–46	Subtilisin/kexin isozyme-1 (SKI1) cleavage site ((RK)-X-(hydrophobic)-(LTKF)-|-X)	
xx(T)xx(DE)x	10–16, 12–18, 53–59, 64–70	Phosphothreonine motif binding a subset of FHA domains that have a preference for an acidic amino acid at the pT+3 position	
x(DE)x(IVL)	20–23, 23–26, 33–36	Class III PDZ domains binding motif	
YxxQ	59–62	Found in the cytoplasmic region of cytokine receptors that bind STAT3 SH2 domain	
(RHK)(STALV)x(ST)x(PESRDIF)	11–16	Consensus derived from reported natural interactors which do not match the mode 1 and mode 2 ligands	
x(DE)x(ST)(ILFWMVA)xx	63–69	Site phosphorylated by the polo-like kinase	
(DER)xxxL(LVI)	39–44	Sorting and internalization signal found in the cytoplasmic juxta-membrane region of type I transmembrane proteins. Targets them from the Trans Golgi Network to the lysosomal–endosomal–melanosomal compartments. Interacts with adaptor protein (AP) complexes	

[a]Upper indexed P denotes phosphate.

(animals, plants, fungi, bacteria, archaea, and viruses) as well as specified species (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Escherichia coli*).

The database queries results allowed us to determine the Ub-subsequences which are over- or underrepresented among certain taxonomic (animals, bacteria, *Homo sapiens*, etc.) or proteomic (e.g., Ub-like proteins) groups. In the whole protein database search, the sequences 8–12 (LTGKT), 50–54 (LEDGR), and 71–74 (LRLR) are the most frequently occurring fragments (Figure 2), whether the representation of sequences 1–4 (MQIF), 38–39 (PD), 59–61 (YNI), and C-terminal glycine is below average. It should be noted that the underrepresentation of the terminal amino acids could result from the method of calculating using the SMA algorithm which affects marginal data points.

The search was also performed using hexapeptide searching frame to find the more specific regions of similarity. The results were comparable to those obtained for the pentapeptides, with fragments 8–14 (LTGKTIT) and 70–75 (VLRLRG) appearing significantly more frequently than others, and the N-terminal part (MQI) being underrepresent-

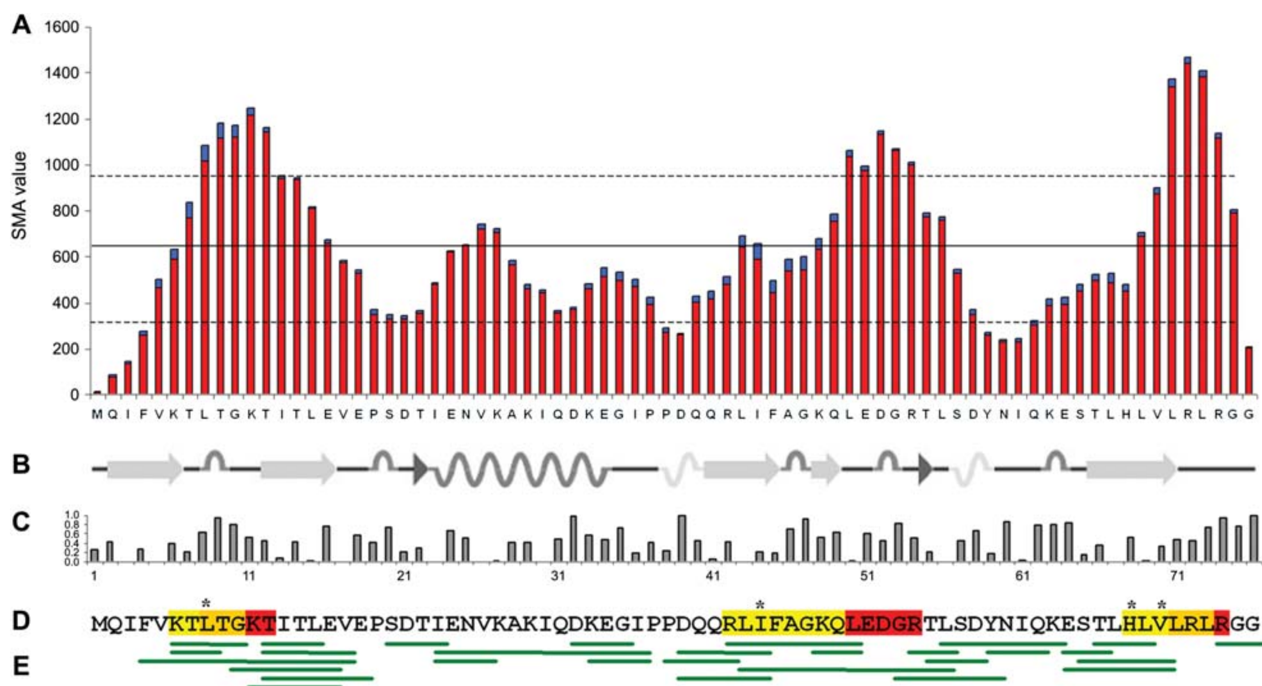

Figure 2 Comparison of the (A) overall pentapeptide queries results with (B) Ub secondary structure, (C) surface accessibility of residues, (D) specific regions of Ub responsible for non-covalent protein–protein interactions, and (E) selected short peptide motifs found through Minimotif Miner and/or ELM searches.
On graph (A) blue bars indicate UbLs hits whether red bars all other protein hits. The SMA values for specific amino acids were calculated according to Eq. (1). Average SMA values are represented with solid lines, standard deviation ranges are marked with dashed lines. Fragments marked over primary structure of Ub responsible for most of the Ub interactions with receptors are given in yellow, the most frequently found in other proteins are given in red, and overlay of both in orange.

ed (Figure 2). The SMA values calculated for other residues do not exceed the range of standard deviation; however the general shape of the graph is similar for penta- and hexapeptide results. A similar method was used to determine the frequently occurring peptide sequences in proteins (80, 81).

The significant differences between the distributions of the under- and overrepresented pentapeptide sequences derived from the Ub were observed among different taxonomic kingdoms (Figure 3). In animals, the fragments 49–54 (QLEDGR) and 69–74 (LVLRLR) were more frequent than in the general database, whereas the 1–4 (MQIF), 59–61 (YNI), P38 and G76 regions occurred less frequently. The result obtained for the animal kingdom database generally resembles the result of the entire database search; however, the significant differences in distribution of the SMA values are visible for the N-terminal part of the Ub sequence (residues 5–24). Another notable difference was observed for plants, with two major overrepresented pentapeptides: 40–44 (QQRLI) and 71–75 (LRLRG). The distribution of SMA values for bacteria is in agreement with the overall results, particularly in the case of the N-terminal half of the Ub sequence. The frequency of the representation of fragment 6–12 (KTLTGKT) is highly increased in viral proteins, whereas the fragment 31–38 (QDKEGIPP), underrepresented in the general database, appears more frequently.

A significant difference exists between the distribution of Ub fragments in animal and specifically in human proteins. Generally, the same fragments are over- and underrepresented except for the fragment 16–20 (EVEPS) in human proteome. Although the SMA values for only two residues (V17 and E18) exceed the standard deviation range, the entire sequence seems to be expressed more often in human than in the entire animal kingdom. This observation can increase the possibility to localize the species specific regions, resulting from protein evolution and phylogenetic distance.

Another interesting result was obtained for the peptide frequency search among UbLs (Figure 3). There is a significant overrepresentation of the fragments 6–10 (KTLTG) and 43–48 (LIFAGK). The first one is generally overrepresented in most of the performed searches, whether the frequent occurrence of the second fragment seems to be unique for UbLs. Furthermore, this fragment of Ub sequence belongs to one of the most crucial regions of Ub involved in protein–protein interactions (Table 1). This finding indicates the strong conservation of this fragment among other UbLs, which could result from participation of this site in the non-covalent interactions of several UbLs.

The search for Ub-derived pentapeptides among human proteins in the Swiss-Prot database resulted in more than 700 hits. The analysis of the proteins sharing a partial sequence

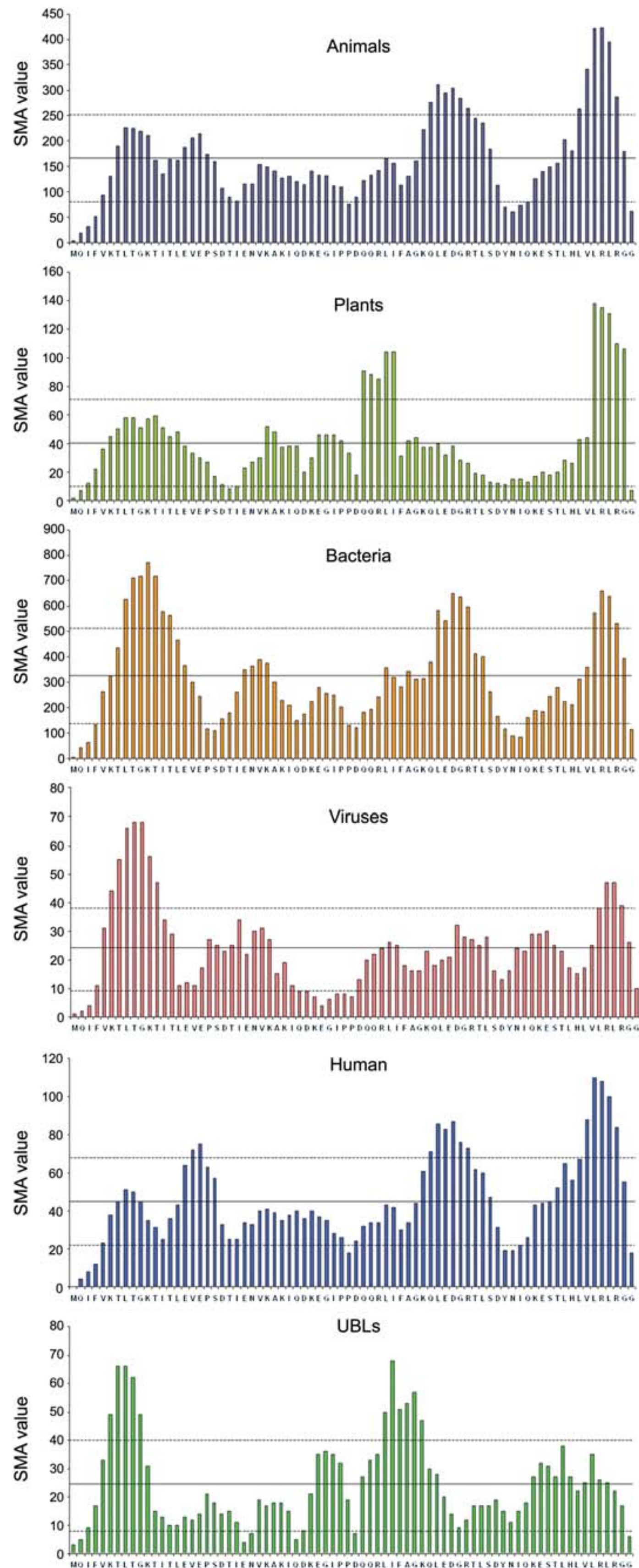

Figure 3 Occurrence of Ub-derived sequences in proteins from certain taxonomic kingdoms, human proteome, and Ub-like protein family. Average SMA values are represented with solid lines, standard deviation ranges are marked with dashed lines.

with Ub revealed that there are several human proteins that have common hexapeptide fragments with Ub, from insulin receptor (P06213.3) (83) to several ubiquilins (103), but proteins were more appealing, which contain more than one pentapeptide fragment identical to Ub (Table 4). The high homology with Ub could be explained by functional similarity in the case of NEDD8, a short Ub-like post-translational modifier, substrate to a complex enzyme system similar to ubiquitination (84). The presence of at least one common pentapeptide might have been expected in titin, the largest known protein (34 350 amino acid residues), but the presence of six different pentapeptide motifs seems to exceed the random occurrence.

However, there are several important proteins of reasonable size, involved, among others, in cell cycle regulation, signal transduction, and cell adhesion, which contain two pentapeptide fragments identical to Ub. Several of these proteins are associated with the immune system. No direct structural comparison was possible owing to size difference (and, in some cases, the lack of reliable structural information), but the fact that in most cases at least one pentapeptide originates from the 50–59 region of Ub seems to confirm the importance of this part of Ub. The phylogenetic tree of human proteins, containing more than one Ub-derived pentapeptide fragment, generated after the multisequence alignment by the Cobalt tool (85), shows the diversity of the whole results set, at the same time indicating some sequence similarities between proteins of different function, brought forth by their relationship with Ub.

The search was performed using a perfect alignment of short sequence to a protein set. The results, particularly in the case of human proteins, could point to the previously undisclosed regions of protein, which could interact with potential Ub receptors, or their partial sequences could be used to design Ub-mimicking ligands.

The comparison of the Ub molecular structure with the occurrence of the Ub-derived peptide fragments reveals an interesting relationship. The fragments present more frequently in the overall proteome dataset and in the proteomes of animals (including human) and bacteria are likely to be located at the surface of the Ub molecule and preferably form loops (residues 7–10, 50–55 and, in the case of human, also 18–21) and unstructured regions (C-terminal region) (Figure 2). By contrast, sequences involved in a formation of larger secondary structures, such as α-helices and β-strands, seem to occur with average frequency among other proteins. Such differentiation of regions can correspond to either their structural or effector function. This observation is supported by the C-terminal region being a substrate for specific enzymes (86) and the suggested involvement of the 50–59 loop in immunosuppressory activity of Ub (59). By contrast, some of the peptides known to possess antibacterial activity, such as 12–27 (TITLEVEPSDTIENVK) and 55–63 (TLSDYNIQK) (76), correspond to regions underexpressed among bacteria, which could be related to their possible regulatory function and disruption of bacterial metabolism. This leads to the assumption that the significantly frequent or rare occurrence of certain peptide fragments, correlated with exposition on the protein surface, can indicate their potential biological importance.

Expert opinion

The search for active fragments of the biologically important proteins is one of the most challenging tasks of protein chemistry. Usually, the short protein-derived peptides can easily cross biological barriers, are less susceptible to proteolysis, do not evoke immune response, and can be administered at higher concentrations, as compared to their intact precursors (87). Therefore, any whole cell digest could be treated as a natural library, a starting point for discovery of bioeffectors (88).

We believe that the Ub molecule can serve as an exploitable source of biologically active peptides, either used directly or as a structural pattern for peptides and peptidomimetics targeting the ubiquitination. In the search for new peptides that could interfere in the interaction of Ub with other proteins, we explored the Ub regions responsible for contacts with domains of known Ub receptors, as well as the presence of the short functional motifs in Ub. The investigation of the occurrence of pentapeptide and hexapeptide Ub motifs within non-ubiquitin proteins led to the observation that several Ub fragments, including 8–12 (LTGKT), 50–54 (LEDGR), and 71–74 (LRLR), are overrepresented in the protein database (Swiss-Prot). It is worth noting that the sequence of the immunosuppressory peptide, originating from Ub, LEDGRTLSDY, overlaps one of the prevalent Ub fragments. Moreover, there are proteins that contain more than one Ub fragment in their molecules. These observations could suggest that the Ub-originating cryptides and perhaps even some digestion products of non-ubiquitin proteins could interfere in the ubiquitination process or disrupt other Ub activities.

Outlook

We suppose that many physiological and functional properties of Ub are attributed to biologically active peptides encrypted in the protein molecule. As Ub and the products of its degradation by proteases are ever-present in the cellular environment, it is hard to believe that such ubiquitous substances of defined structure will not participate in the complex system of cell regulation. The presented results suggest that there are several potentially promising fragments of Ub, which could be useful in the search for novel bioactive peptides and biomimetics.

Highlights

- Ub, the key element of proteasomal protein degradation pathway, is also involved in other intra- and extracellular processes.
- Most of the Ub–protein interactions involve one side of the Ub molecule.

Table 4 Proteins sharing more than one pentapeptide fragment with human ubiquitin.

Ubiquitin fragment	Protein UniProtKB	Protein size[a]	Location	Possible function	References
5–11 (VKTLTGK) 33–38 (KEGIPP) 40–44 (QQRLI) 67–71 (LHLVL)	NEDD8 Q15843.1	81	5–11 33–37 39–44 67–71	Ubiquitin-like protein involved in cell cycle control and embryogenesis, attachment to cullins promotes polyubiquitination and proteasomal degradation of cyclins and other regulatory proteins	(84, 114)
7–11 (TLTGK) 16–20 (EVEPS)	Dual specificity protein phosphatase 19 Q8WTR2.1	217	25–29 45–49	Dual specificity Ser/Thr and Tyr–protein phosphatase	(115)
27–31 (KAKIQ) 70–74 (VLRLR)	Apolipoprotein L domain-containing protein 1 Q96LR9.2	279	227–231 66–70	Involved in angiogenesis	(116)
42–46 (RLIFA) 48–52 (KQLED)	Aminocyclopropane-1-carboxylate synthase-like protein 2 Q4AC99.1	568	529–533 560–564	Putative (class-I pyridoxal–phosphate-dependent aminotransferase family)	–
40–44 (QQRLI) 60–64 (NIQKE)	Glucocorticoid modulatory element-binding protein 1 Q9Y692.2	573	288–292 275–279	Involved in glucocorticoid receptor activation, apoptosis inhibitor	(117, 118)
16–20 (EVEPS) 67–71 (LHLVL)	Thrombopoietin receptor P40238.1	635	562–566 498–502	Receptor for thrombopoietin, could represent a regulatory molecule specific for TPO–R-dependent immune responses	(119)
23–27 (IENVK) 50–54 (LEDGR)	Pseudouridylate synthase 7 homolog-like protein Q9H0K6.1	701	417–421 80–84	Putative Possible pseudouridine synthase	
24–28 (ENVKA) 49–53 (QLEDG)	Far upstream element-binding protein 2 Q92945.3	710	455–459 110–114	Involved in gene expression, interacts with mRNA and DNA	(120)
14–18 (TLEVE) 53–57 (GRTLS)	Coiled-coil domain-containing protein 141 Q6ZP82.1	875	852–856 315–319	Putative Gene transcript	
65–69 (STLHL) 68–72 (HLVLR)	Whirlin Q9P202.2	907	267–271 493–497	Necessary for proper development of stereocilia (hearing)	(121)
11–15 (KTITL) 66–70 (TLHLV)	Protocadherin gamma-B7 Q9Y5F8.1	929	436–440 659–663	Potential calcium-dependent cell-adhesion protein	(122)
57–61 (SDYNI) 69–74 (LVLRLR)	Protocadherin gamma-A5 Q9Y5G8.1	931	416–420 712–717	Potential calcium-dependent cell-adhesion protein	(122)
8–12 (LTGKT) 48–52 (KQLED)	Ribosomal protein S6 kinase delta-1 Q96S38.2	1066	995–999 119–123	Involved in sphingosine-1 phosphate signaling	(123)
25–29 (NVKAK) 54–58 (RTLSD)	Leucine-rich repeat-containing protein 16B Q8ND23.2	1372	519–523 164–168	Putative (ribonuclease inhibitor-like subfamily)	–
24–29 (ENVKAK) 48–52 (KQLED)	Uveal autoantigen with coiled-coil domains and ankyrin repeats (Nucling) Q9BZF9.2	1416	672–677 493–497	Regulates APAF1 expression and plays an important role in the regulation of stress-induced apoptosis	(124, 125)

Table 4 (Continued)

Ubiquitin fragment	Protein UniProtKB	Protein size[a]	Location	Possible function	References
22–26 (TIENV) 30–34 (IQDKE)	Death-associated protein kinase 1 P53355.5	1430	1218–1222 473–477	Calcium/calmodulin-dependent serine/threonine kinase (positive regulator of apoptosis)	(126)
50–54 (LEDGR) 56–60 (LSDYN)	RIMS-binding protein 3A Q9UFD9.3	1545	787–791 1366–1370	Binding of the presynaptic active zone proteins RIMs and voltage-gated Ca(2+) channels	(127)
52–56 (DGRTL) 72–76 (RLRGG)	WD repeat-containing protein 90 Q96KV7.2	1748	1228–1232 532–536	Putative, gene transcript	–
39–43 (DQQRL) 51–56 (EDGRTL)	Dedicator of cytokinesis protein 4 Q8N1I0.2	1966	1308–1312 513–518	Involved in cell migration	(128)
50–54 (LEDGR) 70–74 (VLRLR)	E3 ubiquitin–protein ligase UBR5 O95071.2	2799	62–68 314–318	Involved in ubiquitination, transcriptional regulation of mRNA and the DNA damage response	(129)
3–7 (IFVKT) 39–43 (DQQRL)	PDZ domain-containing protein 2 O15018.4	2839	614–618 2586–2590	Growth and differentiation factor in various cell types	(130)
22–26 (TIENV) 72–76 (RLRGG)	Collagen alpha-3(VI) chain P12111.4	3177	2722–2726 1507–1511	Cell-binding protein	(131)
14–18 (TLEVE) 52–57 (DGRTLS)	Obscurin Q5VST9.3	7968	2006–2010 742–747	Involved in myofibrillogenesis	(132)
4–8 (FVKTL) 12–16 (TITLE) 15–19 (LEVEP) 18–22 (EPSDT) 22–26 (TIENV) 32–36 (DKEGI)	Titin Q8WZ42.2	34 350	12 141–12 145 15 385–15 389 10 007–10 011 7731–7735 14 665–14 669 23 704–23 708	Muscle regulatory protein	(133)

The proteins are arranged by growing number of amino acid residues.
[a]Number of amino acid residues.

- Ub-derived peptides exhibit immunomodulatory and antimicrobial activity.
- There are several short functional motifs in the Ub sequence; the retro-RGD is located on the exposed regions of the Ub molecule.
- Some Ub-derived pentapeptide sequences are overrepresented in the proteome.
- The known immunosuppressory Ub fragment 50–59 overlaps one of the overrepresented Ub sequences.
- In the examined taxonomic units the differences in distribution of the specific fragments might be species-related, possibly resulting from protein evolution and phylogenetic distance.
- The functional variety of human proteins, sharing more than one pentapeptide sequence with Ub, could indicate some unexplored Ub activities or interactions.
- The Ub fragments occurring most frequently in UbLs originate from the Ub regions responsible for most of the Ub–protein interactions.
- The Ub molecule could serve as a source of biologically active peptides.

Computational methods

BLAST searches

The list of all possible penta- and hexapeptides derived from human Ub (UniProtKB P62988.1) was prepared. Swiss-Prot database searches for all listed sequences were performed with BLAST (89) (Basic Local Alignment Search Tool) software accessed through NCBI (90) server using 'blastp' algorithm and BLOSUM62 mutation matrix. Parameters were automatically adjusted to short queries. Separate queries were performed for a whole Swiss-Prot database and specified taxonomic kingdoms (animals, plants, fungi, bacteria, archaea, and viruses) as well as specified species (*Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Escherichia coli*). Maximum number of hits was set to 1000 in the case of the whole database search, 500 for taxonomic kingdoms, and 250 for single species. Expected threshold value was set to 10, word size to 3, gap costs to 'existence: 11, extension: 1', and conditional compositional score matrix adjustment was picked as a parameter. No masks and filters were used.

In calculations, only the direct non-ubiquitin hits were taken into account. The frequency scores for each amino acid in the Ub sequence were calculated using SMA according to Eq. (1):

$$SMA_i = \sum_{j=i-l}^{i} \mathrm{n}_j,\ j>0 \quad (1)$$

where n is the number of penta- or hexapeptide database hits, i is the number of residue in the Ub sequence (from N-terminus), j is the number of fragment peptide (from N-terminus), and l is the length of queried peptides minus 1.

Final SMA values correspond to the total number of direct peptide hits containing specific amino acid residue. For each population of SMA results, the average values and standard deviations were calculated.

Minimotif Miner query

Minimotif Miner application was accessed through the web server (76). The query was performed for human Ub sequence in all subcellular localizations and all organisms. Results were filtered for consensus patterns only.

ELM query

The ELM (Eukaryotic Linear Motif) server was accessed through its webpage (77). A search was performed for human Ub (UBIQ_HUMAN) for *Homo sapiens* without specifying cell compartment.

PepBank searches

The PepBank database (75, 91) searches were performed to determine the occurrence of the theoretical Ub-derived pentapeptide sequences in previously studied synthetic peptides.

Determination of accessibility

Accessibility factors of the amino acid residues of Ubiquitin were obtained from ASAView application (92, 93) which displays information on amino acid exposure on the protein surface transferred from the Protein Data Bank [PDB (48)].

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