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Ubiquitin is a Novel Substrate for Human Insulin-Degrading Enzyme

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Abstract

Insulin-degrading enzyme (IDE) can degrade insulin and amyloid- β (A β), peptides involved in diabetes and Alzheimer's disease, respectively. IDE selects its substrates based on size, charge, and flexibility. From these criteria, we predict that IDE can cleave and inactivate ubiquitin (Ub). Here, we show that IDE cleaves Ub in a biphasic manner, first, by rapidly removing the two C-terminal glycines ($k_{\text{cat}} = 2 \text{ sec}^{-1}$) followed by a slow cleavage between residues 72-73 ($k_{\text{cat}} = 0.07 \text{ sec}^{-1}$), thereby producing the inactive Ub1-74 and Ub1-72. IDE is a ubiquitously expressed cytosolic protein, where monomeric Ub is also present. Thus, Ub degradation by IDE should be regulated. IDE is known to bind the cytoplasmic intermediate filament protein nestin with high affinity. We found that nestin potently inhibits the cleavage of Ub by IDE. In addition, Ub1-72 has a markedly increased affinity for IDE (~ 90 fold). Thus, the association of IDE with cellular regulators and product inhibition by Ub1-72 can prevent inadvertent proteolysis of cellular Ub by IDE. Ub is a highly stable protein. However, IDE instead prefers to degrade peptides with high intrinsic flexibility. Indeed, we demonstrate that IDE is exquisitely sensitive to Ub stability. Mutations that only mildly destabilize Ub ($\Delta\Delta G < 0.6 \text{ kcal/mol}$) render IDE hypersensitive to Ub with rate enhancements greater than 12-fold. The Ub-bound IDE structure and IDE mutants reveal that interaction of the exosite with the N-terminus of Ub guides the unfolding of Ub, allowing its sequential cleavages. Together, our studies link the control of Ub clearance with IDE.

Keywords

ubiquitin turnover; insulin-degrading enzyme; nestin-mediated cleavage regulation; exosite; substrate flexibility

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Introduction

The post-translational modification of countless proteins by ubiquitin (Ub) affects basic cellular processes, which ultimately impact health and disease.¹ Therefore, the mechanisms that govern the stability of Ub are of great interest. In cells, Ub exists as a free monomer or in a conjugated form, and the ratio between these two forms is determined by the balanced activities of conjugation, deubiquitination, and degradation.² Studies in different tissues have shown that although the total level of Ub may vary up to three-to-four-fold, the ratio between free and conjugated Ub is less variable, with approximately 40–60% in the free form.^{3; 4; 5; 6; 7; 8} The levels of Ub can change in different pathophysiological conditions. For example, when cells are exposed to different forms of stress such as chemical, oxidative, or heat stress, aberrant proteins arise at an excessive amount, up-regulating the ubiquitin-proteasome system.^{9; 10; 11} However, the synthesis and degradation of monomeric Ub appear to be tightly regulated. Ub levels can rise under stress, perhaps to provide the cell with sufficient amount of the protein necessary for coping with the increased demand, but are then reduced when cellular needs have been met.^{12; 13; 14} The clearance of Ub occurs with at least two forms of the protein: a free monomeric form and a substrate-conjugated form.² While substantial work has been done to understand the mechanism of poly-Ub conjugation of proteins, the resulting proteasomal degradation, and the recycling of poly-Ub to mono-Ub, little is known about the clearance of mono-Ub.

Insulin-degrading enzyme (IDE), a ubiquitously expressed zinc-metalloprotease, has been reported to selectively bind and degrade a variety of bioactive peptides.^{15; 16} IDE can rapidly degrade insulin with high specificity^{17; 18}, and accumulating evidence supports the notion that IDE is a major enzyme for insulin degradation *in vivo* and is involved in the development of diabetes.^{19; 20; 21; 22; 23} In addition, IDE can effectively degrade amyloid- β (A β), a peptide critical for the progression of Alzheimer's disease.²⁴ Consistent with this notion, IDE gene disruption in mice results in elevated cerebral accumulation of A β , while the overexpression of IDE leads to reduced brain A β levels.^{22; 25} Besides insulin and A β , several other physiologically active peptides have also been identified as high affinity substrates for IDE *in vitro*, such as insulin-like growth factor II (IGF-II), tumor growth factor- α (TGF- α), and atrial natriuretic peptide.^{26; 27}

IDE exhibits unusually high selectivity towards its substrates.²⁸ Recent structural analyses of human IDE offer a model for how IDE utilizes the unique properties of its catalytic chamber to select certain substrates based on size, charge, and the flexibility of peptides.^{15; 29} IDE has two roughly equal-sized N- and C-terminal domains, IDE-N and IDE-C, which form an enclosed catalytic chamber, with an estimated volume of $\sim 16,000 \text{ \AA}^3$. The inner surface of IDE-N domain is mostly neutral or negatively charged, whereas IDE-C is mostly positively charged.¹⁵ This unique feature of the catalytic chamber allows IDE to selectively interact with substrates by charge complementarity. The structures of IDE in complex with insulin, A β , glucagon, amylin, IGF-II, and TGF- α reveal that IDE also contain a highly conserved exosite approximately $30 \text{ \AA}$ away from the catalytic center to anchor the N-terminus of substrates.^{16; 29; 30; 31; 32} Thus, the exosite function is postulated to enhance substrate binding affinity and thereby, facilitate IDE catalysis. Currently, the mechanism of substrate selectivity by IDE is not fully understood. Additionally, other physiological substrates, as well as other functions of IDE still remain to be identified.

In previous studies, Ub was suggested to bind to IDE because it directly competed with the cleavage of insulin by IDE.^{33; 34} Therefore, we exploited the structural features of the catalytic chamber of IDE to predict monomeric Ub as a substrate of IDE. We confirmed that IDE can effectively digest Ub in a biphasic manner and that decreasing the stability of Ub can enhance the rate of its cleavage. The cleavage of Ub by IDE results in inactive Ub, and

the addition of the intermediate filament protein nestin potentially blocks this proteolytic inactivation. Our mutational and crystallographic analyses of IDE suggest a role for the exosite of IDE in the unfolding and the progressive cleavages of Ub by IDE. Together, these results postulate a potential role for IDE in Ub regulation.

Results

Identification of Ub as a substrate of IDE

Ub has been shown to strongly compete with insulin binding to IDE, indicating that insulin and Ub likely interact at the same site.³⁴ However, the molecular basis of this association remains unknown. After careful examination of the available sequence and structural data, we hypothesized that Ub is a candidate substrate of IDE because it shares similar features with other well-studied IDE substrates.

Three such features are the size, the charge distribution, and the location of the N-terminus of Ub. Based on the available structure, Ub (PDB code 1UBQ) is estimated to have a volume of $\sim 16,000 \text{ \AA}^3$, which would fit inside the catalytic chamber of IDE (Fig. 1A). Furthermore, Ub has a dipolar charge distribution; most negative charges are located on one side of the Ub molecule, while positive charges are distributed on the opposite side (Fig. 1A). This polarized surface charge distribution pattern complements the inner surface properties of the IDE catalytic chamber (Fig. 1A). The positively charged patch of Ub will locate proximally to the catalytic center of IDE, and the negatively charged side of Ub will likely face the IDE-C domain. In addition, the N-terminal end of Ub will be near the exosite of IDE in this orientation for the interaction commonly found in substrate-bound IDE structures. The C-terminal end of Ub will be positioned proximal to the catalytic center of IDE, well poised for proteolytic cleavage upon binding to the catalytic cleft of IDE (Fig. 1A). Taken together, all of these observations strongly suggest that Ub is a good candidate for binding to and degradation by IDE.

To test this hypothesis, we used a combination of MALDI-TOF mass spectrometry and Fourier-transform ion cyclotron resonance (FTICR) mass spectrometry to reveal that Ub can be degraded by IDE (Fig. 1B, Table S1). Before IDE incubation, the major peak with observed mass 8598.63 Da was identified as full length Ub, which matched the calculated mass of 8598.57 Da within 10 ppm (Fig. 1B, *top panel*, Table S1). After a 1-min incubation with IDE, a new peak emerged with a mass of 8484.60 Da, corresponding to the Ub fragment 1-74. This result suggests that IDE does indeed cleave Ub and that the first cleavage occurs between Arg-74 and Gly-75, rendering the complete removal of the functionally important C-terminal diglycine from the Ub peptide.³⁵ After a 5-min incubation with IDE, the peak corresponding to full-length Ub completely disappears and the only species present is that of Ub1-74 (Fig. 1B, *top three panels*), thus confirming that Ub can be completely degraded by IDE. With a Ub concentration of 20 μM in the reaction, which is at 200-fold excess of IDE, we estimate that the primary cleavage rate of Ub by IDE is $\sim 2 \text{ sec}^{-1}$.

After a 10-min incubation with IDE, a second Ub product is slowly generated, with a mass 8215.39 Da (Fig. 1B, *lower 2 panels*, Table S1). This peak corresponds to Ub1-72, and occurs only after the production of the first Ub fragment (1-74). We estimate that the rate of this second cleavage, which occurs between Arg-72 and Leu-73, is $\sim 0.07 \text{ sec}^{-1}$. Interestingly, IDE does not cleave Ub further once it generates Ub1-72. Thus, these data clearly indicate that Ub indeed is a substrate of IDE and its cleavage occurs in a biphasic manner by a rapid removal of the two C-terminal glycines and then a slower cleavage between residues 72-73.

We examined the structural basis of the interactions of IDE with Ub by solving the crystal structure of the catalytically less active cysteine-free IDE (IDE-CF-E111Q) in complex with Ub at 2.35 Å resolution (Fig. 1C, Table 1). The structure of this complex is in the closed conformation and virtually identical to the previous structures of substrate-bound IDE-CF-E111Q (rms deviation of 0.1 Å for all C α atoms). The electron density map calculated at 2.35 Å showed peaks in the catalytic chamber in proximity to the exosite. The shape of this density could be modeled well with the first three N-terminal residues of Ub (Met-1, Gln-2, Ile-3) (Fig. 1C). Residues 339, 341, 359, and 361 of IDE are involved in interactions with Ub. The oxygen atoms of the side chain of Glu-341 and of the backbone carbonyls of Gly-339 and Leu-359 are within hydrogen bond distances from the backbone amide of the terminal Met-1 of Ub. Also, residue 361 makes polar interactions with the Ub N-terminus. Hydrogen bonds are mediated by the main-chain amido group and carbonyl oxygen of Gly-361 with the backbone O and N atoms of Met-1 and Ile-3 of Ub, respectively. These same residues of IDE are also involved in binding the N-terminal region of several other substrates.^{16; 31; 32}

Inspection of electron density maps from 5 data sets at resolutions from 2.35 to 3.3 Å revealed peaks of discontinuous electron density in front of the active-site residues (Fig. S2). The observed break in electron density near this site suggested that IDE-CF-E111Q retained enough catalytic activity to cleave the C-terminal region at the first cut site of Ub. To verify this possibility, we analyzed the crystals through MALDI-TOF-MS, and indeed, we found that Ub was fully cleaved between residues 74 and 75 of Ub (Fig. S1). However, despite having this information, we were still unable to model any Ub residues in this region. Thus, it is likely that this discontinuous electron density is indicative of the intrinsic disorder conferred to the Ub C-terminus upon cleavage and removal of the diglycine moiety. Overall, our crystal structure reveals clues about how IDE uses the highly conserved exosite to bind the N-terminus of Ub, while the flexible C-terminal end of Ub can swing to the catalytic site, which leads to its proteolysis.

A nestin tail fragment inhibits the binding and cleavage of Ub by IDE

The interaction between Ub and IDE may indeed occur *in vivo* because both of these proteins co-localize to the cytoplasm. In addition, ubiquitin is present in normal cells at concentrations (5–57 μM) high enough to bind to IDE³⁶, and about half of the total protein exists as a monomer. Since cleavage of Ub by IDE results in the production of inactive Ub, which would be detrimental to the cell, we hypothesized that there must be cellular protein(s) that can regulate this process.

The intermediate filament protein nestin, also located in the cytoplasm, was shown to specifically inhibit the degradation of insulin by IDE yet activate the degradation of the smaller substrate V.³⁷ Nestin is a large protein, spanning 1893 residues in sequence. Truncation experiments revealed that the nestin 641-1177 fragment is responsible for the inhibitory effect on IDE.³⁷ We thus evaluated whether the nestin 641-1177 fragment could also modulate the degradation of Ub by IDE. As shown in Figure 2A, the cleavage of Ub does not occur when IDE (0.1 μM) is pre-incubated with 0.2 μM nestin. This indicates that nestin can potentially block the degradation of Ub by IDE (Fig. 2A, *top panel*).

We then sought to determine whether nestin could also modulate the binding affinity of Ub to IDE. To do so, we first examined the potency of Ub and IDE-digested Ub products, Ub1-74 and Ub1-72, to compete and inhibit the cleavage of the fluorogenic bradykinin-mimetic substrate, Substrate V (4.25 μM), by IDE (90 nM) to assess the affinity of Ub to IDE. The observed IC₅₀ value of ~90 μM (Fig. 2B) is similar to the IC₅₀ value obtained for Ub using radiolabeled insulin as a competitor.³⁴ This suggests Ub binds at a location within IDE, which impacts both short and longer peptide substrates. Ub1-74 has a comparable IC₅₀

value ($\sim 100 \mu\text{M}$). Interestingly, Ub1-72 has higher potency in blocking the degradation of substrate V by IDE with an IC_{50} value of $1 \mu\text{M}$. This interesting difference in IC_{50} values for Ub and Ub 1-72 suggests that ubiquitin may exhibit auto-inhibition with respect to its degradation by IDE. Moreover, we propose that the reported inhibition of IDE by Ub was caused by the full-length Ub and IDE-degraded products of Ub.^{33; 34}

We then took advantage of the high affinity of Ub1-72 to evaluate the effect of nestin on the binding of Ub to IDE (90 nM), using substrate V ($4.25 \mu\text{M}$) as the tracer (Fig. 2C). The order of pre-incubation of IDE with nestin or Ub1-72 resulted in completely different outcomes. When IDE was first incubated with $0.2 \mu\text{M}$ nestin, we found that the later addition of Ub1-72 up to $500 \mu\text{M}$ could not block the degradation of substrate V by IDE (IDE-nestin + Ub1-72). However, when IDE was mixed first with Ub1-72 for 5 min before the addition of nestin, Ub1-72 is a potent inhibitor of IDE (IDE-Ub1-72 + nestin). In fact, nestin was not capable of altering the inhibitory potency of Ub1-72 if IDE was preincubated with Ub1-72. These results suggest that nestin likely allosterically affects the activity of IDE by inducing a conformational change of IDE. This change prevents Ub from entering the catalytic chamber but still allows entrance of small substrates like substrate V. In contrast, if Ub1-72 is already bound to IDE, the addition of nestin does not influence the binding of Ub to IDE.

Role of conformational flexibility of Ub in its degradability by IDE

IDE preferentially degrades amyloidogenic peptides, which exhibit high structural flexibility.³⁸ The substrate-bound IDE structures show that IDE substrates need to undergo a conformational switch in order to access the catalytic cleft of IDE.^{15; 16; 31; 32} Thus, we postulate that the structural flexibility of peptides is a key factor for the substrate selectivity of IDE. However, this hypothesis has never been formally tested. Ub is a highly stable protein, which has a large free energy difference between the folded and unfolded states ($\Delta G = \sim 8-9 \text{ kcal/mol}$).³⁹ We hypothesized that IDE does not cleave Ub beyond the four C-terminal residues because the Ub1-72 fragment lacks the structural flexibility required for the unfolding and subsequent degradation by IDE. Indeed, the conformation of the N-terminus of bound Ub and that of free Ub is virtually identical (Fig. S3), supporting this notion. Ub is an excellent model for protein folding studies, and this available information allowed us to fine-tune the stability of Ub through point mutation(s) without compromising the structural integrity of the protein. We predicted that such Ub mutants would have increased susceptibility to degradation by IDE. To test this, we incubated IDE with mutants of Ub known to maintain the overall fold, but that have reduced stability by $0.5-8.2 \text{ kcal/mol}$ in comparison to Ub.^{40; 41; 42}

In Figure 3, we illustrate a mass spectrometric profile of one such Ub mutant, Ub-F45W/L67A, and its time-dependent degradation by IDE. In this case, L67A causes destabilization to Ub by 2.6 kcal/mol , while F45W does not affect stability. Comparison of the 1-min IDE incubations of Ub-F45W/L67A (1-min *left panel*) and Ub (1-min *right panel*) with IDE shows that the rate of initial cleavage between residues 74 and 75 of Ub-F45W/L67A is comparable in rate to that of Ub ($\sim 2 \text{ sec}^{-1}$). However, we note that there is a striking change in the rate of the subsequent cleavage of Ub-F45W/L67A (3 sec^{-1}) that occurs between residues 72 and 73, as compared to Ub (0.07 sec^{-1}). Furthermore, the 1-72 fragment of Ub-F45W/L67A is further cleaved by IDE into smaller fragments (Fig. 3, 2-min *panel*). After 5 min (Fig. 3, 5-min *panel*, Table S1), the 1-72 fragment of Ub-F45W/L67A is completely degraded. The preferred cut sites mainly occur at the carboxyl side of basic residues. Thus, these data suggest that introducing mutations that decrease the stability of Ub results in its complete fragmentation by IDE.

Analysis of the effect of $\Delta\Delta G$ of Ub mutants on IDE catalysis confirms that the increased local instability of Ub up to 3.1 kcal/mol profoundly enhanced the catalytic activity of IDE up to $\sim 2200 \text{ min}^{-1}$ (Fig. 4, Table S1). However, we note a decline in degradation rate beyond 3.1 kcal/mol. This implies that IDE loses its ability to efficiently recognize and cleave Ub when Ub is too flexible or unstable. With respect to the cleavage mechanism, our results suggest that IDE cleaves Ub sequentially. IDE first cleaves the C-terminal end. With an increase in $\Delta\Delta G$, the cut sites progressively move from the C-terminal end of Ub toward its N-terminus. However, the specific cleavage sites vary depending upon the specific mutation(s) in Ub and may be dictated by the flexibility of Ub caused by the given mutation(s) (Fig. 4).

Mutations at the IDE exosite prevent subsequent fragmentation of Ub1-74

The degradation of Ub and Ub mutants exhibits at least two steps, the first being a fast C-terminal diglycine removal followed by slow cleavage(s). Our structure shows that the N-terminus of Ub intimately interacts with the exosite (Fig. 1C). We then examined whether mutations at the exosite would affect this process. We first mutated three exosite residues that make important contacts with Ub: Glu-341, Gly-339, and Gly-361 (Fig. 1C). The negatively charged glutamate at 341 was mutated to the smaller, aliphatic alanine, the positively charged lysine, and the neutral glutamine. The glycines at 339 and 361 were replaced by prolines to disrupt the hydrogen bond network between the exosite of IDE and Ub. The mutant enzymes were constructed, expressed, and purified to homogeneity (Fig. 5A). These mutants are active in degrading a short peptide substrate, Substrate V (Fig. 5B). Their activities range from 20-100% of the wild-type counterpart (1.7 min^{-1}), indicating that these exosite residues are not required for the proteolytic activity of IDE. We then incubated Ub-F45W/L67A with each of the IDE exosite mutants and found that each enzyme can still cleave the C-terminal diglycine at the same rate as IDE-WT ($\sim 2 \text{ sec}^{-1}$) (Fig. 5C). However, in contrast to IDE-WT, all of the IDE exosite mutants are unable to further degrade the Ub1-74 fragment of Ub-F45W/L67A (Fig. 5C). Thus, while the exosite of IDE does not contribute in the cleavage of the diglycine moiety of Ub, it does profoundly affect the ability of IDE to further cleave Ub.

Discussion

In this study, we found that monomeric Ub is a novel substrate of IDE. After short incubations, IDE completely cleaves the crucial C-terminal diglycine motif of Ub that is required to attach Ub to its target proteins. While the physiological relevance of the cleavages of Ub by IDE remains to be established, this interaction is likely to occur *in vivo* because both of these proteins compartmentalize to the cytoplasm, Ub concentrations are high enough for binding to IDE, and a truncated form of Ub was co-purified with IDE in leukemic splenocyte extracts.³⁴ In line with this, early purification strategies of native Ub led to the isolation of Ub1-74 rather than full-length Ub, indicating that Ub is susceptible to proteolysis, perhaps by proteases such as IDE.^{43; 44} In addition, circumstantial evidence also suggests that IDE and Ub could interact in a pathological context. For example, immunocytochemical studies of the brain tissues obtained from patients that suffered from Alzheimer's disease demonstrated that both IDE⁴⁵ and Ub⁴⁶ are present in intracellular (neurofibrillary tangles) as well as extracellular (senile plaques) characteristics of the disease. However, it remains to be established whether IDE causes functional alterations to Ub (or vice versa) in these lesions.

It should be emphasized that our findings may have additional connotations. In addition to cleaving Ub, it is conceivable that IDE can also digest proteins that are homologous to Ub. Several of these functionally and structurally homologous proteins have been categorized into two functional classes called ubiquitin-like modifiers and ubiquitin-domain proteins.⁴⁷

The ubiquitin-like modifiers such as small ubiquitin-related modifier (SUMO1) or proteins of the NEDD8 family are, similar to Ub, covalently attached to other proteins, but only as single molecules per target.⁴⁸ In contrast, the ubiquitin-domain proteins (such as Parkin, RAD23, and BAG-1) contain domains that are related in sequence to Ub but are not covalently conjugated to other proteins.⁴⁷ Instead, they associate with their protein partners non-covalently. Thus, our findings suggest the possibility that ubiquitin-like proteins might be recognized by IDE.

Together, our mass spectrometric, crystallographic, and mutagenic analyses allow us to put forth a model of how the size, charge, exosite anchoring, and structural flexibility of Ub contribute to the binding of Ub to IDE and to its subsequent digestion (Fig. 6A). The size and charge distribution of Ub match well with the catalytic chamber of IDE (Fig. 1A). This allows the proper orientation of the N-terminus of Ub to the exosite of IDE as well as the initial cleavage site of Ub (scissile bond between Ub 74-75) to be near the catalytic site of IDE (Fig. 6A). The charge complementation of the catalytic chamber of IDE with Ub is similar to that with insulin.³¹ In contrast to the insulin-bound IDE structure, which shows that the initial cleavages are about 15 Å away from the catalytic site, our Ub-bound IDE model places the initial cleavage site of Ub proximal to the catalytic site. Thus, instead of the major conformational switch required for insulin to be cut by IDE, a relatively small repositioning of the C-terminal tail of Ub is needed for IDE to cut the C-terminal diglycine of Ub.

Our structural and mutational analyses reveal the key role of the exosite in unfolding Ub for its degradation. Similar to other substrates, the N-terminal segment of Ub is found anchored by the IDE exosite, which is extended 30 Å away from the catalytic zinc ion (Fig. 1C). The anchoring of the N-terminus of Ub to the exosite of IDE triggers a partial conformational switch in Ub to allow access of the next cleavage site of Ub (72-73) to the catalytic cleft of IDE (Fig. 6A). Such binding would promote the unfolding of Ub that has destabilizing mutation(s), leading to further cleavages by IDE (Fig. 6A). The anchoring of the N-terminus of Ub to the exosite of IDE also explains how Ub mutants are degraded from their C-termini toward their N-termini by IDE (Fig. 4).

Our studies on the cleavage of Ub by IDE reveal a new exosite-dependent mechanism of substrate degradation by IDE. Based on the accumulated structural and biochemical evidence, exosite-anchored substrates can be cleaved either processively, as illustrated with insulin or stochastically, as is the case with Aβ.^{16; 31} Here, we show that IDE can also cleave its exosite-anchored substrates in a *sequential, biphasic* manner. Following binding, IDE degrades Ub in three sequential steps (Fig. 6A). (1) The flexible C-terminal tail of Ub swings toward the catalytic cleft without considerable disruption of the Ub fold. An initial cut occurs between residues Arg-74 and Gly-75 at a rate of 2 sec⁻¹. (2) The exosite, which interacts with the N-terminus of Ub, then facilitates the slight unfolding of Ub, aligning residues Arg-72 and Leu-73 at the catalytic cleft. This slower cleavage occurs at a rate of 0.07 sec⁻¹. (3) IDE can then further degrade Ub sequentially from the C- to N-terminal direction, depending on the stability of Ub. For wild-type Ub, IDE acts as a C-terminal exopeptidase by cutting two C-terminal residues each time for two cycles.

The mechanism of nestin-mediated inhibition of the degradation of Ub likely involves restriction of the open state of IDE and modulation of the switch rate between the open and closed states of IDE (Fig. 6B). Without substrate, IDE is postulated to exist in a stable equilibrium between closed and open state. When Ub proteins are present (Fig. 6B), the charge complementarities will lead the substrate in binding to the inner surface of the IDE C-terminal domain first. Subsequently, the flexible N-terminus of Ub may form a tight interaction with the exosite of IDE. This interaction will induce a conformational change of

IDE from open to closed state, which traps Ub inside the catalytic chamber. Alternatively, thermodynamic motion can trigger the conformational switch of IDE from the open state to a closed state. The proximity of the N-terminus of Ub allows it to be anchored at the exosite of IDE. Once IDE is in the closed state, the substrate needs to undergo secondary structural rearrangements to allow the exposure of *all* of the cleavage sites of Ub. The binding of nestin could allosterically modulate the extent of opening of IDE and/or the switch rate between the closed and open states. This modulation may, on the one hand, facilitate the degradation of short peptide substrates, such as bradykinin-mimetic substrate V and, on the other hand, slow down the cleavage of larger substrates, such as Ub. Therefore, we postulate that cellular proteins like nestin serve a crucial role in tightly regulating the ability of IDE to degrade important physiological peptides like Ub.

In summary, we described the discovery of Ub as a novel substrate for IDE, which greatly implicates a potential role of IDE in the modulation of mono-Ub levels in the cell. Our findings also provide insights about the mechanism of IDE substrate recognition and degradation. As a ubiquitously expressed protein, IDE may have a more general role in regulating human physiology by degrading other substrates. Our structure-based modeling identifies Ub as an IDE substrate, instead of an inhibitor as previously reported.^{33; 34} A similar strategy predicts macrophage inflammatory protein (MIP)-1 α and MIP-1 β as substrates for IDE *de novo*.⁴⁹ Due to its role in the clearance of insulin and A β , IDE is a potential therapeutic target for the treatment of diabetes and Alzheimer's disease. Our ability to effectively identify physiologically-relevant substrates of IDE will not only broaden the therapeutic potential of IDE, but will also offer knowledge that may help improve such therapeutic strategies.

Materials and Methods

Site-directed mutagenesis, expression, and purification of IDE and Ub proteins

The Stratagene QuikChange site-directed mutagenesis kit was used to introduce single base mutations to the pProExhIDEWT plasmid, which encodes human IDE. The oligonucleotides shown below and their complementary sequences were used to introduce the mutations into the cDNA: *E341A*, GGT CAT CTC ATT GGG CAT CAA GGT CCT GGA AGT C; *E341Q*, GGT CAT CTC ATT GGG CAT GCA GGT CCT GGA AGT C; *E341K*, GGT CAT CTC ATT GGG CAT AAA GGT CCT GGA AGT C; *G339P*, T GGT CAT TAT CTT GGT CAT CTC ATT CCG CAT GAA GGT CC; and *G361P*, TCA AAG GGC TGG GTT AAT ACT CTT GTT CCG GGG CAG AAG GAA GG. IDE-CF-E111Q mutant was constructed as previously described.¹⁶ The mutations were confirmed by DNA sequencing. Wild-type IDE (IDE-WT) and mutated IDE were expressed as N-terminal His₆-tagged proteins in *E. coli* Rosetta (DE3) cells. The IDE-WT and mutated IDE were purified to homogeneity using chromatography on Ni-NTA agarose (Qiagen) followed by Source-Q anion exchange and then Superdex-200 gel filtration (GE Healthcare).³⁰ The purity of the enzymes was assessed using SDS-PAGE. Wild-type Ub (Ub) was purchased from Sigma, and the Ub mutants were constructed, expressed, and purified according to the previously described methods.^{39; 40; 41; 42; 50} The nestin 641-1177 fragment was purified as described.³⁷

Mass spectrometry (MS) analysis of Ub degradation by IDE

A mass spectrometry analysis was carried out, as previously described, with some modifications.^{31; 51} Briefly, enzyme reactions were performed at 37 °C by mixing equal volumes of 20 μ M Ub with buffer alone or containing 0.1-1 μ M IDE in the absence or presence of 200 nM nestin fragment. An aliquot of the reaction mixture (20 μ L) was removed at the indicated times and stopped with the addition of 80 mM EDTA and 0.03%

TFA. For analysis by MALDI-TOF/MS, the reaction mixture was purified on a Zip-Tip C₁₈ column (Millipore). The purified reaction mixture was then spotted on a metal plate with an equal volume of α -cyano-4-hydroxycinnamic acid matrix (Sigma). Mass spectra were collected on an ABI 4700 MALDI-TOF/TOF MS or a Voyager De-Pro MALDI-TOF. The data were visualized and analyzed with Data Explorer.

For LC-ESI-FTICR-MS, Ub incubation mixtures (12 μ l) were injected into a nano RP-HPLC system (Dionex), with a C₈ analytical column (Agilent). Peptides were eluted from the nano column with a linear gradient of 5-95% acetonitrile in 0.1% formic acid and sprayed into a LTQ-FT tandem MS instrument (Thermo Scientific). Spectra were acquired using positive ion nano ESI mode with the FT-ICR acquiring precursor spectra from 200-2000 m/z. For tandem MS, precursor ions were fragmented by collision-induced dissociation (CID). MS/MS spectra were acquired in a data dependent manner from the 5 most intense precursor ions of each FT-ICR MS scan. The RAW data files were processed by Xtract™ function in Xcalibur™ (Thermo Fisher Scientific), which generated reduced data files containing the deconvoluted masses and intensities for MS spectra. For a reduced data file, all deconvoluted MS spectra are summed to create a single pseudo-MALDI TOF mass spectrum by use of an in-house developed program written in Python. In brief, the peaks in all MS spectra are merged and peaks with m/z values matching within 10 ppm are summed into a single peak with the summed intensities.

Enzymatic competition assay

IDE enzyme activities were assayed using the bradykinin mimetic substrate V (7-methoxycoumarin-4-yl-acetyl-NPPGFSAFK-2,4-dinitrophenyl).^{16; 31} 85 μ l of 5 μ M substrate V was mixed with 10 μ l of Ub (WT and mutants) at various concentrations. The reactions were initiated by addition of 5 μ l of IDE alone (1.8 μ M) or after pre-incubation with nestin fragment (0.2 μ M). The substrate V degradation was monitored by fluorescence intensity on a Tecan Safire microplate reader at 37 °C for 15 min with excitation and emission wavelengths at 327 nm and 395 nm, respectively. Data were plotted and analyzed with EXCEL and SIGMAPLOT software.

Protein crystallization

The complex of the catalytically less active cysteine-free IDE mutant, IDE-CF-E111Q with Ub was purified by gel filtration (Superdex 200) and immediately used after purification. 1 μ l of protein (15–20 mg/ml) and 1 μ l of crystallization solution (10–13% PEG MME 5000, 100 mM HEPES, pH 7.0, 10–14% Tacsimate, 10% dioxane) were mixed and equilibrated with 500 μ l of well solution at 18 °C by the hanging drop method. Clusters of needle crystals appeared in 3–5 days (Fig. S1). For data collection, crystals were sequentially equilibrated in 15% and 30% glycerol cryo-protective solutions containing reservoir buffer and then flash-frozen in liquid nitrogen. The presence of Ub in the IDE-Ub crystal was verified by MALDI-TOF MS (Fig. S1).

X-ray data collection and structure determination

Diffraction data were collected at 100 K at the Structural Biology Center 19-ID beamline of the Argonne National Laboratory Advanced Photon Source. The data sets were processed with HKL2000. Structure determinations were performed by molecular replacement using the previously known IDE-E111Q structure (PDB: 2G47) as the search model. Structure refinement and rebuilding were performed by CNS and COOT. The absence of extra electron density in the catalytic chamber was assessed by inspection of both σ_A -weighted $2F_o - F_c$ and $F_o - F_c$ difference electron density maps, as well as by inspection of the $2F_o - F_c$ -simulated annealing omit map. The final model had R_{work} and R_{free} of 21.0% and 24.0%,

respectively. The extra electron density in the exosite was fitted by residues 1-3 of Ub. The refinement statistics are summarized in Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IDE	insulin-degrading enzyme
CF	cysteine-free
IDE-N	N-terminal-domain of insulin-degrading enzyme
IDE-C	C-terminal domain of insulin-degrading enzyme
WT	wild-type
Ub	ubiquitin

Ub1-74	1-74 fragment of ubiquitin
Ub1-72	1-72 fragment of ubiquitin
MS	mass spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
FT-ICR	Fourier-transform ion cyclotron resonance
LITQ	linear trap quadrupole
ESI	electrospray ionization
CID	collision-induced dissociation
IGF-II	insulin-like growth factor-II
TGF-α	tumor growth factor- α
PEG MME	polyethylene glycol monomethyl ether
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SUMO1	small ubiquitin-related modifier-1
NEDD8	neural precursor cell expressed developmentally downregulated 8
BAG-1	BAG family molecular chaperone regulator 1

Fig. 1. Identification of Ub as a substrate of IDE. (A) Model of Ub inside the catalytic chamber of IDE. IDE is depicted with the N- and C-terminal domains colored red and blue, respectively. The proposed orientation of Ub within the IDE catalytic chamber shows the Ub N-terminus facing the exosite and the Ub C-terminal glycines facing the catalytic site. In this orientation, we also show the molecular surface of Ub (1UBQ) as calculated by APBS (<-6 kT in red, 0 kT in white, and >+6 kT in blue)⁵². The “+” “-” depict the chamber charge prosperity of IDE. (B) Representative MALDI-TOF mass spectra of Ub before (*top panel*) and after IDE digestion (*lower panels*). (C) Crystal structure of IDE-CF-E111Q in complex with Ub. IDE-N and IDE-C are shown as red and blue cartoon, respectively. Zn²⁺ and Ub are depicted as pink sphere and green sticks, respectively. The residues of the exosite – Gly-339, Glu-341, Leu-359, and Gly-361 – are shown as cyan sticks. The simulated-annealing omit map is shown around Ub as grey mesh contoured at 2.0σ.

Fig. 2.

Nestin inhibits Ub binding to and degradation by IDE. (A) Representative MALDI-TOF mass spectra of Ub before (*bottom*) and after incubation with IDE in the absence (*middle panel*) and presence (*top panel*) of nestin fragment. (B) Inhibition of IDE-mediated degradation of fluorogenic substrate V by Ub (○), Ub1-74 (Δ), and Ub1-72 (□). (C) Inhibition of IDE-mediated degradation of fluorogenic substrate V by Ub1-72 after no IDE preincubation (□), after IDE preincubation with Ub1-72 followed by addition of 200 nM nestin (Δ), or after preincubation with 200 nM nestin followed by the addition of Ub1-72 (○).

Fig. 3.

Representative MALDI-TOF mass spectra of the time dependent reaction of Ub-F45W/L67A (*control shown in the top panel*) with IDE in the absence (*middle three panels*) or presence of 200 nM nestin fragment (*bottom panel*). Ub and IDE were mixed in a 200:1 molar ratio at 37 °C in 25 mM HEPES, pH 7.0. For comparison, a portion of the spectra is shown for Ub alone and after 1-min and 5-min incubations with IDE.

Fig. 4. The stability of Ub affects its degradation by IDE. The primary, secondary and tertiary structures of Ub are depicted with the seven major structural elements of Ub highlighted from the N-terminal end as: β₁, red; β₂, orange; α₁, yellow; β₃, green; β₄, blue; α₂, purple; β₅, magenta. Core residues were mutated on each of the seven major structural elements of Ub, which destabilized the protein by 0.5-8.2 kcal/mol [indicated by ΔΔ*G*^{40; 42; 50}]. The cleavage rates of Ub mutants of the second phase of cleavage by IDE are shown followed by the location of their first IDE cut sites (shown as black triangles or arrows) and the later IDE cut sites (shown as maroon triangles or arrows).

Fig. 5. Mutations at the exosite render IDE insensitive to changes in Ub stability. (A) SDS-PAGE of recombinant WT and mutant human IDE. (B) Relative activities are reported for each mutant of IDE assuming as 100% the activity of IDE-WT. Activity was monitored by measuring the fluorescence resulting from the cleavage of 4.25 μ M substrate V. Data are mean $\pm$ SEM for two independent experiments with two or three replications per condition. (C) Representative MALDI-TOF mass spectra of Ub-F45W/L67A before (bottom panels) and after WT and mutant IDE incubations (upper three panels). Ub and IDE were mixed in a 200:1 molar ratio for the proteolytic assay.

Fig. 6. Model of Ub degradation by IDE and nestin-mediated regulation. (A) Three-step mechanism of Ub degradation by IDE. (1) The flexible C-terminal tail of Ub swings toward the catalytic cleft without considerable disruption of the Ub fold. (2) The exosite binds the N-terminus of Ub and facilitates in slight unfolding of the peptide and alignment of residues 72 and 73 at the active site. (3) IDE may further degrade the Ub protein processively along the C- to N-terminal direction, depending on Ub instability. (B) Inhibition of Ub degradation by nestin-mediated modulation of the open/closed state of IDE. IDE exists in equilibrium between an open and closed state. While IDE is open, both Ub and small substrates may enter the chamber. However, nestin binds to the exterior surface of IDE, which likely restricts IDE to a partially open state. This partially open state provides enough entry space for small substrates. Ub is denied access to the chamber and is thus not cleaved.

Table 1

Data statistics for the crystal of IDE-Ub complex:

	IDE-Ub
Data Collection	
Beamline	APS 19ID
Space group	P6 ₅
Cell dimension(Å)	
a	262.9
b	262.9
c	91.0
Resolution (Å)	50-2.35
R _{sym} (%) ^a	16.2(53.3) ^e
I/sigma	21.3(2.6) ^e
Redundancy ^b	5.6(3.7) ^e
Completeness (%)	100.0(99.8) ^e
FOM(Figure of Merit)	
Unique reflections	148306
Refinement	
R _{work} ^c	21.0 %
R _{free} ^d	24.0 %
No. atoms	
Protein	15668
Water	333
B-factors	
IDE	32.2
Substrate	44.8
Water	28.6
r.m.s. deviations	
Bond lengths (Å)	0.02
Bond angles (°)	1.98
Ramachandran plot (%)	
Favorable region	92.4
Allowed region	7.5
Generously allowed region	0.1
Disallowed region	0
PDB code	3OFI

$$^a R_{\text{merge}} = \Sigma(I - \langle I \rangle) / \Sigma \langle I \rangle$$

$$^b N_{\text{Obs}}/N_{\text{unique}}$$

$$^c R_{\text{work}} = \Sigma_{hkl} \|F_{\text{Obs}}| - k |F_{\text{Calc}}\| / \Sigma_{hkl} |F_{\text{Obs}}|$$

^d R_{free} , calculated the same as for R_{work} but on the 5% data excluded from the refinement calculation.

^ethe outer resolution shell. Values in parentheses indicate the highest resolution shell