Characterization of the structural determinants of the ubiquitin-dependent proteasomal degradation of human hepatic tryptophan 2,3-dioxygenase

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Abstract

Human hepatic tryptophan 2,3-dioxygenase (hTDO) is a homotetrameric hemoprotein. It is one of the most rapidly degraded liver proteins with a half-life ($t_{1/2}$) of ~2.3 h, relative to an average $t_{1/2}$ of ~2–3 days for total liver protein. The molecular mechanism underlying the poor longevity of hTDO remains elusive. Previously, we showed that hTDO could be recognized and ubiquitinated by two E3 ubiquitin (Ub) ligases, gp78/AMFR and CHIP, and subsequently degraded via Ub-dependent proteasomal degradation pathway. Additionally, we identified 15 ubiquitination

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

CRediT Author Contribution

Maria Almira Correia: Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing — original draft, Project administration, Writing — review and editing. Yi Liu: Resources, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing — review and editing. Sung-Mi Kim: Conceptualization, Resources, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing — original draft, Writing — review and editing. YongQiang Wang: Conceptualization, Resources, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing — original draft, Writing — review and editing. Shay Karkashon: Resources, Data curation, Formal analysis, Validation, Investigation, Methodology, Writing — review and editing. Ariel Lewis-Ballester: Resources, Formal analysis, Supervision, Investigation, Methodology. Syun-Ru Yeh: Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing — original draft, Writing — review and editing.

Preprint
An earlier version of this manuscript was published as a preprint [81].
K-sites and demonstrated that Trp-binding to an exosite impeded its proteolytic degradation. Here, we further established autophagic-lysosomal degradation as an alternative back-up pathway for cellular hTDO degradation. In addition, with protein kinases A and C, we identified 13 phosphorylated Ser/Thr (pS/pT) sites. Mapping these pS/pT sites on the hTDO surface revealed their propinquity to acidic Asp/Glu (D/E) residues engendering negatively charged DEpSpT clusters vicinal to the ubiquitination K-sites over the entire protein surface. Through site-directed mutagenesis of positively charged patches of gp78, previously documented to interact with the DEpSpT clusters in other target proteins, we uncovered the likely role of the DEpSpT clusters in the molecular recognition of hTDO by gp78 and plausibly other E3 Ub-ligases. Furthermore, cycloheximide-chase analyses revealed the critical structural relevance of the disordered N- and C-termini not only in the Ub-ligase recognition, but also in the proteasome engagement. Together, the surface DEpSpT clusters and the N- and C-termini constitute an intrinsic bipartite degron for hTDO physiological turnover.

Introduction

Tryptophan 2,3-dioxygenase (TDO) is the rate-limiting enzyme in the kynurenine pathway. It catalyzes the oxidative breakdown of the essential amino acid, L-tryptophan (Trp) to N-formylkynurenine (NFK) [1–8]. This reaction is also carried out by an analogous hemoprotein, indoleamine 2,3-dioxygenase (IDO), albeit with a much lower substrate selectivity [4–6,8]. Unlike TDO, which is largely localized in the liver and brain, IDO exhibits a much broader tissue distribution [4–6,9]. The product generated from either TDO or IDO-mediated Trp breakdown is then further converted into various physiologically and pathophysiologically relevant derivatives, including the essential cofactor NAD⁺, along the kynurenine pathway [4–9]. TDO is known to be critical for regulating the Trp flux into the kynureninic versus serotonergic pathways, thereby controlling the serotonergic tone in the central and peripheral nervous systems [9–13]. In addition, it is pathophysiologically relevant in a variety of human diseases, such as cancer, central nervous system depression, immunocompromise diseases, and schizophrenia [9–25]. As such, TDO, along with IDO, is a key target of chemotherapeutic drug development [25–31].

The recently reported crystal structure of the human TDO (hTDO) verifies that it is a homotetrameric hemoprotein formed by a dimer of dimers (Figure 1) [32]. Each of the four subunits comprises a four-helix bundle constituting the tetrameric interface. One end of the four-helix bundle binds a prosthetic heme moiety that is co-ordinated by His328, as its proximal ligand, and an O₂ molecule, as its distal ligand. A Trp molecule is nestled above this heme-bound O₂, which is strategically placed for the insertion of the O₂ into the C₂=C₃ bond of its indole ring. This Trp molecule is stabilized by the prosthetic heme, as well as its surrounding residues, via a variety of hydrophobic and hydrophilic interactions [32]. Although the hTDO structure shows that all four of its active sites are complexed with heme, the endogenous hepatic heme availability permits only two of the four subunits to be saturated with heme at any given time in vivo. Accordingly, the protein normally exhibits ~50% of its full catalytic capacity [34,35]. The addition of heme in vitro or injection of exogenous heme in vivo can restore TDO to its full catalytic potential [34,35].
Physiologically, TDO is in equilibrium with the hepatic free-heme pool [34], thereby serving as a hepatic heme-sensor.

Early studies with purified *Pseudomonas* TDO have revealed that the Michaelis–Menten plot of the enzyme activity exhibits an unusual sigmoidal behavior [36,37] and that inclusion of α-methyltryptophan (αMTrp, a Trp derivative that cannot bind to the active site due to steric clashes of its α-methyl moiety with the heme) in the enzyme assay converts the sigmoidal behavior to hyperbolic behavior. This observation engendered the hypothesis that the enzyme contains a second Trp-binding site [36,37]. The hTDO crystal structure firmly verified the presence of this second Trp-binding site (referred to as exosite hereafter) at one end of the four-helix bundle opposite to the active site (Figure 1) [32]. The Trp-affinity of the exosite was found to be 100-fold higher than that of the active site (with a $K_d$ of ~0.5 μM versus 54 μM) [32], which provides a molecular explanation for the sigmoidal behavior of the enzyme activity and the ability of αMTrp (which binds to the exosite, but not the active site) to revert the sigmoidal behavior to hyperbolic behavior. At this exosite, the indole ring of the Trp is sandwiched between Trp$^{208}$ and Pro$^{213}$, and is in direct contact with several other residues, enabling H-bonding interactions of its carboxylate and ammonium groups with the sidechains of Arg$^{211}$, Arg$^{103}$ and Glu$^{105}$ [32]. Site-directed mutagenesis of Glu$^{105}$/Trp$^{208}$/Arg$^{211}$ to Leu/Val/Leu (referred to as the EWR mutant hereafter) abolishes the high-affinity Trp-binding, but retained the low-affinity Trp-binding, as well as hTDO enzymatic proficiency [32].

Classic studies of Schimke et al. [38–40] documented that the proteolytic stability of hepatic TDO is enhanced by the administration of its substrate Trp to rats, thereby establishing the then radical concept of ‘substrate-mediated enzyme induction via protein stabilization’ in mammals. These earlier findings, combined with our structural discovery of the exosite, led us to examine whether Trp-binding to the exosite was involved in the substrate-mediated stabilization of hTDO in human liver HepG2 cells [32]. We found that treating the cells with αMTrp prolonged the lifetime of the wild type protein, but not the EWR mutant, and that the EWR mutation shortened the lifespan of the protein, which verified the relevance of the hTDO exosite to its substrate-elicited cellular stabilization. Additionally, we discovered that hTDO could be degraded via the ubiquitin (Ub)-dependent proteasomal degradation pathway (UPD) and that three E2/E3 Ub-ligase complexes could ubiquitinate hTDO with the following efficiency: Ubc7/gp78 > UbcH5a/CHIP > Ubc7/Hrd1. With Ubc7/gp78 and UbcH5a/CHIP, the two major E2/E3 Ub-ligase complexes involved in the UPD pathway of hTDO and several other hepatic proteins, we further identified 15 ubiquitinated Lys (K)-sites in the protein [32].

In this current study, we further interrogated the relative contribution of the autophagic-lysosomal degradation (ALD) pathway, with respect to the UPD pathway, in hTDO cellular stability. We characterized UPD as the major cellular disposal route for hTDO, with ALD as an alternative back-up pathway. We identified 13 phosphorylated Ser/Thr (pS/pT) sites in close proximity to acidic Asp/Glu (D/E) residues on the hTDO surface, which effectively constitutes negatively charged clusters (referred to as DEpSpT or DEST for the phosphorylated and unphosphorylated clusters, respectively, hereafter) surrounding or vicinal to the previously identified ubiquitinated K-sites. Through site-directed mutagenesis
of two positively charged gp78-subdomains, known to interact with similar DEpSpT clusters in P450 enzymes [41], we infer that charge–charge interactions between gp78 and the negatively charged DEpSpT surface clusters is likely critical for hTDO ubiquitination. We further systematically examined the relevance of several critical hTDO structural elements, including its N- and C-termini and the active site, in addition to the exosite, to its proteolytic stability.

**Materials and methods**

**Materials**

PKA was purchased from New England BioLabs (Ipswich, MA, Cat. # P6000S). PKC-α (Cat. # SRP5251), PKC-βII (Cat. # P3287), PKC-γ (Cat. # K4518), PS (1,2-Diacyl-sn-glycero-3-phospho-L-serine, Cat. # P6641), DAG (1,2-Dioleoyl-sn-glycerol, Cat. # D0138) and Trp (Cat. #45ZU64) were acquired from Sigma–Aldrich (St. Louis, MO). Anti-Ub (Cat. # 43124), anti-His (Cat. # 12698), anti-GAPDH (Cat. # 5174) and anti-HA (Cat. # 3724) were purchased from Cell Signaling Technology (Danvers, MA).

**Plasmids**

The expression plasmid of hTDO (pcDNA6-hTDO) was constructed by inserting a DNA fragment encoding the wild type full-length (FL) or mutants of human TDO into the pcDNA6-(His)₆ vector. The His₆-tag was present at the C-terminus in all hTDO constructs. In the EWR mutant construct, Glu¹⁰⁵/Trp²⁰⁸/Arg³¹¹ were mutated to Leu/Val/Leu by QuickChange Lightning mutagenesis kit (Agilent Technologies, Santa Clara, CA, U.S.A.). In the truncation mutant constructs, ΔN, ΔC and ΔNC, the N-terminal fragment (1–38), C-terminal fragment (383–406) or both were similarly deleted. In the DE and 5S mutant constructs, 5 Asp/Glu residues in an acidic D/E patch or 5 Ser residues in potential phosphorylation sites in the C-terminus, respectively, were mutated to Gly (the mutated residues are underlined in the sequence shown at the bottom of Figure 4). In the active site mutant constructs, His³²⁸ and His⁷⁶ were replaced with Ala and Ser, respectively, using a site-directed mutagenesis kit as described [41]. The expression plasmid for the wild type gp78 (pcDNA6-gp78WT) was constructed as reported previously [42]. Its structural mutant (pcDNA6-gp78PT), wherein the positively charged (Arg³¹⁰/Arg³¹¹/His³¹²/Lys³¹³) and (Gln⁵⁸⁴/Arg⁵⁸⁵/Lys⁵⁸⁶) residues, documented to interact with negatively charged clusters in P450s [41], were mutated to Ala, and synthesized as a 309–643 residue encoding DNA, followed by a stop codon (Genewiz, South Plainfield, NJ) and ligated into the pcDNA6-HA vector. All constructs were verified through DNA sequencing analyses.

**Assessment of cellular hTDO degradation via UPD and ALD pathways**

Human hepatocellular HepG2 cells were maintained at 37°C under 5% CO₂ in minimum essential high glucose medium (MEM) containing nonessential and essential amino acids (EAA, including 0.050 mM L-Trp), 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were transfected with a plasmid encoding hTDO-(His)₆ and cultured for 48 h, and then treated with the UPD inhibitor, MG262 (20 μM), or ALD inhibitors, 3-methyladenine (3-MA; 5 mM) and NH₄Cl (10 mM).
Cycloheximide (CHX)-chase analyses

HepG2 cells were transfected with a plasmid encoding the wild type or mutant of hTDO-(His)_6 and cultured for 48 h. CHX (50 μg/ml) was then added to block de novo protein synthesis, and cells cultured and harvested at various time points thereafter. The cell lysate at each time point was collected and subjected to Western immunoblotting with anti-His_6 and anti-TDO IgGs and anti-GAPDH IgG (loading control). Transfection of each mutant construct and CHX-treatment were performed in 3–6 individual cell cultures to obtain 3–6 biological replicates at each time point. Cell lysates were subjected to Western immunoblotting analyses, and images were quantified using ImageStudioLite (Licor Corp.) and normalized to their corresponding GAPDH-loading control, and expressed as % of their corresponding 0 min control for each individual experiment. The Mean ± SD values from the 3 to 6 biological replicates were determined and the data were then fitted to one-phase exponential decay model using non-linear least squares (ordinary) fit using Prism Graphpad Version 6.07 as described previously [43]. This computation analysis does not yield ± SD values of the Mean t_{1/2} of the biological replicates, just the 95% confidence intervals for each t_{1/2}, which are listed in Table 2.

35S-L-Met/Cys-pulse-chase analyses of FL-TDO, and its structural mutants

HepG2 cells were transfected with a plasmid encoding the wild type or mutant of hTDO-(His)_6 and cultured in DMEM (Dulbecco’s modified Eagle medium) for 48 h. The culture medium was then removed and replaced with methionine/cysteine-free MEM (containing 0.05 mM L-Trp along with other EAA), with or without added αMTrp (2.5 mM) for 1 h, and subsequently pulsed with 35S-L-Met/Cys (75 μCi) for 1 h at 37°C as described previously [32]. After two washes with ice-cold PBS containing Met (0.2 mM)/Cys (1.4 mM), cells were further incubated with MEM containing cold Met (5 mM)/Cys (5 mM) with or without added αMTrp (2.5 mM), for 0, 30, 60 and 90 min at 37°C. The cells were lysed with Cell-Signaling Lysis buffer, containing general protease/phosphatase inhibitors, and N-ethylmaleimide (10 mM) to inhibit cellular deubiquitinases. The lysate was sedimented at 10 000 g at 4°C for 10 min to remove insoluble cell debris. Its protein concentration was determined by the bicinchoninic assay (BCA). Lysate protein (200 μg) was then diluted (1 : 4, v : v) in Dynabead pull-down buffer and mixed with Dynabeads (50 μl), and incubated at 4°C with rotation overnight. The Dynabeads-His_6-tagged hTDO protein complexes were then collected using a magnetic stand and washed five times with Dynabead-washing buffer. The His_6-tagged hTDO proteins were eluted by heating the complexes for 5 min in an SDS-PAGE sample-loading buffer (40 μl, 62.5 mM Tris buffer containing 25% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue). A 10 μl- aliquot of the eluate was collected, whose radioactivity was monitored in 4 ml of Ecolmune using a Beckman LS3801 liquid scintillation counter. The radioactivity counts of the various time points were plotted as a function of time. The half-life (t_{1/2}) of each sample was calculated based on single exponential fit of the data with Prism Graphpad Version 6.07 as described previously [43].
**35S-pulse-chase analyses of FL-TDO, ΔNC and ΔNC/EWR ubiquitination**

Plasmids containing hTDO [pcDNA6-hTDO-His<sub>6</sub>] or its corresponding deletion (ΔNC) and/or site-directed (ΔNC/EWR) mutants were transfected in HepG2 cells for 48 h, at the end of which DMEM was replaced with a Met/Cys-free DMEM with or without αMeTrp (2.5 mM), pulse-chased with 35S-L-Met/Cys EXPRESS mixture (75 μCi) for 1 h at 37°C exactly as described above [32]. After extensive cold Met (5 mM)/Cys (5 mM) chases, cells were harvested at the times indicated. His<sub>6</sub>-tagged 35S-hTDO, its ΔNC and/or ΔNC/EWR mutants were isolated from the cell lysates by Talon-Dynabeads pull-down analyses. After extensive washes of the beads, the hTDO species were eluted and equal volume aliquots of the pull-down isolates subjected to SDS-PAGE/fluorography with Typhoon scanning as described above.

**hTDO phosphorylation by PKA and/or PKC**

Purified recombinant FL-hTDO (400 pmol) was incubated separately with PKA (2 ku) or one of the three isoforms of PKC (α, βII, γ) (0.2 μg, activity 200 pmol/min/μg) or a mixture of all kinase isoforms in a Tris buffer, containing 0.5 mM ATP, ATP-generating system, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2 mM DTT, 1 mM CaCl<sub>2</sub> and a lipid mixture (PS 100 μg/ml and DAG 20 μg/ml), at 30°C for 30 min as described previously [44]. An additional sample was incubated without ATP as a parallel non-phosphorylated control. hTDO-(His)<sub>6</sub> protein was isolated with Dynabeads, reduced, alkylated, and subjected to SDS–PAGE as described previously [44–46]. Protein bands were excised from the gel, followed by in-gel digestion first with trypsin, and then with lysyl endoprotease C (Lys-C) as detailed previously [45,46]. The digested peptides were analyzed by LC–MS/MS analyses on LTQ Orbitrap or LTQ Velos Orbitrap mass spectrometers (ThermoFisher Scientific, San Jose, CA), both equipped with a Waters nanoAcquity UPLC system (Milford, MA) as described previously [45,46]. The peak lists from all MS runs were generated using an in-house script, PAVA [47] based on the Raw Extract script in Xcalibur v3.1 (Thermo Scientific). The peak lists were first searched against the SwissProt.2019.4.8 database using the in-house ProteinProspector search engine (v 6.2.23) with no organism restrictions to identify proteins present in the samples. Constant modification of carboxymethyl on cysteine and various modifications of N-terminal methionine loss and acetylation, N-terminal glutamine conversion to pyroglutamate, oxidation of methionine, were employed as the default setting, with a mass tolerance of 20 ppm for the parent/precursor ion as well as a maximal allowance of one missed cleavage. A second search was carried out that included variable phosphorylations on serine and threonine against the same SwissProt database using the protein list identified from the first search (150/559228 entries searched). The phosphopeptide identification and the site assignment were generated and annotated by ProsPector itself [48], and manually verified by inspection of the raw MS/MS spectra. False discovery rates (FDRs) for the phosphorylation assays were estimated to be 0.1% corresponding to the maximum expectation values of 0.001, and a minimum peptide score of 25 was used in the assays. Mass tolerance for parent/precursor ions was 20 ppm and fragment mass tolerances of 0.2 Da/300 ppm for CID ions detected in LTQ Orbitrap and 30 ppm for HCD ions detected in LTQ Velos Orbitrap mass spectrometers, were respectively employed. A maximum of two missed and/or non-specific cleavages were also permitted.
The TDO phosphorylation stoichiometry was estimated by the application of a label-free semi-quantitative analysis as described [45,46].

**Effects of PKA/PKC inhibitors on cellular hTDO ubiquitination**

HepG2 cells were transfected with a plasmid encoding the wild type hTDO-(His)\(_6\) and cultured with the diagnostic PKA inhibitor (KT5720, 5 \(\mu\)M) and/or PKC inhibitor (Ro-32-0432/Bis XI, 1 \(\mu\)M) for 30 min. They were then treated with the proteasomal inhibitor, MG-262 (1 \(\mu\)M), to optimize the detection of the ubiquitinated species, and further cultured for 16 h and then harvested. The cell lysates were collected and blotted against anti-His\(_6\) antibody (input) or pulled down with magnetic Dynabeads to isolate the His\(_6\)-tagged hTDO protein. The isolated hTDO protein was then solubilized and subjected to Western immunoblotting against an anti-Ub antibody.

**Effect of gp78 mutation on hTDO ubiquitination**

HepG2 cells were co-transfected with a plasmid encoding hTDO-(His)\(_6\) and a plasmid encoding wild type HA-gp78 or its mutant (pcDNA6-gp78PT), wherein the positively charged K, R or Q residues interacting with the P450 DEpSpT clusters [41,49] were mutated to Ala. The cells were cultured for 40 h, and then treated with the UPD proteasomal inhibitor, MG262 (1 \(\mu\)M), and cultured for additional 8 h. Cells were then lysed with RIPA buffer, containing proteinase/phosphatase inhibitors and N-ethylmaleimide (20 mM). The lysates were clarified by centrifugation at 10,000 \(g\) at 4°C for 10 min. The lysate (500 \(\mu\)g) was collected and treated with magnetic Dynabeads to isolate the His\(_6\)-tagged hTDO protein, which was then solubilized and subjected to Western immunoblotting against anti-Ub, anti-His\(_6\), anti-HA or anti-GAPDH antibody.

**Results and discussion**

**Relative contribution of ALD versus UPD pathway to intracellular hTDO degradation**

We have previously reported that hTDO incurs ubiquitination that targets it to UPD in HepG2 cells, using the proteasomal inhibitor MG132 as the diagnostic probe [32]. Herein we examined the relative contribution of ALD versus UPD to the proteolytic turnover of hTDO by employing diagnostic inhibitors of each pathway in HepG2 cells transfected with a plasmid encoding a six histidine-tagged hTDO. We found that upon treating the cells with the proteasomal inhibitor MG262, both the parent protein and its ubiquitinated species were greatly stabilized (Figure 2). Similarly, upon treating the cells with ALD inhibitors, 3-methyladenine (3-MA, which inhibits autophagosomal formation) coupled with \(\text{NH}_4\)Cl (which inhibits lysosomal proteases), the parent hTDO protein was stabilized, albeit to a lesser extent. These findings suggested UPD as the major intracellular degradation pathway, with ALD as a back-up pathway.

Although hepatic hTDO is a cytoplasmic enzyme, it is ubiquitinated largely by gp78/AMFR, an endoplasmic reticulum (ER) E3 Ub-ligase, and CHIP, a cytoplasmic E3 Ub-ligase, but not Hrd1, a significant ER-associated degradation contributor in yeast [32]. Herein, in addition to those of gp78, CHIP, and Hrd1, we also examined the relative contributions of FL-TEB4/MARCH IV (a mammalian homolog of yeast Doa10, another ER E3 Ub-ligase involved in
the ubiquitination of both cytoplasmic and ER-proteins [50]) in the UPD-dependent hTDO degradation, by co-expressing each ligase with hTDO in HepG2 cells. We found that TEB4/ MARCH VI and Hrd1 were considerably more sluggish in catalyzing hTDO degradation relative to gp78 and CHIP (Supplementary materials, Figure S1). Our current understanding of the hTDO UPD pathway is depicted (Scheme 1).

**Role of phosphorylation**

The role of protein phosphorylation in the ubiquitination of various short-lived regulatory proteins has been well documented [51–57]. However, it is unclear how precisely protein phosphorylation promotes protein ubiquitination. Multiphosphorylation sites within conserved primary sequence motifs, known as ‘phosphodegrons’, are a common feature of target protein recognition by the E3 Ub-ligase, SKP1-CUL1-F-Box protein complex [58–60]. Likewise, CHIP-mediated protein ubiquitination involves distributed phosphodegrons, consisting of phosphorylation sites dispersed over the entire protein sequence [61–63] that function cooperatively to promote target protein recognition in a highly phosphorylation- and sequence-dependent manner [61–63]. On the other hand, tetra-glutamate residues adjacent to a K-residue in HMGCoA reductase have been proposed to be critical for its gp78-mediated ubiquitination [64]. Similarly, K residues in the E/D-enriched sequences of a yeast integral membrane protein have been reported as preferred ubiquitination sites for other E3 Ub-ligases [65]. Previously, we found that (i) gp78 and CHIP ubiquitinate K-residues in two P450s (CYP3A4 and CYP2E1) within linear or spatially organized DEpSpT clusters situated on the external surface, loops or disordered regions [41, 45, 46], and (ii) two relatively promiscuous protein kinases, protein kinase A (PKA) and protein kinase C (PKC), phosphorylate certain S/T residues within P450 DEST clusters, thereby enhancing their ubiquitination and accelerating their UPD-dependent degradation [41, 44–46].

To examine the role of PKA- and PKC-mediated phosphorylation in regulating the cellular ubiquitination of hTDO, we transfected HepG2 cells with a plasmid encoding hTDO and treated them with the diagnostic PKA inhibitor, KT5720, and/or PKC inhibitor, Ro-32-0432/Bis XI. We found that either PKA or PKC inhibitor resulted in a small, albeit appreciable, impairment of hTDO ubiquitination, and that co-treatment with both inhibitors significantly enhanced this impairment (Figure 3A). These data support the relevance of these kinases in promoting hTDO ubiquitination in intact cells. We next used LC-MS/MS, combined with peptide mapping [45, 46] to determine the specific phosphorylation sites upon incubation of purified hTDO protein with PKA and various PKC-isomers. We identified 13 hTDO phosphorylation pS/pT sites (Table 1). It is notable that three of these 13 sites (S23, S155, and S345) have been previously documented (https://www.phosphosite.org//proteinAction?id=8460&showAllSites=true).

Although the phosphorylation levels of some of these S/T residues are somewhat low (Table 1), it is important to note that post-translational protein modifications are dynamic regulatory processes. At best 1–3% of a given protein is estimated to be phosphorylated or ubiquitinated at any given time, due to the existence of abundant cellular phosphatases and deubiquitinases. Given this dynamic nature, phosphorylation of the S/T residues is transient and need not be stoichiometric. For instance, the impairment in CYP3A4 ubiquitination

Biochem J. Author manuscript; available in PMC 2022 March 03.
elicited by the Ala mutation of its \text{S}^{178}, \text{ which was phosphorylated merely to an extent of 1.29\% by PKA}, was far greater than that elicited by the double Ala mutation of \text{T}^{264} \text{ and S}^{420}, \text{ which were phosphorylated to an extent of 23.2\% and 14.1\%, respectively [44,46]. Similarly, in CYP2E1, the Ala mutation of \text{S}^{129}, \text{ which was >95\% phosphorylated, had no influence on its UPD [45,66]. Such a dynamic phosphorylation process could transiently alter the intrinsic negative charge character of each DEpSpT cluster, thereby promoting its productive molecular interactions with the positively charged patches of the Ub-ligases. In turn, this would enable progressive waves of E2/E3-recognition of individual K-residues within or vicinal to the DEpSpT clusters variously dispersed on the target protein surface. Our findings that such post-translational modifications occur largely on the external surfaces of the \text{hTDO} dimers, but not on their internal self-interacting/apposing surfaces, reveal that intracellularly, PKA/PKC and the E3 Ub-ligases target the intact homotetrameric \text{hTDO} protein, rather than the monomeric or dimeric species whose internal surfaces would be solvent exposed and accessible to post-translational modification (Supplementary materials, Figure S2).

**Role of surface DEpSpT clusters**

The 13 phosphorylation pS/pT sites, along with the 15 previously identified ubiquitination K-sites [32], were mapped on a \text{hTDO-Trp} complex structure (Figure 4). Visual inspection of this structure reveals that the ubiquitinated K-sites not only lie largely on the external surface, but also in close proximity to the pS/pT sites as well as negatively charged D/E residues. We have previously discovered that, in CYP3A4 and CYP2E1, the negatively charged residues in the ‘DEpSpT’ clusters within disordered loops or clustered together on the surface by the tertiary protein fold, constituted phosphodegrons that were important for the molecular recognition by the positively charged domains of gp78 and CHIP Ub-ligases [41,45,49]. The similar distribution of the DEpSpT clusters, vicinal to the ubiquitinated K-sites, on the \text{hTDO} surface (Figure 4) suggests that such negatively charged clusters also function as recognition sites for the gp78 E3 Ub-ligase.

To evaluate this possibility, we co-transfected HepG2 cells with a plasmid encoding \text{hTDO} and a plasmid encoding either wild type gp78 or its mutant, with the positively charged K, R, H or Q residues in the 310–313- and 584–586-subdomains (previously documented to interact with specific P450 DEpSpT clusters [41,49]), mutated to Ala. Cells were cultured in the presence or absence of the UPD proteasomal inhibitor MG262, in order to enable the detection of \text{hTDO} ubiquitinated species. We found that unlike wild type gp78, the gp78 mutant failed to enhance \text{hTDO} ubiquitination above basal levels (Figure 3B; + MG262). Correspondingly, the gp78 mutant also impaired \text{hTDO} proteasomal degradation (see the relative parent \text{hTDO} bands at ~47 kDa in the presence or absence of MG262). These findings confirm the importance of the positively charged gp78-domains in its plausible interactions with the acidic \text{hTDO} DEpSpT clusters, in determining its proteolytic stability. In addition, they expand the molecular gp78 substrate-recognition paradigm beyond P450s and suggest that it may extend to other gp78-targets.
Effects of N- and C-terminal truncations

The structural features highlighted in Figure 4 show that the surface DEpSpT clusters vicinal to the ubiquitination K-sites in hTDO are enriched in two structural regions: (i) the N-terminal region involving the N-terminal tails of two adjacent subunits, the active site of one of the two subunits, and the helix-loop-helix domain of the 3rd subunit, and (ii) the C-terminal region involving the C-terminal tails of two adjacent subunits and the exosites and helix-loop-helix domains of the same subunits. Previously, we found that the truncation of the intrinsically disordered N- and C-termini greatly improves the solubility and crystallization of hTDO [32], indicating their importance in defining the native conformation of the protein. Here we examined whether the truncation of the N- and/or C-termini affects the proteolytic stability of hTDO. We first transfected HepG2 cells with a plasmid encoding the wild type or a hTDO mutant, wherein the N-terminal subdomain (1–38), C-terminal subdomain (383–406) or both were truncated (hereafter denoted as ΔN, ΔC and ΔNC, respectively). The cells were then subjected to CHX-chase analyses. We found that the ΔN- and ΔC-truncations greatly lengthened the $t_{1/2}$ of hTDO from 45.2 min to 199.3 min and 212.7 min, respectively; while the coupling of the two truncations (ΔNC) further prolonged the $t_{1/2}$ to 247.2 min (Figure 5A and Table 2). The enormous enhancement in the hTDO cellular stability indicates the crucial role of the disordered/unstructured N- and C-termini in promoting its proteolytic instability.

The importance of the N-terminus is evident, as it contains three phosphorylation sites (T$^{14}$, S$^{23}$ and S$^{36}$) (Table 1) vicinal to the three ubiquitination K-sites (K$^{16}$ [67], K$^{17}$ and K$^{37}$ [32]) and that S$^{23}$ lies within an E/D cluster in the linear sequence (see the sequence at the top of Figure 4). The structural role of the C-terminus, on the other hand, is less clear. The truncated subdomain contains no ubiquitination K-sites, although a vicinal K$^{380}$ is found to be ubiquitinated [32], along with a vicinal phosphorylation site, T$^{384}$ (see the sequence at the bottom of Figure 4). To further interrogate the role of the C-terminus, we generated two mutants, DE and 5S, wherein 5 Asp/Glu residues in an acidic D/E patch or 5 Ser residues as potential phosphorylation sites were mutated to Gly. These residues are underlined in the sequence shown at the bottom of Figure 4. Our data showed that the 5S and DE mutations, like the ΔN- and/or ΔC-truncations, lengthened the $t_{1/2}$ from 45.2 min to 171.9 min and 191.7 min, respectively (Figure 5B and Table 2), indicating that the C-terminus, like the N-terminus, also regulates hTDO proteolytic stability plausibly by controlling the surface distribution of its DEpSpT clusters.

The phosphorylation S/T sites and D/E residues in the N- and C-termini are in close proximity to those in other structural elements within the same subunits or in neighboring subunits, gathered together by the three-dimensional fold of the quaternary assembly of the tetrameric protein (Figure 4). These negatively charged DEpSpT patches, vicinal to the ubiquitination K-sites, are irregularly distributed throughout the hTDO surface, thereby serving as recognition sites for the Ub-ligases. Previous studies revealed that unstructured/disordered regions in proteins can serve not only as Ub-ligase recognition sites [41, 68], but also as proteolytic initiation sites by engaging the proteasome through insertion into the narrow central aperture of its substrate-translocation channel [69–71]. Accordingly, we
propose that hTDO N- and C-termini are an integral part of its Ub-ligase recognition sites as well as important engagement sites for its proteasomal degradation.

We have previously documented employing $^{35}$S-pulse-chase analyses, that aMTrp-binding to the exosite rescues the poor longevity of hTDO in HepG2 cells, while EWR mutation in the exosite shortens its lifespan [32]. These findings, confirmed herein through CHX-chase analyses (Supplementary materials, Figure S3), underscore the crucial role of the exosite in stabilizing hTDO against proteasomal degradation. In the hTDO tetrameric structure, the C-terminal ends of two adjacent subunits lie right next to their exosites (Figure 4), indicating potential cross-talk between these two structural elements. Indeed, upon CHX-chase analyses, comparison of the proteolytic stability of the wild type protein versus that of the ΔNC-mutant upon EWR mutation (ΔNC/EWR) (Figure 6A and Table 2) revealed that the EWR mutation of the wild type protein shortened its $t_{1/2}$ from 45.2 to 27.0 min, consistent with that reported previously [32]. Surprisingly, the EWR mutation in the ΔNC-mutant also dramatically reduced its enhanced proteolytic stability shortening its $t_{1/2}$ from 246.2 to 67.0 min (Figure 6A and Table 2). Consistent with the significantly shortened $t_{1/2}$, $^{35}$S-pulse-chase analyses showed that the instability induced by the EWR mutation of the ΔNC-mutant was associated with the dramatically enhanced hTDO ubiquitination in HepG2 cells (Figure 6B). This cellular ubiquitination of the ΔNC/EWR mutant, unlike that of either the wild type protein or its ΔNC-mutant, as expected could not be mitigated by aMTrp-treatment of the cells. Thus, although the ΔNC-mutant is per se relatively stable to ubiquitination, this stability is further enhanced by aMeTrp (Figure 6B). In contrast, this stability is greatly lost upon its EWR mutation (Figure 6A). Indeed, 48 h post-transfection, very little of the ΔNC/EWR mutant exists as the parent 47 kDa species (Figure 6A) but is largely found as its ubiquitinated species (Figure 6B). Together, these findings underscore the pivotal structural relevance of an intact exosite to overall hTDO structural integrity, even in the absence of its L-Trp- or aMTrp-occupancy. These findings also rationalize the successful crystallization of hTDO/ΔNC in the absence of both L-Trp and heme [72]. Furthermore, the remarkably enhanced proteolytic instability introduced by the EWR mutation in the ΔNC-mutant with respect to that in the wild type protein indicates that there is a strong positive cooperativity between the N/C-termini and the exosite in regulating hTDO proteolytic stability, both in the presence and absence of L-Trp.

**Effect of active site mutation**

The substrate Trp in the hTDO active site is stabilized by a variety of interactions, including hydrophobic interaction between its indole ring and the porphyrin ring of the heme (which is co-ordinated by H$^{328}$ on the proximal side) and a H-bond between its indoleamine group and H$^{76}$ [32]. It is further secured by an extended H-bonding network between its ammonium and carboxylate groups, a propionate group of the heme, and the fully conserved ‘GTGG’ motif at the tip of the JK-Loop with a reversed turn structure. The JK-Loop contains three phosphorylation S/T sites (T$^{342}$, S$^{345}$, S$^{346}$) and a ubiquitination K site (K$^{339}$), and is located in the N-terminal structural region, where DEST clusters are enriched (Figure 4). The fact that the JK-Loop is totally disordered in the absence of Trp [72] suggests that Trp-binding to the active site might perturb DEST clusters critical for Ub-ligase recognition. To evaluate this possibility, we examined the proteolytic stability of the H328A and H76S mutants,
using CHX-chase or $^{35}$S-pulse-chase analyses. These two mutants were selected because we previously found that H328A mutation abolished spectrally detectable heme binding and catalytic function [73], plausibly also Trp-binding; whereas H76S mutation directly disrupts Trp-binding [33]. Our data indicated that the H328A or H76S mutation significantly lengthened their $t_{1/2}$ from 45.2 to 106.1 min, and from 90 to 230 min (Figure 7, Table 2). The observation that the two mutations introduced similar outcomes suggests that the enhanced proteolytic stability is a result of the disordering of the JK-Loop due to their inability to bind Trp at the active site. The disordering of the JK-Loop possibly leads to the redistribution of the DEST clusters on hTDO surface that weakens their interactions with Ub-ligases and renders the protein more proteolytically stable.

Collectively, our data reveal that the proteolytic stability of hTDO is negatively regulated by Trp-binding to the active site, while it is positively regulated by Trp-binding to the exosite, although the latter largely prevails given its relatively avid L-Trp-affinity. It raises an important question: Is there any cross-talk between the active site at one end of its 4-helical bundle and the exosite at the other end? To address this question, we examined whether EWR mutation perturbs the proteolytic stability of the H328A and H76S mutants with respect to that of the wild type protein. We found that the EWR mutation in the H328A and H76S mutants shortened their $t_{1/2}$ to 51.7 min and 118 min, respectively, just like it did in the wild type protein, indicating that the two structural elements are roughly orthogonal to each other (Figure 7, Table 2).

Conclusions

Protein levels in animal tissues are modulated by a wide variety of cellular factors. It has long been known that hepatic TDO level in rat liver is inducible by its substrate, Trp, [74–79] and that Trp does so by retarding TDO protein degradation [38–40]. The molecular basis for such Trp-mediated protein stabilization remained elusive for over 60 years. The recently solved crystal structure of hTDO enabled us to identify the long-suspected 2nd Trp-binding site (the exosite) in hTDO and determine its role in retarding hTDO proteolytic degradation upon Trp-binding [32]. It provided a molecular explanation for the ‘substrate-mediated enzyme induction via protein stabilization’ paradigm. Our current findings that H328A and H76S mutations boost hTDO proteolytic stability indicates that Trp-binding to the active site also contributes to the regulation of the proteasomal degradation, albeit in an opposite fashion (i.e. while Trp-binding to the exosite retards its proteolytic degradation, that to the active site may promote it). Nevertheless, given that hTDO is greatly stabilized upon exogenous Trp-administration [38–40,74–76], it appears that the exosite-binding of Trp ($K_d \approx 0.5 \mu M$) must prevail as a predominant Trp-mediated regulatory determinant of hTDO turnover. This regulation manifests not only at or below physiological Trp-levels (~50–100 $\mu M$), but even at greatly elevated cellular Trp-levels that would certainly exceed the requirements of its relatively low-affinity (54 $\mu M$) Trp-binding active site as well as prolong the Trp-saturation of the exosite.

Beyond the biological significance of the exosite to hTDO proteolytic stability, its disruption is also of clinical relevance. Genetic polymorphisms of hTDO exist in the human population with allelic variants resulting either in a prematurely truncated protein that is functionally
inactive, or a M108I mutation (80). M108 is a residue at the exosite whose mutation to Ile can perturb its Trp-binding. Although the M108I mutant can be expressed in vitro in a functionally active homotetrmeric form, its Trp-affinity to the exosite is two-orders of magnitude lower than that of wild type protein (80), which contributes to its accelerated proteolytic degradation and consequently to clinical hypertryptophanemia in affected individuals (80).

In summary, our current study identified UPD as the major pathway for hTDO degradation and ALD as a back-up pathway. It revealed the importance of the negatively charged DEpSpT clusters in Ub-ligase recognition and the critical role of the unstructured N- and C-termini in controlling hTDO proteolytic stability, by serving as recognition sites for Ub-ligases and possibly as initiation sites for proteasomal degradation. Additionally, it demonstrated not only the importance of the active site and the exosite in regulating hTDO proteolytic stability through binding the substrate Trp, but also underscored the critical role of an intact exosite to the overall hTDO structural integrity. Our current finding of gp78-mediated hTDO ubiquitination, coupled with our similar findings of hepatic P450s [41,45,49], reveal a common paradigm for protein recognition by gp78 through profound reliance on charge complementarity between its positively charged domains and the negatively charged DEpSpT surface clusters, within or vicinal to potential ubiquitination K-sites, in target proteins. Future study of other known gp78 substrates may reveal the universality of these gp78-molecular interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Prof. Mark Hochstrasser, Yale Univ, for a full-length TEB4/MARCH VI expression plasmid. We also acknowledge the use of the UCSF Bio-Organic Biomedical Mass Spectrometry Resource (Prof. A. L. Burlingame, Director) supported by the Adelson Medical Research Foundation.

Funding

Supported by NIH grants GM44037 (MAC), and GM 115773 (SRY). We also acknowledge the UCSF Biomedical Mass Spectrometry and Proteomics Resource Center (Prof. A. L. Burlingame, Director) supported by the Adelson Medical Research Foundation.

Data Availability

Our mass spectrometric analyses of the phosphorylation sites are available as Supplementary material, Figures S1–S3.

Abbreviations

\[\alpha\text{-MeTrp}\] α-methyltryptophan
3-MA 3-methyladenine
ALD autophagic-lysosomal degradation
AMFR  autocrine motility factor receptor
CHIP  C-terminus of Hsc70-interacting protein
CHX  cycloheximide
ER  endoplasmic reticulum
ERAD  endoplasmic reticulum-associated degradation
FL  full-length
hTDO  human TDO
IDO  indoleamine 2,3-dioxygenase
L-Trp  L-tryptophan
NFK  N-formylkynurenine
TDO  tryptophan 2,3-dioxygenase
Ub  ubiquitin
UPD  Ub-dependent proteasomal degradation

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Figure 1. hTDO crystal structure.

hTDO is a homotetramer constituted of a dimer of dimers. Each monomer comprises a four-helix bundle domain and a helix–loop–helix domain. One end of the four-helix bundle binds the heme prosthetic moiety and the substrate Trp (in the active site); while the other end of the bundle binds a second substrate Trp (in the exosite). In the active site, the heme is co-ordinated by H$^{328}$ on the proximal side; Trp sits on top of it next to the O$_2$ binding site in the distal pocket. The heme, Trp and H$^{328}$ are displayed as dark magenta sticks. The N-terminal fragment from the neighboring subunit that forms the roof of the active site is depicted as green cartoon. The residues important for stabilizing Trp in the exosite, E$^{105}$, W$^{208}$ and R$^{211}$, are shown as green sticks. The specific interactions stabilizing Trp in the exosite are illustrated in the inset (i). The tetrameric hTDO assembly is shown in the inset (ii), where the Trp bound in the exosites in subunit B and C are shown as red spheres. The N and C-terminal fragments (−1–38 and 383–406, respectively) are not shown as they are totally disordered and not resolved in the structure. The structure is based on a Trp-bound complex of hTDO (PDB code: 5TIA).
Figure 2. Relative contribution of ALD and UPD pathways to hTDO proteolytic degradation. HepG2 cells were transfected with a plasmid encoding hTDO-(His)$_6$ and cultured for 48 h. Cells were then further cultured with or without the proteasomal inhibitor, MG262, or the ALD inhibitor, 3-MA/NH$_4$Cl, and harvested at 6, 16 and 24 h thereafter. The cell lysate was collected and treated with magnetic Dynabeads to isolate the His$_6$-tagged hTDO protein, which was then solubilized and subjected to Western immunoblotting against an anti-Ub antibody (top), anti-His$_6$ antibody (to monitor the parent hTDO) (middle) or anti-GAPDH antibody (as a loading control) (bottom).
Figure 3. Effects of PKA and PKC inhibitors (A) or gp78 mutation (B) on hTDO proteolytic degradation.

(A) HepG2 cells were transfected with a plasmid encoding hTDO-(His)_6 and cultured with the PKA inhibitor, KT5720 (5 μM) and/or the PKC inhibitor, Ro-32-0432/Bis XI (1 μM) for 30 min. They were then treated with the proteasomal inhibitor, MG262, to maximize the detection of the ubiquitinated species, and further cultured for 16 h and then harvested. The cell lysate was collected and immunoblotted against anti-His_6 antibody or pulled-down with magnetic Dynabeads to isolate the His_6-tagged hTDO protein. The isolated protein was then solubilized and subjected to Western immunoblotting against an anti-Ub antibody.

(B) HepG2 cells were co-transfected with a plasmid encoding hTDO-(His)_6 and a plasmid encoding wild type HA-gp78 or its mutant, where the positively charged K, R or Q residues interacting the DEpSpT clusters in P450s [41,64] were mutated to Ala. The cells were cultured for 40 h and then treated with a UPD proteasomal inhibitor, MG262 (1 μM), and cultured for additional 8 h. The cell lysate was collected and treated with magnetic Dynabeads to isolate the His_6-tagged hTDO protein, which was then solubilized and subjected to Western immunoblotting against anti-Ub, anti-His_6, anti-HA or anti-GAPDH antibody. Immunoblotting with anti-His_6 antibody (α.His) is shown at low (LE) and high (HE) exposures, respectively.
Figure 4. hTDO surface regions enriched with the negatively charged DEpSpT clusters. Surface view of hTDO tetramer showing the DEpSpT clusters, consisting of the phosphorylated S/T sites identified in this work (green) and surface acidic D/E residues (blue), vicinal to the ubiquitinated K-residues (magenta) identified previously [32]. The DEpSpT clusters are enriched in the disordered N and C-terminal regions. The N- and C-termini are highlighted in yellow, while their phosphorylated S/T sites and ubiquitinated K-sites are depicted as magenta and green spheres, respectively. The sequences of the structurally unresolved N- and C-termini are shown at the top and bottom of the structure. The Trp bound in the exosites and the heme bound in the active sites are shown as red spheres. The inset highlights the reverse turn structure of the JK-Loop (highlighted in yellow background). The sequence of the JK-Loop is shown at the bottom of the inset; the Trp and the heme are shown as red sticks, while the histidine H-bonding with the Trp (H^76) and the proximal heme ligand (H^328) are shown as gray sticks. hTDO structure is based on a Trp-bound complex (PDB code: 5TIA).
Figure 5. Effects of N- and C-terminal truncations (ΔN, ΔC or ΔNC) (A) or C-terminal mutation (DE or 5S) (B) on hTDO proteolytic stability determined by CHX-chase analyses. In the N- and C-terminal truncation mutants (Δν, DC or ΔNC), the N-terminal fragment (1–38), C-terminal fragment (383–406) or both were truncated. In the DE and 5S mutants, 5 Asp/Glu residues in an acidic D/E patch or 5 Ser residues in potential phosphorylation sites in the C-terminal were mutated to Gly (the mutated residues are underlined in the sequence shown at the bottom of Figure 4). HepG2 cells were transfected with a plasmid encoding the wild type (FL) or mutant hTDO-(His)6 and cultured for 48 h. The left panels show representative immunoblots against anti-His6 or anti-GAPDH antibody, with hTDO ≈ 48 kDa, and GAPDH ≈ 37 kDa. The right panels show the time-dependent changes in the protein level. The exponential fits of the data are shown as the solid lines. The $t_{1/2}$ values derived from the fits are listed in Table 2.
Figure 6. Effect of exosite mutation (EWR) on the proteolytic stability of the hTDO ΔNC-mutant determined by CHX-chase or $^{35}$S-pulse-chase analyses. 

(A) HepG2 cells were transfected with a plasmid encoding the wild type (FL) or mutant hTDO-(His)$_6$ and cultured for 48 h. The left panel shows representative immunoblots against anti-His$_6$ or anti-GAPDH antibody, with hTDO $\approx$ 48 kDa, and GAPDH $\approx$ 37 kDa. The right panel shows the time-dependent changes in the protein level. The exponential fits of the data are shown as the solid lines. The $t_{1/2}$ values derived from the fits are listed in Table 2. 

(B) The relative ubiquitination of the wild type or mutant hTDO species were determined in the presence or absence of αMTrp (2.5 mM) by $^{35}$S-pulse-chase analyses. The intracellular ubiquitination color wheel intensity code is: red > orange > yellow > green > blue > indigo > violet.
Figure 7. Effect of active site mutation (H328A or H76A) and/or exosite mutation (EWR) on hTDO proteolytic stability determined by CHX-chase analyses or $^{35}S$-pulse-chase analyses. 

(A) HepG2 cells were transfected with a plasmid encoding the wild type (FL) or H328A mutant hTDO-(His)$_6$ or their corresponding EWR mutants and cultured for 48 h. The left panels show representative immunoblots against anti-His$_6$ or anti-GAPDH antibody, with hTDO ~ 48 kDa, and GAPDH ~ 37 kDa. The right panel shows the time-dependent changes in the protein level. The exponential fits of the data determined by Prism-Graph Pad are shown as the solid lines. The $t_{1/2}$ values derived from the fits are listed in Table 2. 

(B) The time-dependent changes in the protein level of H76S versus H76S/EWR or FL versus FL/EWR were determined by $^{35}S$-pulse-chase analyses and mean values of two individual replicates were subjected to exponential fits. The $t_{1/2}$ values derived from the fits are listed in Table 2.
Scheme 1. hTDO UPD degradation pathway.
Our envisaged scheme for the hTDO-UPD pathway based on our current observations and those of other UPD-targets in the literature, follows these steps: 1. hTDO homotetrameric external surface in the absence of Trp is ‘structurally floppy’ (72) and recognized by the cytosolic CHIP-Hsp70/Hsp40 complex and ER-anchored gp78/AMFR E3-Ub-ligases and ubiquitinated (hTDO[Ub]n). The findings that the post-translationally modified S/T and K-residues lie largely on the external surface of the hTDO A/B and/or C/D-dimers, and the failure to detect any similarly modified residues on the internal dimer-interacting juxtaposed surfaces suggest that hTDO is most likely recognized and ubiquitinated as its homotetrameric species (Supplementary material, Figure S2). 2. gp78 is known to concomitantly recruit the cytosolic AAA ATPase p97/valosin-containing protein (VCP)/Npl4/Ufd1 complex through its VIM (VCP-interacting motif), thereby enabling the p97-complex to closely interact with the ubiquitinated hTDO proximally bound to the substrate-interacting positively charged gp78-patches via its DEpSpT clusters. This dual recruitment, in turn, enables the p97-complex to recruit the ubiquitinated hTDO and to deliver it to the 19S lid Ub-receptors (Rpn10 and Rpn13 subunits) of the 26S proteasome. Upon recognition and tethering of the ubiquitinated hTDO, the 19S lid subunit Rpn11 deubiquitinase proceeds to deubiquitinate hTDO, enabling Ub-chain dismantling and restoration of the Ub-molecules to the cellular pool for fresh Ub-recycling (Step 5). 4. The disordered hTDO Nor C-termini then engages the narrow central aperture into the 20S proteasomal core, while the 19S base Rpt AAA ATPases unravel the deubiquitinated hTDO monomeric protein for insertion of the unfolded protein into the 20S core for degradation by the 20S cavity-lining β-subunit proteases into peptides of various lengths.

Biochem J. Author manuscript; available in PMC 2022 March 03.
Although no direct evidence currently exists, given the relatively large molecular size of the homotetrameric hTDO protein, too large to be accommodated and ‘devoured’ \textit{in toto} by the 20S proteasomal proteolytic core, we presume that the dissociation of the hTDO homotetramer occurs at some stage post-ubiquitination, with subsequent unfolding and internalization of each hTDO monomeric subunit at a time. The resulting hTDO peptides (commonly 8–10 residue long) could either further serve in antigenic presentation or be digested and reincorporated into the cellular amino acid pool for recycling.
Table 1.

hTDO phosphorylation sites determined upon incubation with various isoforms of PKA and PKC, combined with LC–MS/MS analyses

| Site | Peptide sequence | m/z      | z  | Error (ppm) | Score | Expect value | Kinase                  | Extent (n = 2) (%) |
|------|------------------|----------|----|-------------|-------|--------------|-------------------------|-------------------|
| T14  | M (Met-loss)SGC (Car) PFLGNFpTYPF | 894.8815 | 2  | 5.9         | 51.0  | 27 × 10⁻¹¹  | PKC-βII                | 42.34             |
| S23  | KLPVEGpSEEDK     | 655.7963 | 2  | -1.7        | 30.8  | 2.1 × 10⁻⁷  | Mixed PKA/PKCs          | 2.94              |
| S36  | SQTVNRApSK       | 564.2632 | 2  | -1.2        | 16.5  | 6.6 × 10⁻⁶  | Mixed PKA/PKCs          | 0.18              |
| S59  | VLNAQELQpSETK    | 720.3402 | 2  | -3.2        | 34.8  | 6.7 × 10⁻⁹  | Mixed PKA/PKCs          | 0.21              |
| T61  | VLNAQELQSEpTK    | 720.3431 | 2  | 0.8         | 34.2  | 4.4 × 10⁻⁸  | Mixed PKA/PKCs          | 0.30              |
| S155 | EYLSAGpQpSLQFR   | 905.4178 | 2  | 4.2         | 41.0  | 9.6 × 10⁻¹¹ | Mixed PKA/PKCs          | 3.10              |
| S266 | EVLLpSLFDEK      | 636.8113 | 2  | 2.4         | 31.7  | 8.0 × 10⁻⁷  | Mixed PKA/PKCs          | 0.78              |
| S278 | RHEHLLpSK        | 367.1855 | 3  | 0.75        | 15.9  | 8.6 × 10⁻⁶  | PKA, PKC-α,γ           | 1.95              |
| S285 | RLPpSYR          | 387.6877 | 2  | 3.0         | 18.8  | 9.2 × 10⁻⁶  | PKA                     | 11.76             |
| T342 | AGpTGGSSGYHLYRSTVSDR | 684.3075 | 3  | 4.9         | 14.8  | 3.5 × 10⁻³  | PKA                     | 1.62              |
| S345 | GTGGpSGGYHYL      | 703.3018 | 2  | 0.79        | 52.5  | 5.7 × 10⁻⁸  | PKC-α,γ/PKC-βII         | 15.34             |
| S346 | GTGGSpSGGYHYL     | 703.3018 | 2  | 4.8         | 59.0  | 7.4 × 10⁻⁹  | PKC-α,γ/PKC-βII         | 1.62              |
| T384 | MNpTHK           | 460.7066 | 2  | 0.045       | 27.0  | 1.6 × 10⁻⁴  | PKC-γ                   | 5.12              |

1 A full-length hTDO was used for this study. Phosphorylation and ubiquitination sites were determined as described previously [32,45,46]. Phosphorylated residues are shown as pS or pT, while the K-residues ubiquitinated within the peptide are shown as K. Because Trypsin/Lys-C were used to digest the peptides, the ubiquitinated K residues preceding the peptide sequence were not captured.

2 The extent of phosphorylation was estimated by the application of a label-free semi-quantitative analysis, as detailed previously [45,46].
Table 2.

Half-lives ($t_{1/2}$, min) of the wild type and hTDO mutants upon CHX-chase and/or $^{35}$S-pulse-chase analyses.

|                | CHX-chase $^2$ | $^{35}$S-pulse-chase $^3$ | $^{35}$S-pulse-chase +αMTrp |
|----------------|----------------|---------------------------|----------------------------|
|                |                | $^{35}$S-pulse-chase −αMTrp |                            |
| Control        | FL             | 45.2 (35.4–62.5)           | 90 $^4$                    |
|                |                |                            | 170 $^4$                   |
| Exosite        | EWR            | 27.0 (21.8–35.5)           | 60                         |
|                |                |                            | 68                         |
|                | ΔN             | 199.3 (143.3–327.5)        | ND                         |
|                |                |                            | ND                         |
|                | ΔC             | 212.7 (144.5–402.8)        | 252                        |
|                |                |                            | 360                        |
|                | ΔNC            | 247.2 (184.9–373.1)        | 210                        |
|                |                |                            | 275                        |
|                | ΔNC/EWR        | 67.0 (50.4–99.7)           | 60                         |
|                |                |                            | 70                         |
|                | ΔE → G         | 171.9 (131.4–248.8)        | ND                         |
|                |                |                            | ND                         |
|                | 5S → G         | 191.7 (138.6–310.9)        | ND                         |
|                |                |                            | ND                         |
| Active site    | H328A          | 106.1 (77.2–169.6)         | ND                         |
|                |                |                            | ND                         |
|                | H328A/EWR      | 51.7 (41.5–68.5)           | ND                         |
|                |                |                            | ND                         |
|                | H76S           | ND                         | 230                        |
|                |                |                            | ND                         |
|                | H76S/EWR       | ND                         | 118                        |

$^1$The $t_{1/2}$ values obtained by $^{35}$S-pulse-chase analyses are relatively longer than the corresponding values obtained by CHX-chase analyses because the Met/Cys-deficient MEM contained 0.05 mM Trp (required as an EAA for optimal de novo protein synthesis), which was 100-fold higher than the $K_d$ (Trp) of the exosite (0.5 μM). Hence, the introduction of the EWR mutation in every case resulted in a marked reduction in the $t_{1/2}$ values of the TDO mutants. Whenever αMTrp (2.5 mM) was included in the cultures, it further prolonged the $t_{1/2}$ if the exosite was intact. The values listed are Means of two biological replicates.

$^2$The $t_{1/2}$ values were obtained based on the exponential fit of the data using Prism Graphpad. Each data point was averaged from at least three separate cell cultures; the 95% confidence interval of the $t_{1/2}$ values is indicated in the parentheses.