Minimal requirements for ubiquitination-mediated regulation of thyroid hormone activation

Péter Egrı¹² and Balázs Gereben¹
¹Department of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Szigony Street 43, Budapest H-1083, Hungary
²János Szentágothai PhD School of Neurosciences, Semmelweis University, Budapest H-1085, Hungary

Abstract

Activation of thyroxine by outer ring deiodination is the crucial first step of thyroid hormone action. Substrate-induced ubiquitination of type 2 deiodinase (D2) is the most rapid and sensitive mechanism known to regulate thyroid hormone activation. While the molecular machinery responsible for D2 ubiquitination has been extensively studied, the combination of molecular features sufficient and required to allow D2 ubiquitination have not previously been determined. To address this question, we constructed chimeric deiodinases by introducing different combinations of D2-specific elements into type 1 deiodinase (D1), another member of the deiodinase enzyme family, which, however, does not undergo ubiquitination in its native form. Studies on the chimeric proteins expressed transiently in HEK-293T cells revealed that combined insertion of the D2-specific instability loop and the K237/K244 D2 ubiquitin carrier lysines into the corresponding positions of D1 could not ubiquitinate D1 unless the chimera was directed to the endoplasmic reticulum (ER). Fluorescence resonance energy transfer measurements demonstrated that the C-terminal globular domain of the ER-directed chimera was able to interact with the E3 ligase subunit WSB1. However, this interaction did not occur between the chimera and the TEB4 (MARCH6) E3 ligase, although a native D2 could readily interact with the N-terminus of TEB4. In conclusion, insertion of the instability loop and ubiquitin carrier lysines in combination with direction to the ER are sufficient and required to govern WSB1-mediated ubiquitination of an activating deiodinase enzyme.

Introduction

Thyroid hormone activation, the first step of thyroid hormone action, is catalyzed by activating deiodinases, type 1 and type 2 deiodinases (D1 and D2 respectively) via outer ring deiodination (Gereben et al. 2008). In a physiological setting, D2 is the major activating deiodinase (Maia et al. 2005, Schneider et al. 2006) and its activity is subjected to complex controls, which also involves ubiquitination of the D2 protein (Gereben et al. 2000). Selective proteolysis is driven by the ubiquitin–proteasome system (UPS) and represents a crucial regulatory mechanism of cell function (Hershko & Ciechanover 1998). Targeting proteins into the proteasome for degradation is one of the most heavily studied phenomena among the diverse set of ubiquitination-controlled cellular functions. Ubiquitination is a three-step process involving ubiquitin activation by the E1 enzyme,
conjugation of ubiquitin to E2 followed by specific protein targeting by the E3 ubiquitin ligase complex responsible for substrate recognition and specificity (Hershko & Ciechanover 1998). Proteins carrying degradative ubiquitin signal (e.g., lysine 48-linked polyubiquitin chain) are transferred into the proteasome to be cleaved into short oligopeptides or amino acids.

D2 is intrinsically unstable and is degraded in the 26S proteasome (Steinsapir et al. 1998). D2 was identified as the first endoplasmic reticulum (ER)-resident enzyme undergoing substrate-induced ubiquitination (Gereben et al. 2000). The ubiquitination of the D2 protein also involves a degradation-independent mechanism via ubiquitination-induced conformational changes of D2 homodimers that result in quick decline of D2 activity (Sagar et al. 2007). As a consequence, ubiquitination is currently considered the most rapid and efficient way to regulate D2-mediated T₃ generation (Bianco & Larsen 2005).

Specific molecular elements have been shown to be important for D2 ubiquitination, e.g., an instability loop between amino acids 92 and 97 and the K237/K244 lysines in the human D2 protein (Dentice et al. 2005, Zeold et al. 2006a, Sagar et al. 2007). Presently, two E3 ligases are known, which contribute to D2 degradation. First, the SOCS-box-containing WD40 protein (WSB1), a sonic hedgehog-induced protein, was recognized as a D2-interacting substrate-recognition subunit of an E3 ligase catalytic core complex (ECSWSB1) that consists of Elongin BC-Cullin5-Rbx1 subunits (Dentice et al. 2005). Then, TEB4 (MARCH6), the mammalian ortholog of yeast Doa10, was identified as a functional E3 ligase for the ubiquitination of D2 (Zavacki et al. 2009), but molecular details of the TEB4–D2 interaction remain to be determined.

In contrast to D2, D1 is a long-lived plasma membrane-located activating deiodinase that is not subjected to ubiquitination (Baqui et al. 2000, Gereben et al. 2000).

Despite accumulating data, the minimal requirements for ubiquitination-mediated deiodinase regulation have not yet been defined. We aimed to determine the combination of molecular features required and sufficient to allow an activating deiodinase to be targeted by E3 ubiquitin ligases, a pre-requisite for proteins processed along the ubiquitin–proteasome pathway. We inserted D2-specific molecular elements into D1 to generate chimeric proteins that allow assessment of the power of specific D2-ubiquitinating elements in the context of a natively non-ubiquitinated deiodinase protein.

### Materials and methods

#### Generation of DNA constructs

FLAG-tagged chimeric deiodinases were constructed using standard recombinant DNA techniques. Site-directed mutagenesis was performed with Vent polymerase PCR on templates containing the human D2- or rat D1-coding region with a cysteine-mutant active center followed by cloning into a D10 expression vector (Gossen & Bujard 1992). For fluorescence resonance energy transfer (FRET) experiments, the generated fragments were subcloned into pEYFP–N1 (Clontech), resulting in the fusion of EYFP to the C-terminus of chimeras.

The human TEB4-coding region was amplified with Vent polymerase on a pcDNA3.1-GFP-TEB4 template (a kind gift from Dr M Hochstrasser, Yale University). The product was subcloned into pEYFP–N1, pEYFP–C1, and pECFP–C1 fusion vectors (Clontech) that resulted in TEB4-Y (in pEYFP–N1), Y–TEB4 (in pEYFP–C1), and C–TEB4 (in pECFP–C1). C or Y indicate ECFP or EYFP respectively. Construct nomenclature also provides information on the position of the fluorescent protein in the translated fusion protein (e.g., TEB4–Y indicates that EYFP was fused to the C-terminus of TEB4 protein, while the C–TEB4 construct contains ECFP at the N-terminus of TEB4). All constructs were confirmed by sequencing. The D2–Y, D2–C, and C–D2 constructs (Vivek Sagar et al. 2007) and the WSB1–C and SEC62–D1 constructs have been described previously (Zeold et al. 2006a, Vivek Sagar et al. 2007).

#### Cell culture and transfection

HEK-293T (Zeold et al. 2006b) cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin–streptomycin solution (Sigma). One day before transfection, cells were plated in a 35 mm dish at a density of 2×10⁵ cells/hole concentration. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. Secreted alkaline phosphatase (SEAP) was used as a transfection internal control. At 48 h after transfection, cells were harvested and processed for western blotting or deiodinase activity measurement.

#### Reagents and treatments

Tetracycline (Sigma) treatment (6- and 12-h, 1 mg/l final concentration) was performed on the second day after transfection (Gossen & Bujard 1992). MG132 (Calbiochem, DOI: 10.1530/JME-14-0156 Printed in Great Britain.
Darmstadt, Germany), a protease inhibitor for proteasome activity, was dissolved in DMSO and cells were treated for 4 h using 2 μmol/l final concentration vs vehicle. Hormone-free medium for T₄-treatment was prepared with charcoal-stripped FBS. In brief, 100 mg charcoal (Sigma) and 50 mg dextran (Sigma) were preincubated overnight in 0.01 mol/l Tris buffer (pH = 7.6). After centrifugation, 40 ml FBS were added and incubated for 1 h. The suspension was recentrifugated and the supernatant was added to DMEM in 1:10 dilution followed by membrane filtration and 1 mmol/l T₄ (Sigma) stock solution was kept in NaOH.

**SEAP assay**

Media was removed from cells before treatment and processed for SEAP measurement using Novabright Chemiluminescent SEAP Reporter Gene Assays (Invitrogen) followed by measurement with Luminoscan Ascent (Thermo, Waltham, MA, USA) according to the manufacturer’s instructions.

**Western blot**

Western blots were performed as described previously (Gereben et al. 2000). For the detection of high-molecular-weight ubiquitinated bands, samples were run on 4–20% gradient gels (Bio-Rad). The blots were incubated with an M2 anti-FLAG MAB (Sigma) in 1:3000 dilution.

**Deiodinase activity assay**

HEK-293T cells were processed for in vitro deiodinase assay to measure the activity of D1 containing a cysteine-mutant active center according to the American Thyroid Association Guide to investigating thyroid hormone economy and action in rodent and cell models (Bianco et al. 2014). The basic assay procedure was the same as described previously (Curcio-Morelli et al. 2003). In this study, 125I-T₄ and 1 μmol/l T₄ substrate were used for 3 h.

**FRET and confocal microscopy**

Transfection was carried out as described above, but cells were plated in 35-mm glass-bottomed dishes (MatTek Co., Ashland, MA, USA). FRET was performed according to our current protocol (Arrojo et al. 2013). FRET measurement was performed on the second day after transfection using acceptor photobleaching on a Nikon A1R laser scanning confocal system in spectral detector mode equipped with the Tokai Hit stage-top incubator and Supertech temperature controller. The following parameters were applied in FRET experiments: 457 nm argon laser for ECFP excitation and 464–500 nm range for detection, and 514 nm argon laser for EYFP excitation and 516–540 nm range for detection. Cells with at least 80% bleach efficiency (decrease in EYFP intensity) were selected for analysis. At least 20 cells were measured per group.

Calculation of FRET efficiency was based on the increase in the ECFP donor signal after photobleaching the EYFP acceptor using the following equation:

\[
\text{FRET} = \frac{(\text{ECFP (postbleach)} - \text{ECFP (prebleach)})}{\text{ECFP (prebleach)}}
\]

Data normalization was performed by expressing FRET efficiency of specific FRET pairs as a percentage of that of the ECFP–EYFP (C–Y) tandem construct (Cicchetti et al. 2004). ECFP and EYFP (C and Y) monomers were used to determine the background.

**Statistical analysis**

Deiodinase activities of MG132-treated samples were analyzed by the t-test. FRET results were analyzed by the t-test or one-way ANOVA followed by Tukey’s post-hoc test.

**Results**

Specific D2 elements with a known effect on D2 ubiquitination were introduced into rat D1 in homologous positions. The K237/K244 ubiquitin-binding lysines of D2 are phylogenetically conserved but these residues are absent in D1 proteins (Fig. 1A). The K237 and K244 lysine residues of D2 were inserted into rat D1 by generating R223K and P230K D1 mutants respectively. The instability loop of D2 (amino acids 92–97 of human D2) was inserted between amino acids 102 and 103 of D1 (Fig. 1A). Direction of the chimera to stable retention in the ER was achieved by deleting its N-terminal 33 amino acids to remove the transmembrane domain, and the resulting fragment was fused to the C-terminus of human SEC62, an ER-resident protein using our approach described previously (Zeold et al. 2006a). The constructs are depicted in Fig. 1B.

The half-life of chimeras was tested in HEK-293T cells using the Tet-off expression system that allows transcriptional suppression of the transfected constructs (Fig. 2A; Gossen & Bujard 1992). Insertion of the ubiquitin carrier lysines into D1 (D1–K and D1–2K) did not result in detectable changes in protein half-life and high-molecular-weight ubiquitinated bands could not be
observed on western blot (Fig. 2B). Insertion of the D2-specific 6-aa. loop into D1–K and D1–2K (D1–K–loop and D1–2K–loop) remarkably destabilized the chimera but ubiquitinated forms did not appear. Importantly, direction of the D1–2K–loop chimera into the ER via fusion with SEC62 (SEC62–D1–2K–loop) resulted in both destabilization and generation of high-molecular-weight ubiquitinated forms (Fig. 2B).

Proteasomal uptake of the chimeric proteins was also studied by treating the cultures with 2 μmol/l MG132 for 4 h followed by the measurement of deiodinase activity. While activity of the D2 control was readily increased, the D1–K, D1–2K, D1–K–loop, and D1–2K–loop chimeras were not sensitive to MG132 similar to native D1, indicating that these proteins are not processed by the proteasome (Fig. 2C).

Figure 1
(A) Alignment of amino acid sequences of D1 and D2 portions in different species. Amino acid positions indicated using the positions of amino acids in human D2, the first six amino acids of the D2-specific instability loop and the conserved lysine residues are boxed. Arrows indicate the position of ubiquitin carrier lysines of D2. (B) Schematic of the applied D1–D2 recombinant chimeric proteins tagged with a FLAG epitope on the N-terminus.
To test whether the generated chimeric proteins can bind with the WSB1 E3 ligase subunit or the TEB4 E3 ligase, FRET was performed (Fig. 3A). While the D2-binding domain of WSB1 has already been resolved (Vivek Sagar et al. 2007), localization of the substrate recognition surface of TEB4 has not been described yet. Therefore, first, we aimed to identify the D2-binding domain of TEB4. FRET pairs were constructed fusing EYFP either to the N-terminus or to the C-terminus of TEB4 and tested in the presence of the ECFP-tagged D2 protein. As membrane topology and localization of the TEB4 C-terminal portion are controversial (Hassink et al. 2005, Kreft et al. 2006), we tested both possibilities, i.e., whether the C-terminus would be located in the ER lumen or in the cytosol. Therefore, we measured the interaction of the C-terminus of TEB4 (TEB4–Y) with both the C- and N-termini of D2 (D2–C and C–D2), which are localized to the cytosol and ER lumen respectively (Fig. 3B and E).
Figure 3
Topology and substrate dependence of the D2–TEB4 interaction assessed with FRET. (A) Experimental design. (B) Schematic of FRET pairs. Owing to the controversial topology of the C-terminus of TEB4, located positions in both the cytosol and ER lumen were depicted (C) FRET-assisted detection of the topology of TEB4–D2 interaction. Data are expressed as percentages of the level for the fused ECFP–EYFP (C–Y) tandem positive control, while cotransfected monomeric ECFP (C) and EYFP (Y) were applied to detect non-specific background (mean ± S.E.M.; n ≥ 15 per group) ***P < 0.001; **P < 0.01 by one-way ANOVA vs monomer followed by Tukey’s post-hoc test). (D) Effect of 1 μmol/l T4 on the D2–TEB4 interaction. T4-sensitive D2 homodimers were used as positive controls (mean ± S.E.M.; n ≥ 15 per group) ***P < 0.001; **P < 0.01 by the two-tailed t-test. (E) Photomicrography of individual HEK-293T cells demonstrating acceptor photobleaching FRET to detect the interaction between TEB4 and D2. Left-top: prebleach (pre) acceptor; right-top: postbleach (post) acceptor; left-bottom: prebleached donor; right-bottom: postbleached donor. The order of the fluorescent protein (C or Y) and the tagged protein in the name of the constructs reflects their position in the fusion protein. A full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0156.
tested in the presence of either the cytosolic C-terminus (TEB4–Y vs D2–C) or the ER lumen-localized N-terminus (TEB4–Y vs C–D2) of D2 (0.73 ± 1.78%) (Fig. 3C). T₄, the substrate known to accelerate D2 ubiquitination, increased the strength of the interaction between the N-terminus of TEB4 and the C-terminus of D2 (Y–TEB4 vs D2–C) by approximately 40% after 4 h of incubation with 10 μmol/l T₄ in hormone-free media. However, no significant change occurred when the C-terminus of TEB4 (TEB4–Y) was tested, although the FRET signal of the D2–D2 homodimer control decreased, as expected (Fig. 3D).

We then moved to test the binding of the chimeras to the WSB1 E3 ligase subunit and to the TEB4 E3 ligase. The chimeras were tagged with EYFP on the C-terminus and energy transfer was measured in the presence of WSB1 (tagged on the C-terminus with ECFP, WSB1–C) or TEB4 (ECFP on its N-terminus, C–TEB4) following the scheme depicted in Fig. 4A. Inserting the ubiquitin carrier lysine residues of D2 into D1 by R223K and P230K mutations in combination with the 6-aa loop (D1–2K-loop–Y) did not result in detectable interaction between the chimeras and WSB1–C or C–TEB4 and gave a result similar to native, or ER-inserted, D1 (SEC62–D1–Y) (Fig. 4B and C). Interestingly, the ER-localized lysines and 6-aa-loop-containing mutant (SEC62–D1–2K–loop–Y) showed a measurable FRET signal when paired with WSB1–C (53.14 ± 14.63%) but not with C–TEB4 (Fig. 4B and D).

Discussion

Thyroid hormone metabolism catalyzed by selenodeiodinase enzymes allows the accurate and flexible regulation of tissue-specific thyroid hormone levels (Gereben et al. 2008). Although both D1 and D2 are capable to generate T₃ from thyroxin, under physiological conditions, D2 is the major T₃ producer due to its high substrate affinity (Maia et al. 2005). D2 is subjected to tight and multilevel control (Gereben et al. 2008) that includes substrate-mediated ubiquitination, a process representing the fastest and most sensitive regulation of thyroid hormone activation. The efficiency of this process is explained by the complex nature of D2 ubiquitination, that not only occurs via the degradation of the D2 protein, but also involves D2 inactivation in a degradation-independent manner due to ubiquitination-induced conformational changes of the globular domain of the D2 homodimers allowing for a fast and reversible control of D2 activity (Sagar et al. 2007). This is especially useful for the cell taking into account both the energy-consuming multistep process required to incorporate the specific amino acid selenocysteine into the D2 protein and the need for rapid regulation of D2-mediated T₃ generation. In contrast, the D1 protein is not processed through this pathway and represents stability (Gereben et al. 2000). Subcellular localization of the two enzymes is also different, as D2 is subjected to stable retention in the ER while D1 is located in the plasma membrane (Baqui et al. 2000, Zeold et al. 2006b).

Results from previous studies have identified the molecular elements involved in the maintenance of metabolic instability of the D2 molecule (Dentice et al. 2005, Zeold et al. 2006b). However, the combination of elements required and sufficient for D2 ubiquitination has not been resolved. In this study, we used a reversed strategy by incorporating D2-specific elements into the long-lived deiodinase D1 in order to test the power of specific molecular combinations to destabilize an otherwise non-ubiquitinated protein.

Insertion of the ubiquitin-binding K237/K244 lysines into homologous positions of D1 (D1–K and D1–2K) was not sufficient to destabilize or ubiquitinate the chimera. Interestingly, insertion of the lysines in combination with the D2 instability loop (D1–loop–K and D1–loop–2K) decreased the stability without the appearance of detectable amounts of ubiquitinated forms and remained insensitive to MG132, proving that they were not subjected to proteasomal uptake. This observation raised the possibility that the inherent instability of D2 is not exclusively dependent on the UPS but might be also affected by other, proteasome-independent mechanisms, as shown for iron regulatory protein 2 and IκBζ (Shumway & Miyamoto 2004, Chang et al. 2011). However, SEC62-mediated direction of the loop- and lysine-containing D1 into the ER resulted in intense ubiquitination of the chimera, indicating that this combination is sufficient to drive the chimera to the ubiquitin–proteasome pathway. The SEC62-fused deiodinases are inactive due to the absence of the transmembrane domain, thus their activities cannot be tested (Zeold et al. 2006a).

As ubiquitin ligase binding is crucial for the substrate specificity of ubiquitination, we studied whether the chimeric proteins could be recognized by the D2-specific E3 ligase WSB1 and TEB4 in live HEK-293T cells. WSB1 is a WD40 repeat and SOCS box-containing protein (Hilton et al. 1998). It was demonstrated that WSB1 works as a substrate-recognizing subunit of an ECSWSB1 E3 ligase complex and mediates substrate-induced ubiquitination of D2 (Dentice et al. 2005) binding the C-terminus of D2 via its SOCS-box domain (Sagar et al. 2007). However, the recognition of D2 by TEB4 is less understood. Therefore, first, we had to identify the D2-interacting domain of TEB4 and showed that...
Figure 4

Interaction of deiodinase chimeras with the WSB1 and TEB4 E3 ligases assessed with FRET. (A) Experimental design. (B) FRET efficiency of chimeric deiodinases coexpressed with WSB1–C or C–TEB4 in HEK-293T cells. Data are expressed as percentages of the level for the fused CFP–YFP (C–Y) tandem positive control, while cotransfected monomeric CFP (C) and YFP (Y) were applied to detect non-specific background (mean ± S.E.M.; n ≥ 30 per group); ***p<0.001; **p<0.01; one-way ANOVA vs monomer followed by Tukey's post-hoc test. (C) Photomicrography of individual HEK-293T cells demonstrating FRET between D1–D2 chimeric proteins and WSB1. (D) Same as (C) with TEB4. Each panel contains the following order of pictures: left-top: prebleach (pre) acceptor; right-top: postbleach (post) acceptor; left-bottom: prebleached donor; right-bottom: postbleached donor. The order of the fluorescent protein (C or Y) and the tagged protein in the name of the constructs reflects their position in the fusion protein. A full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0156.
its N-terminus is responsible for this action. Importantly, this region contains the catalytically active RING domain that represents a common structural unit in an E3 ligase subclass (Deshaies & Joazeiro 2009). Our results indicate that the TEB4 protein integrates the ability of recognition and ubiquitination of D2 and its interaction with D2 is increased upon T4 exposure that results in substrate-mediated downregulation of D2 activity.

Having identified the basic topology of the D2-TEB4 interaction, we used this information to perform FRET studies of the interaction between the chimeras and TEB4 and also WSB1. We obtained evidence that the ubiquitination of D2 lysine- and loop-containing ER-localized chimera (SEC62–D1–2K–loop) binds WSB1. This demonstrates that the ubiquitination of the SEC62–D1–2K–loop protein is carried out by an E3-ligase-driven specific process rather than an ERAD-driven clearance mechanism of the exogenous protein. Lack of WSB1 binding of the D1–2K–loop chimera is in accordance with the lack of ubiquitinated forms observed when studying this chimera by western blotting. Therefore, we conclude that insertion of the instability loop and ubiquitin carrier lysines into an ER-located activating deiodinase is sufficient and required to govern WSB1-mediated ubiquitination. We could not detect the interaction between TEB4 and the SEC62-fused chimera, although the fusion allowed direction of the chimera into the ER-linked location of native D2, and the C-terminus of D2 was able to interact with the N-terminus of TEB4 (Fig. 3C). These findings indicate that the lack of interaction between the chimera and TEB4 cannot be explained by topology and TEB4 binding would still require some unidentified molecular elements in the deiodinase protein.

It has been shown that D2 undergoes classical K48-linked ubiquitination targeting the protein for proteasomal degradation (Arrojo et al. 2013). While there are accumulating data on the biological significance of unconventional polyubiquitin chains not composed of K48-linked ubiquitin (Kulathu & Komander 2012), presently no data are available on such alternatively linked ubiquitin on the D2 protein. Consequently, it is also not known whether D2-specific E3 ligases would be differently involved in the generation of alternatively linked ubiquitin chains, e.g., via the formation of non-proteasomal signals. These signals could be relevant for D2, as beyond proteasomal degradation, D2 ubiquitination also drives proteasome-independent conformational changes of the D2 homodimers, resulting in transient loss of D2 activity (Sagar et al. 2007). It has also been demonstrated that Doa10 (the yeast ortholog of mammalian TEB4), in combination with the Ubc6 (the yeast ortholog of mammalian UBE2J) ubiquitin-conjugating E2 enzyme, could be involved in the K11-linked polyubiquitin chain synthesis (Xu et al. 2009). As UBE2J has also been shown to be involved in the ubiquitination of D2 (Botero et al. 2002, Kim et al. 2003), these results raise the possibility that TEB4 could facilitate K11-linked polyubiquitin chain formation on D2.

In conclusion, the obtained data identified a combined set of molecular elements and intracellular localization necessary for WSB1-mediated regulation of thyroid hormone activation and demonstrated distinct requirements for WSB1- and TEB4-mediated ubiquitination of D2. In vivo, the complex regulation of deiodination via WSB1 and TEB4 could play a role in D2-expressing tanycytes of the mediobasal hypothalamus known to coexpress WSB1, TEB4 and deubiquitination enzymes (Fekete et al. 2007, Zavacki et al. 2009).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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