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An Ubiquitin Ligase Recognizing a Protein Oxidized by Iron: Implications for the Turnover of Oxidatively Damaged Proteins

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Protein oxidation is a natural consequence of aerobic metabolism in cells. Oxidative modification of amino acid residues of proteins causes to lose activity or function of proteins. Organisms have thus developed pathways to remove oxidized proteins by rapid protein degradation. These pathways are important components in cellular quality control mechanisms. It has been suggested that oxidized proteins are degraded by the proteasome. However, whether ubiquitylation is necessary for the degradation of oxidized proteins remains a controversial issue. We have recently identified HOIL-1 (heme-oxidized IRP2 ubiquitin ligase-1) as an E3 ligase that recognizes a protein that has been oxidized by iron. This review describes the recent progress made in understanding the ubiquitin-proteolytic pathway and the regulation of iron metabolism. The process involved in eliminating oxidized proteins and the possible roles that HOIL-1 ubiquitin ligase may play in these processes are discussed.

Key words: iron, iron regulatory protein, oxidation, protein degradation, ubiquitin.

Overview of the ubiquitin system

The half-lives of proteins range from a few minutes to several days. In general, regulatory proteins have shorter half-lives compared to those of constitutively expressed proteins. The mechanism that determines protein half-lives was not discussed until the discovery of the ubiquitin-proteolytic pathway. This pathway involves the tagging of proteins with ubiquitin, which targets the tagged proteins for degradation.

The ubiquitin system was first identified as a part of ATP-dependent protein degradation machinery by Hershko and co-workers in 1978 (*1*). Ubiquitin is a small globular protein consisting of 76 amino acids and is one of the most conserved proteins among eukaryotes. The process used to tag target proteins with ubiquitin is termed ubiquitylation and it is a multi-step process that depends on the activities of three kinds of enzymes, denoted E1, E2 and E3. First, ubiquitin is activated by ATP to form a high-energy thiol-ester intermediate with the conserved cysteine residue of E1 (ubiquitin activating enzyme). The activated ubiquitin is then transferred to one of several E2s (ubiquitin conjugating enzymes) from E1 to form high-energy thio-ester bond. In the presence of an E3 (ubiquitin-protein ligase), E2 transfers ubiquitin to the specific protein substrate recognized by the E3. Therefore, the E3s are the components of the ubiquitin system that largely determine its substrate specificity.

A number of different E3 ligase families have been identified. In the case of the HECT (homology to the E6-AP C-terminus) E3 ligases, ubiquitin is first transferred to E3 and, then to the substrate. In contrast, E3s bearing a RING finger domain transfer ubiquitin to target proteins directly from E2s, which are bound to the RING finger domain. This family contains both monomeric (*e.g.* Mdm2, c-Cbl) and hetero-oligomeric ligases (*e.g.* APC/C, SCF, and VBC-Cul2). Several other ligase families such as U-box and PHD families have been identified, but their modes of ubiquitylation has not been fully elucidated.

Through the functions of E1, E2 and E3 enzymes, ubiquitin forms an isopeptide bond between its C-terminal glycine and an ε-amino group of a lysine residue of the target proteins (*2–4*) (Fig. 1). Successive isopeptide bond formation between the C-terminal glycine and lysine-48 of the conjugated ubiquitin molecules then generates a polyubiquitin chain that functions as a recognition signal for the 26S proteasome. This multisubunit protease then degrades the ubiquitylated proteins to small polypeptides (Fig. 1A). Recent works have shown that there is a variation to this general system in that the formation of polyubiquitin chains can also employ lysine residues of ubiquitin apart from lysine-48 (*3*, *5*). However, the roles of those ubiquitin chains will not be discussed in this review.

The 26S proteasome is composed of the 20S proteasome and the 19S regulatory particle. The 20S proteasome is a barrel-shaped protease composed of four ring structures, namely two outer α rings and two inner β rings, that are arranged in the order αββα. Each ring consists of seven small subunits. Some β subunits have catalytic sites that face the inside of the barrel. The crystal structure of the yeast 20S proteasome has revealed that the center of the outer α ring is closed to prevent unrelated proteins from entering the barrel (*6*). The binding of the regulatory particle to the outer α ring of the 20S proteasome opens the center of the ring thereby allowing substrates into the barrel, and enhances the protease activity (*7*).

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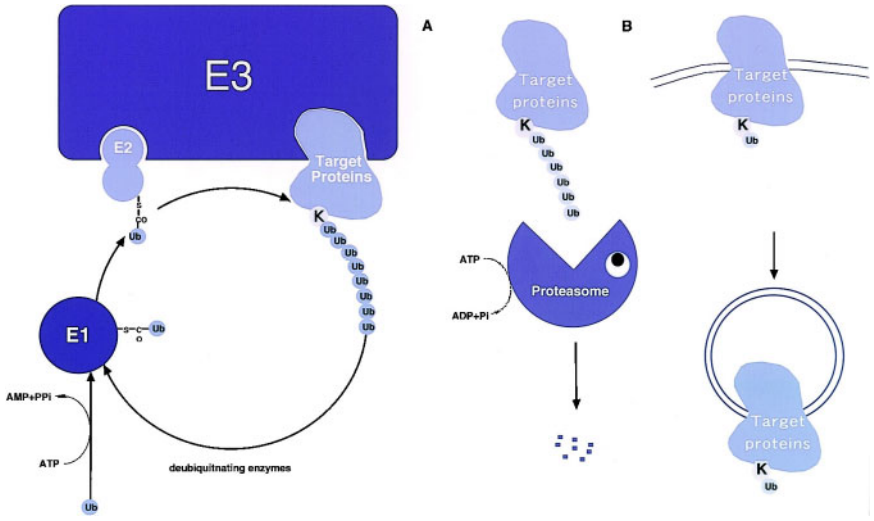

Fig. 1. **The ubiquitin system.** The tagging of target proteins with ubiquitin is termed ubiquitylation and it involves a multi-step process that depends on the activities of three kinds of enzymes denoted E1, E2, and E3. Ubiquitin is first activated by ATP to form a high-energy thiol-ester intermediate with the conserved cysteine residue of E1 (ubiquitin activating enzyme). The activated ubiquitin is then transferred to one of several E2s (ubiquitin conjugating enzymes) to form a high-energy thio-ester bond. In the presence of an E3 (ubiquitin-protein ligase), E2 transfers ubiquitin to the specific substrate recognized by the E3. The functions of these three enzymes thus cause ubiquitin to form an isopeptide bond between its C-terminal glycine and an ε-amino group of a lysine residue of the target proteins. There are also enzymes that can remove ubiquitin from target proteins (deubiquitinating enzymes) and thus the ubiquitin system is now recognized to be a reversible ubiquitin-conjugation system that regulates protein functions. The formation of successive isopeptide bonds between the C-terminal glycine and lysine-48 of the conjugated ubiquitin molecules generates a polyubiquitin chain, which functions as a recognition signal for the 26S proteasome (A). This is a multisubunit protease that degrades ubiquitylated proteins into small polypeptides. (B) The ubiquitin system also appears to regulate protein functions by mechanisms that do not involve proteasomal degradation. For example, ligand binding to membrane receptors triggers the tagging of the receptors with ubiquitin, which provokes the internalization of the receptor molecules and their targeting to lysosomes, where they are degraded. This lysosomal targeting of the receptors seems to be signaled by monoubiquitylation.

It should be noted that ubiquitylation regulates function of proteins by non-proteasomal degradation processes. For example, ligand binding to membrane receptors triggers the tagging of the receptors with ubiquitin, which provokes the internalization of these molecules and their targeting to the lysosome, where they are degraded (Fig. 1B). This lysosomal targeting of receptors seems to be signaled by monoubiquitylation (*5*).

In the past decade, the physiological roles played by the ubiquitin-mediated protein degradation system have been extensively studied. The system is now recognized to be involved in the regulation of a broad array of cellular functions, including cell cycle progression, signal transduction, metabolism, and stress responses (*2–4*). The ability of the ubiquitin system to regulate so many diverse cellular processes is largely due to its ability to recognize target molecules in a timely and selective manner. As mentioned, the E3s are the key molecules that are responsible for this selective substrate recognition. However, E3 recognition of target proteins is also regulated, as they do not recognize their targets in a constant fashion. Several mechanisms regulate this selective recognition by E3s. In some cases, the activity of the E3 is itself regulated by post-translational modification. In most cases, however, the target proteins must be modified before the appropriate E3s can recognize them. These modifications include the binding of chaperone proteins, the dissociation from appropriate oligomeric partner proteins, and post-translational modifications.

With regard to the latter, several different types of post-translational modifications function as recognition signals for ubiquitin ligases. One is phosphorylation, which is one of the most common modifications of proteins. For example, phosphorylation of IκBα and β-catenin is needed before $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase will ubiquitylate these proteins (*8*). The identification of the specific recognition signals that are recognized by particular ubiquitin ligases has led to the elucidation of some unexpected and novel post-translational modification mechanisms that regulate protein fates. One example is *N*-linked glycosylation. SCF^{Fbx2} ligase specifically recognizes *N*-linked glycosylation (*9*), particularly the two GlcNAC residues at the base of the *N*-linked sugar tree. Consequently, SCF^{Fbx2} ligase has been suggested to be involved in the degradation of unfolded proteins in the endoplasmic reticulum because *N*-glycosylation occurs exclusively in membrane compartments. Another important post-translational modification is the oxidative alteration of target proteins involved in the oxygen-sensing pathway. Hypoxia inducible factors (HIFs) are transcriptional regulators of hypoxic responses that are themselves regulated by the oxygen-dependent degradation of their α (HIF-α) subunits. This degradation involves the hydroxylation of a specific proline residue of HIF-α by proline hydroxylases. The hydroxylated protein is then recognized by the von Hippel-Lindau protein, a tumor-suppressor protein for clear cell renal carcinoma that is also a substrate recognition subunit of the VBC-Cul2 ubiquitin ligase. Thus, HIF-α is ubiquitylated and degraded in an oxygen-dependent manner (*10*, *11*).

The studies on the HIFs revealed the importance of novel post-translational modifications in ubiquitin-mediated regulatory systems that participate in the oxygen-sensing pathway in cells. In our research, we have identified another type of oxidative modification. We found that iron regulatory protein 2 (IRP2), which is a regulator of iron metabolism in mammalian cells, is itself regulated by iron-dependent degradation via the ubiquitin-proteasome pathway (*12*). We postulated that this iron-mediated oxidative modification functions as a specific signal for selective ubiquitylation (*13*). Here I will review our recent progress in elucidating the mechanism underlying the iron-dependent ubiquitylation of IRP2 (*14*). I will also discuss how this research broadens our understanding of

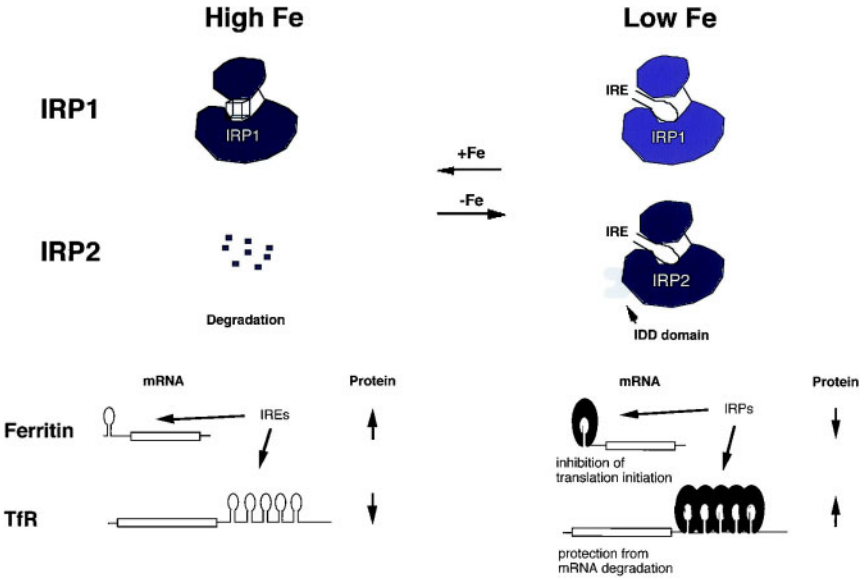

Fig. 2. **Regulation of iron metabolism by the IRE/IRP system.** The expression of proteins involved in iron metabolism is regulated at a post-transcriptional level. In mammalian cells, the transcripts encoding molecules involved in iron metabolism contain RNA stem-loop structures known as iron responsive elements (IREs) that are recognized by a small family of IRE-binding proteins referred to as iron regulatory proteins (IRPs). Two members of this family, IRP1 and IRP2, only bind IREs with high affinity in iron-depleted conditions. The binding of IRPs to IREs located near the 5′ cap site of ferritin transcripts prevents the initiation of translation. The binding of IRPs to IREs in the 3′ UTR of the transferrin receptor (TfR) mRNA inhibits the endonucleolytic cleavage of the TfR transcript at a site flanked by IREs, thereby increasing the half-life of TfR mRNA and elevating TfR biosynthesis.

the oxidation-induced ubiquitin system and the turnover of oxidized proteins.

Overview of the regulation of iron metabolism in mammalian cells

First, I will briefly describe cellular iron metabolism. Iron is an essential nutrient for almost all organisms, often because it functions as a redox center of enzymes or regulatory proteins. However, iron simultaneously acts as a potentially cytotoxic element by generating hydroxyl radicals in conjunction with oxygen (*15*). Its potentially harmful effects mean that the metabolic pathways of iron uptake and storage are highly regulated.

The proteins involved in iron metabolism are regulated at a post-transcriptional level. This is unlike most other proteins, whose functions are generally regulated by controlling the transcription of genes, which determines the protein levels. In mammalian cells, the transcripts encoding molecules involved in iron metabolism carry RNA stem-loop structures known as iron-responsive elements (IREs). These are recognized by a small family of IRE-binding proteins referred to as iron regulatory proteins (IRPs) (*16*, *17*). IRP2 belongs to this family. Like another member of this family, IRP1, IRP2 binds to IREs with high affinity only in the iron-depleted condition (Fig. 2). The binding of IRPs to IREs located near the 5′ cap site of the transcripts prevents the initiation of translation of several transcripts, including ferritin, erythroid δ-aminolevulinate synthase, and mitochondrial aconitase (Fig. 2). In contrast, the binding of IRPs to IREs in the 3′UTR of the transferrin receptor (TfR) mRNA inhibits the endonucleolytic cleavage of the TfR transcript at a site flanked by IREs, thereby increasing the half-life of TfR mRNA and elevating TfR biosynthesis (Fig. 2) (*16*, *17*). Thus, IREs and IRPs can positively and negatively regulate the levels of proteins involved in iron metabolism to control cellular iron concentration.

There is also evidence that IREs may also be involved in regulating other iron-metabolizing proteins. Recently, molecules involved in iron uptake from the intestinal lumen were found to include DMT1, originally denoted as DCT1 or Nramp2. DMT1 is a divalent cation transporter located at the apical surface of duodenal epithelial cells (*18*). Several alternative transcripts of DMT1 have been reported, some of which contain an IRE in the 3′ UT. This IRE has been shown to be involved in the iron-regulated expression of the protein in some settings (*19*). In addition, the mRNA of Ferroportin/IREG1/MTP1, a basolateral transporter of duodenal epithelial cells, has also been found to contain an IRE, but its function has not yet been clarified (*20*).

As mentioned, IRP1 and IRP2 both bind to IREs in iron-depleted cells but not in cells with adequate iron levels. This observation suggests that the elucidation of the underlying mechanism that regulates the IRE-binding activity of these IRPs may help us to understand the iron-sensing mechanism of cells. IRP1 was first identified in 1988 as a protein that binds to ferritin mRNA (*21*). cDNA cloning of IRP1 revealed that IRP1 not only exhibits significant homology to mitochondrial aconitase, it also bears all the critical residues needed for aconitase activity (*22*). Mitochondrial aconitase is one of the TCA cycle enzymes and bears a cubane [4Fe-4S] iron-sulfur cluster. Further analyses have revealed that IRP1 is a bifunctional protein, as in iron-replete cells, IRP1 bears an iron-sulfur cluster and functions as a cytoplasmic aconitase with no IRE-binding activity, while in iron-depleted cells, IRP1 lacks an iron-sulfur cluster and exhibits high affinity IRE-binding activity with no aconitase activity. Thus, the assembly of an iron-sulfur cluster in IRP1 controls the IRE-binding activity of this protein (*23*).

IRP2 was reported by Guo *et al.* and Samaniego *et al.* in 1994 (*24*, *25*). IRP2 exhibits approximately 58% identity with IRP1 in amino acid sequence, and has the same sensitivity to IREs as IRP1. However, unlike IRP1, which is a stable bi-functional protein, the IRE-binding activity of IRP2 is regulated by the iron-dependent proteasomal degradation of the protein in iron-replete cells (*25*, *26*). A unique domain composed of 73 amino acids in IRP2 termed the iron-dependent degradation (IDD) domain appears to be essential for the iron-dependent degradation of IRP2. Supporting this is that the insertion of the

IDD domain in the corresponding position of IRP1 is sufficient to transfer the phenotype of iron-dependent degradation to the otherwise stable IRP1 (*12*). Consequently, it has been hypothesized that the binding of iron to the degradation domain is the initial iron-sensing step of IRP2.

IRP2 is ubiquitylated in iron-replete cells but not in iron-depleted cells. As mentioned above, it is well documented that post-translational modifications of target proteins create signals for their selective recognition by ubiquitin ligases prior to their removal by the proteasome. Further analyses were performed to identify the nature of the signal delivered by IRP2 in iron-replete cells and they revealed that IRP2 is oxidatively modified *in vivo* in iron-replete cells. Moreover, we showed that IRP2 oxidized by iron and oxygen *in vitro* is ubiquitylated more strongly than its unmodified counterpart. These observations suggest that the oxidative modification of proteins creates motifs that can be recognized by the ubiquitin system and that allows their ubiquitylation and proteasomal degradation (*13*). Supporting this notion is a report showing that IRP2 levels are stabilized when cells are cultivated in hypoxic conditions (*27*).

Isolation of the ubiquitin ligase recognizing oxidized IRP2

To elucidate the mechanism behind the ubiquitylation of IRP2, several questions must be asked. First, what is the form of iron that binds to the IDD domain? Second, what is the oxidative modification created by iron and oxygen? Third, what is the ubiquitin ligase recognizing the oxidized form of IRP2? We first tried to identify the putative ubiquitin ligase that recognizes oxidized IRP2 (IRP2-E3). As the IDD domain is involved in the iron-dependent degradation of the protein, it may be that the IDD domain is the recognition site for IRP2-E3. Indeed, the ubiquitylation of oxidized IRP2 *in vitro* was inhibited by adding *in vitro*–oxidized IDD domain expressed in *E. coli* as a source of E3 enzymes to the *in vitro* ubiquitylation assay of oxidized IRP2. In striking contrast however, the addition of *in vitro*-oxidized IDD domain derived from insect cells enhanced IRP2 ubiquitylation in a dose-dependent manner. Insect cells but not bacteria contain an active ubiquitin system. Therefore, we hypothesized that the IRP2-E3 associates with the IDD domain from insect cells. Further analyses suggested that the IDD domain indeed serves as the recognition site for IRP2-E3 and that IRP2-E3 only associates with the IDD domain in iron-rich cells. Thus, the IDD domain appears to serve as the recognition site for IRP2-E3 in iron-rich conditions.

As IRP2 is stabilized in hypoxic conditions within cells, it appears that iron and oxygen are both necessary for the iron-dependent ubiquitylation of the protein. This feature was used to isolate IRP2-E3 by functional 2-hybrid screening. Thus, in the screening assay, yeast cells were cultured in either aerobic or anaerobic conditions and the IDD domain was used as the bait (*14*). A cDNA clone containing the entire coding region of a RING finger protein was identified. It was designated as HOIL-1 (heme-oxidized IRP2 ubiquitin ligase-1). HOIL-1 consists of 468 amino acids and possesses an ubiquitin-like domain at its N-terminus and the RING finger domain in its C-terminal half. HOIL-1 was cloned previously as a protein that associates with the hepatitis B virus X protein (XAP3) (*28*), protein kinase C (RBCK) (*29*), and UbcM4 (UIP28) (*30*). As RING finger proteins constitute an important family of ubiquitin ligases, we assessed the involvement of HOIL-1 in the iron-dependent ubiquitylation of IRP2. We found that HOIL-1 associates with IRP2 only in iron-rich conditions and that a HOIL-1 mutant lacking the ubiquitin-like domain could not bind to IRP2. Moreover, the introduction of a dominant negative HOIL-1 mutant that carries mutations in the RING finger domain inhibits the iron-dependent down-regulation of IRP2 levels. This mutant protein functions as a dominant negative protein because the mutation of the RING domain abolishes its E2 binding but not its substrate binding.

We also characterized the form of the iron that binds to the IDD domain. It had been suggested previously that heme is involved in IRP2 degradation (*31*), and a motif homologous to HRM (heme regulatory motif) exists in the IDD domain. HRM (also called the CP motif) has been found in heme-binding regulatory proteins, including the yeast transcriptional activator Hap1 (heme activator protein 1) (*32*) and the mammalian transcriptional activator Bach1 (*33*). Thus, we speculated that iron might bind to IDD in the form of heme. *In vitro* loading of heme to purified IRP2 showed that heme does bind to IRP2 (*14*). Heme-binding of IRP2 was also observed *in vivo*. The molar-ratio of heme to IRP2 was approximately 1 (Ishikawa, H., and Iwai, K., unpublished observations). Thus, heme may bind to the IDD domain of IRP2, possibly *via* its HRM-like motif, in iron-replete cells.

The next question to be addressed was whether heme binding to IRP2 triggers the ubiquitylation of IRP2. We found that succinyl acetone, an inhibitor of heme synthesis, suppresses the down-regulation in cells of IRP2 protein levels caused by the presence of iron. However, when heme was added exogenously, the effect of succinyl acetone was ameliorated. An *in vitro* ubiquitylation assay also showed that heme-loaded IRP2 is ubiquitylated by HOIL-1 while it is not ubiquitylated by VBC-Cul2 ubiquitin ligase, which recognizes prolyl-hydroxylated HIF-α (*10*, *11*). We also found that the ubiquitylation of heme-bound IRP2 was strongly suppressed when the reaction was carried out anaerobically. These results suggest a model for the iron-induced degradation of IRP2 that is depicted in Fig. 3. This model contends that heme binds to the IDD domain in iron-rich cells, followed by the generation of a superoxide radical through the reaction of heme with oxygen. Upon the oxidation by the superoxide radical, the IDD domain is subsequently recognized by HOIL-1 and IRP2 is ubiquitylated (*14*).

There are two questions that need to be addressed to further understand the heme-dependent degradation of IRP2. First, what is the oxidative modification of IRP2 that is generated by heme and oxygen? Second, what roles does HOIL-1 play in the turnover of oxidized proteins? These questions will be assessed in the penultimate section below where I describe the role HOIL-1 plays in the turnover of oxidized cells. Before I do this, however, I want to discuss the role heme plays in the iron-sensing of cells.

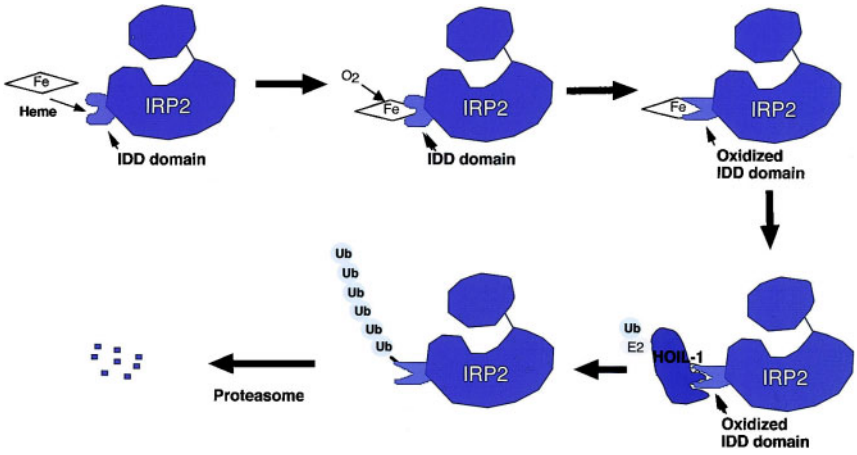

Fig. 3. **The proposed model of the iron-induced degradation of IRP2.** In iron-rich cells, heme synthesis is induced. The heme then binds to the IDD domain, followed by the generation of a superoxide radical through the reaction of heme with oxygen. Upon oxidation by the superoxide radical, the IDD domain is subsequently recognized by HOIL-1 and IRP2 is ubiquitylated, followed by proteasomal degradation.

Involvement of heme in the iron-sensing of cells

That IRP2 is a heme-binding protein and its stability is regulated by heme suggests that heme is involved in the iron-sensing of cells. As mentioned, IRPs are the central regulators of iron metabolism in cells, and the iron-induced regulation of their IRE-binding is the key feature of iron regulation. It has also been established that IRP1 is regulated by the assembly/disassembly of its iron-sulfur cluster. These observations together indicate that the availability of iron to cells is sensed by two different systems, namely, the system that assembles iron-sulfur clusters and the system that makes heme available. Heme is generated in mitochondria and in yeast at least, iron-sulfur clusters are also formed in mitochondria. Therefore, it seems reasonable to propose that IRP2 is regulated by a different mechanism from that used to regulate IRP1 because heme synthesis and iron-sulfur cluster assembly are both regulated by mitochondrial iron availability. Supporting this notion is that we have observed that the iron concentration required to down-regulate IRP2 is identical to that required to disassemble the iron-sulfur cluster of IRP1 (Iwai, K., unpublished observations). Arguing against this notion, however, is that it has been suggested that in metazoans, iron-sulfur clusters are generated both in the mitochondria and the cytoplasm (*34*).

Heme is known to function as a cofactor of many proteins. For example, it serves as an oxygen-binding center of hemoglobin and myoglobin. It also functions as a redox active center of proteins. In addition, heme modulates the functions of regulatory proteins involved in gene expression. For example, when the transcriptional activator Hap1 in *S. cerevisiae* binds to heme, it activates the transcription of genes involved in oxygen utilization or oxidative damage responses (*32*).

Moreover, heme binding to the mammalian transcriptional repressor Bach1 suppresses the repressor activity of the protein (*33*). In addition, the activity of Irr, the transcriptional regulator of heme synthesis in bacteria, has been shown to be regulated by heme. The binding of heme to these regulatory proteins is mediated via the conserved amino acid HRM sequence. As mentioned above, IRP2 is also believed to bind to heme via its HRM-like motif in the IDD domain. These observations indicate that with regard to understanding the role heme plays in iron-sensing, it is of interest to determine the role HRM plays in heme-binding.

As mentioned, the activity of Irr is regulated by heme-mediated degradation of the protein (*35*, *36*). The heme-dependent binding of Irr to ferrocheletase has shown to be critical for its degradation (*37*), suggesting that both Irr and IRP2 share common features in the iron-mediated regulation of their protein activity. However, it has not been shown that oxidation is critical for the degradation of Irr. Indeed, since bacteria do not have an active ubiquitin system or mitochondria (which is where ferrocheletase is located in eukaryotic cells), the degradation mechanism of Irr must be different from that of IRP2.

Role of the ubiquitin system in the turnover of oxidized proteins

That HOIL-1 recognizes oxidized IRP2 suggests that this ubiquitin ligase may participate in the broader metabolic process of removing oxidized proteins. Protein oxidation is a natural consequence of aerobic metabolism in cells. Oxygen radicals oxidatively modify amino acid residues of proteins, which cause these proteins to lose their activity or function. It has been shown that oxidized proteins tend to form protein aggregates and are often toxic to cells (*38*). Moreover, such aggregates participate in the pathogenesis of several neurodegenerative disorders, and oxidation has been suggested to be involved in their formation (*38*). Therefore, organisms have developed pathways to remove oxidized proteins by rapid protein degradation. These pathways thus serve as important components in cellular quality control mechanisms. Little is known about the mechanisms that underlie this selective removal of oxidized proteins. However, to understand the potential role HOIL-1 could play in the elimination of oxidized proteins, it will be necessary to briefly review what is known about the mechanisms used by cells to degrade oxidized proteins.

Most studies agree that oxidized proteins are degraded by the proteasome (*39*, *40*). However, it remains unclear whether ubiquitylation is involved in this process. Some studies have revealed that oxidized proteins are ubiquitylated. The susceptibility of RNase A to *in vitro* ubiquitylation is increased by oxidation (*41*). However, other researchers have shown that oxidized proteins are degraded by the 20S proteasome and no ubiquitylation is

necessary for their degradation (*42*). Oxidized proteins turn over normally in temperature-sensitive E1 cells at the non-permissive temperature and *in vitro* oxidized proteins are degraded in the lysates prepared from the ts-E1 cells at non-permissive temperatures (*42*). No ubiquitylation carry out in ts-E1 cells at non-permissive temperature because of the lack of the E1 activity at non-permissive temperature. Moreover, ubiquitin conjugation activity in cells has been shown to be impaired in strongly oxidized conditions (*38*).

Normally, the 20S proteasome is controlled by the 19S regulatory particle. Ubiquitylated proteins are recognized by the 19S regulatory particle and then degraded by 20S proteasome. The crystal structure of the yeast 20S proteasome has revealed that the center of the α ring that constitutes part of the proteasome barrel is closed to prevent unrelated proteins from entering the barrel (*6*). This suggests that nonubiqutylated proteins cannot enter the barrel of the protease, which contains the catalytic sites. However, as several reports have suggested that the 20S proteasome can degrade unfolded proteins (*43*), and oxidation often causes protein unfolding, it may be that under strongly oxidized conditions, the activity of the 20S proteasome is stimulated and nonubiquitylated unfolded proteins can be degraded. Supporting this is that the activity of the 20S proteasome is enhanced when it is treated with low doses of SDS *in vitro* (*7*). This suggests that oxidative modification could promote the ability of the 20S proteasome to degrade nonubiquitylated, oxidatively-damaged proteins.

There are also suggestions that the 26S proteasome degrades unfolded nonubiqutylated proteins. For example, the Ca^{2+} binding protein calmodulin is degraded by the 26S proteasome without ubiquitylation *in vitro* when Ca^{2+} is removed from the protein and it unfolds (*44*). Moreover, it appears that the 19S regulatory particle preferentially interacts with unfolded proteins (*40*). Analysis of the archeal 20S proteasome and the PAN (proteasome-activating nucleotidase) regulatory complex, which is a homolog of the basal part of the eukaryotic 19S regulatory particle, revealed that unfolded proteins are preferentially recognized by PAN and degraded by the proteasome (*40*). It is likely that the ATPases at the base of the 19S regulatory particle participate in its unfolding of substrates as well as in other 19S activities such as substrate recognition, the opening of the gate of the 20S proteasome, and the translocation of unfolded peptides to the 20S proteasome (*45*). ATPase activity is also necessary for the degradation of the unfolded proteins (*40*). Thus, it is possible that physiologically unfolded proteins could be degraded in an ubiquitin-independent manner by the 26S proteasome.

There are also some specific exceptions to the rule that ubiquitin conjugation is necessary for degradation by the 26S proteasome. For example, ornitine decarboxylase has been shown to be degraded by the 26S proteasome without ubiquitylation (*46*). In this case, a specific ancillary protein called antizyme targets ornitine decarboxylase to the 26S proteasome. The CDK inhibitor p21 is also degraded by the 26S proteasome without ubiquitylation, although the involvement of an ancillary protein in this process has not been reported (*47*).

These observations can be reconciled by the hypothesis of the degradation of oxidized proteins: in that lightly oxidized proteins, where the oxidation creates a specific motif that can be recognized by a ubiquitin ligase, are ubiquitylated and degraded by the 26S proteasome, while heavily oxidized proteins may be degraded by the 20S or the 26S proteasome in the absence of ubiquitylation, probably because the extensive oxidation unfolds these proteins.

What roles could HOIL-1 play in the removal of oxidized proteins in general? To address this issue, it is necessary to identify the oxidative modification that leads to IRP2 ubiquitylation. Is it a site-specific modification? In other words, is it similar to the oxygen-dependent hydroxylation of the specific proline residue in HIF-α that leads to its recognition by VBC-Cul2 ubiquitin ligase and proteasomal degradation (*10*, *11*). Or does HOIL-1 recognize random oxidative protein damage? With regard to this, it is interesting that IRP2 maintains its activity in iron-rich cells treated with proteasome inhibitors, which indicates that the oxidized IRP2 still possesses its physiological activity (*12*). Thus, it appears that the protein is not inactivated during the oxidation event that generates the specific signal for ubiquitylation. This in turn suggests that the oxidation of IRP2 creates a site-specific motif rather than random damage.

What could be the nature of the site-specific oxidative IRP2 modification? It has been hypothesized previously that since the ubiquitylation of HIF-α requires both oxygen and iron, HIF-α and IRP2 may be modified in an identical manner and are recognized by the same ligase (*48*). However, that we found that oxidized IRP2 is not recognized by VBC-Cul2 ligase (*14*) challenges this possibility. Moreover, IRP2 is degraded in an iron-dependent fashion in renal cell carcinoma cells lacking the von Hipplel-Lindau protein (Iwai, K., unpublished observation). Thus, the oxidative modification of IRP2 does not seem to involve the hydroxylation of proline residues. However, it cannot be ruled out that the oxidative modification event may involve the specific oxidation of residues other than prolines.

It is possible that HOIL-1 recognizes the IDD domain that has become unfolded or otherwise damaged due to the oxidation mediated by the heme and the oxidative modification does not affect the overall structure or the function of the protein. This would explain why oxidized IRP2 still possesses its physiological activity (*12*). Supporting this is that the IDD domain, which is the site for heme binding and the site subjected to oxidation, is not needed for the IRE-binding of the protein, as an IRP2 mutant lacking the IDD domain and an IRP1 mutant possessing the domain at the corresponding position of the protein both possess IRE-binding activity (*12*). Thus, the oxidatively unfolded IDD domain may be recognized by HOIL-1 ubiquitin ligase and thereby be destined for degradation.

On the basis of the evidence to date, it is likely that HOIL-1 ubiquitin ligase participates in the removal of physiologically oxidized proteins rather than eliminating proteins subjected to moderate or heavy oxidation.

Concluding remarks

Whether ubiquitylation is necessary for the degradation of oxidized proteins remains a controversial issue. Our isolation of the HOIL-1 ubiquitin ligase, which recognizes a physiologically oxidized protein, suggests that the ubiquitin system is involved in degrading oxidized proteins. Analysis of the function of HOIL-1 will shed more light on this issue.

HOIL-1 may play diverse roles in biology and thus could be useful in medicine. For example, it may participate in carcinogenesis. As mentioned previously, HOIL-1 has been cloned as an associated protein of the hepatitis B virus X protein (HBx) (*28*), HBx has been shown to possess cell-transforming potential and to be a critical factor in hepatocarcinogenesis. However, the precise mechanism behind its cell-transforming potential is not yet clear, although it is known that HBx is involved in numerous cellular events including gene transcription, signal transduction, DNA repair, apoptosis and cell proliferation (*49*). We speculate that HBx may function as an ancillary protein of HOIL-1 ligase, similar to the papilloma E6 protein, which helps the E6-AP ligase to ubiquitylate p53 (*50*). Further work is needed to clarify the role HOIL-1 plays in HBx-mediated carcinogenesis. Thus, the characterization of HOIL-1 function may reveal new aspects of ubiquitin-mediated regulation.

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