Identification of Determinants in E2 Ubiquitin-conjugating Enzymes Required for hect E3 Ubiquitin-Protein Ligase Interaction*

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Members of the hect domain protein family are characterized by sequence similarity of their C-terminal regions to the C terminus of E6-AP, an E3 ubiquitin-protein ligase. An essential intermediate step in E6-AP-dependent ubiquitination is the formation of a thioester complex between E6-AP and ubiquitin in the presence of distinct E2 ubiquitin-conjugating enzymes including human UbcH5, a member of the UBC4/UBC5 subfamily of E2s. Similarly, several hect domain proteins, including Saccharomyces cerevisiae RSP5, form ubiquitin thioester complexes, indicating that hect domain proteins in general have E3 activity. We show here, by the use of chimeric E2s generated between UbcH5 and other E2s, that a region of UbcH5 encompassing the catalytic site cysteine residue is critical for its ability to interact with E6-AP and RSP5. Of particular importance is a phenylalanine residue at position 62 of UbcH5 that is conserved among the members of the UBC4/UBC5 subfamily but is not present in any of the other known E2s, whereas the N-terminal 60 amino acids do not contribute significantly to the specificity of these interactions. The conservation of this phenylalanine residue throughout evolution underlines the importance of the ability to interact with hect domain proteins for the cellular function of UBC4/UBC5 subfamily members.

Modification of proteins by the covalent attachment of ubiquitin in many cases targets these proteins for proteasome-mediated degradation (reviewed in Refs. 1–5) or, as shown for some membrane proteins, for internalization and degradation via the lysosomal pathway (6, 7). Ubiquitin conjugation (ubiquitination) requires the combined action of ubiquitin-activating enzymes E11 (or UBA), ubiquitin-conjugating enzymes E2 (or UBC), and, probably in most cases, ubiquitin-protein ligases E3 (or UBR). In the first step, ubiquitin is activated by E1 at the expense of ATP via the formation of a high energy thioester bond. Finally, ubiquitin is covalently linked to a substrate protein either by the E2 alone or in conjunction with an E3. This sequential mode of ubiquitin transfer indicates that E2s and in particular E3s play a major role in substrate recognition.

Proteins that have been identified with E3 activity include yeast UBR1 (8), mammalian E6-AP (9), members of an E6-AP related family of putative E3s termed hect (homologous to E6-AP C terminus) domain proteins (10, 11), a protein complex termed APC (anaphase promoting complex) or cyclosome (reviewed in Refs. 12 and 13), and the recently identified SCF complexes (14–17). Based on their mode of action, it appears that E3s can be classified into at least two categories. Some E3s may function as docking proteins by binding specifically to E2s and substrate proteins, thereby facilitating E2-catalyzed ubiquitination of the substrates. Such E3s appear to be represented by the SCF complexes (14, 15, 18). Other E3s are loaded with ubiquitin by E2s via thioester formation, indicating that these E3s directly catalyze the attachment of ubiquitin to substrate proteins. E3s with the capacity to form ubiquitin thioester complexes include E6-AP and hect domain proteins (10, 11, 19, 20). Despite their different mode of action, a common feature of all known E3s is that each E3 appears to specifically interact with a distinct E2 or distinct subsets of E2s. For instance, SCF complexes bind specifically to UBC5CDC34 (14–16, 18), whereas E6-AP and other hect domain proteins, including Saccharomyces cerevisiae RSP5, have been shown in vitro to interact with UbcH5 and/or UbcH7 and related E2s (9–11, 21–23). UbcH5 belongs to an evolutionarily conserved subfamily of E2s that are highly similar to S. cerevisiae UBC4/UBC5 (9, 21, 24), whereas based on amino acid sequence comparisons, the genome of S. cerevisiae does not encode an obvious ortholog to UbcH7 (22).

Members of the hect domain family are large proteins ranging in size from 90 kDa to more than 500 kDa and are found in all eukaryotic organisms examined (10, 11, 19). They are characterized by a C-terminal region of approximately 350 amino acids in length, which shows significant similarity to the C terminus of E6-AP, and therefore has been termed the hect domain (10). The hect domain appears to represent the catalytic domain of these otherwise unrelated proteins because the hect domain is necessary and sufficient to form ubiquitin thioester complexes in the presence of its cognate E2s (11). Similar to E2s, it appears that a cell contains a number of different hect domain proteins. Based on data base searches, the genome of S. cerevisiae encodes five different hect domain proteins (4), whereas the human genome codes for at least 20 hect domain proteins (11).

All known E2s are characterized by a conserved core domain of approximately 150 amino acids in size, termed the UBC domain, and can be grouped into four classes based on the presence or absence of additional N-terminal and/or C-terminal sequences (25). Because UbcH5 belongs to the so-called class I

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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; SCF, SKP1, CDC53, F-box protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; nt, nucleotide(s).
E2 enzymes, which consist of the UBC domain only, were interested to determine the regions of UbcH5 that specify its ability to interact with members of the UbcD family. Here we report that a region surrounding the catalytic site cysteine residue, as well as a phenylalanine residue at position 62 that is characteristic for members of the UBC4/UBC5 subfamily, are critical for the ability of UbcH5 to interact with E6-AP and RSP5 in vitro. This indicates that the structural conformation of the thioester-forming region of E2s determines whether or not a given E2 can functionally interact with UbcD proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The pET-3a expression plasmid for UbcH5 has been described previously (21). Expression plasmids for UbcM3 and UbcH1 were constructed by ligating PCR products into pET-3a (UbcH1 and UbcM3) and pGEX-2T (UbcM3). The valine residue at position 129 of UbcM3 (or position 83 with respect to the amino acid sequence of UbcH5; see Fig. 1A), the serine residue at position 83 of the cmissive E2 35 (see Fig. 1B), and the asparagine residue at position 65 of UbcH1 (see Fig. 1A) were altered to serine (3-V83), valine (35-S83V), and phenylalanine (1-N65F), respectively, by PCR-directed mutagenesis. The respective cDNAs were cloned into pGEX-2T. The cDNAs encoding the various chimeric E2s were constructed by inserting two different chimeric E2s between UbcH5 and UbcM3 into the bacterial pET expression vector system. The respective cDNAs were termed 353 and 355 (the nomenclature reflects the relative contribution of the UBC domain of UbcH5 and UbcM3 to the UBC domain of the respective chimeric E2; Fig. 1B) and consist of the N-terminal 62 amino acids of UbcH5 fused to the C-terminal 85 amino acids of UbcM3 and of the UbcH5 amino acids of UbcM3 fused to the C-terminal 85 amino acids of UbcH5, respectively.

**Protein Expression**—UbcH1, UbcH5, and UbcM3 were expressed in Sf9 cells infected with the respective recombinant baculoviruses (RSP5 and wheat germ E1 were prepared from Sf9 cells infected with the respective recombinant baculoviruses (RSP5 and wheat germ E1 encoding recombinant baculoviruses were kindly provided by J. M. Huibregtse and W. Krek, respectively). Partial purification of E6-AP, RSP5, and RSP5C6 was performed as described previously (9, 10, 27), and partial purification of E1 was performed as described for bacterially expressed E1 (9).

**Ubiquitination Assays**—Formation of thioester adducts of the different E2s, E6-AP, RSP5, and RSP5C6 with ubiquitin was determined essentially as described previously (19). Reaction mixtures contained approximately 20–50 ng of E1, 300 ng of radiolabeled ubiquitin, the respective E2s, and 300 ng of E6-AP or 100–300 ng of RSP5 as indicated. With respect to their enzymatic activity, similar amounts of the different E2s were used as assessed by their ability to form thioester complexes with ubiquitin in the presence of E1.

**RESULTS**

**Generation of Chimeric E2s Consisting of Different Parts of UbcH5 and UbcM3**—Similar to UbcH5, murine UbcM3 and its putative human homolog UbcH6 are members of the UBC4/UBC5 subfamily of E2s (22, 26). Unlike UbcH5, however, UbcM3 and UbcH6 contain an N-terminal extension of 46 amino acids in addition to the UBC domain (Fig. 1A). Despite its similarity to UbcH5 at the amino acid sequence level, it was shown that UbcH6 is only weakly active in E6-AP-dependent ubiquitination (22). Furthermore, this difference between UbcH5 and UbcH6 was not due to the N-terminal extension of UbcH6 because a respective deletion mutant of UbcH6 was as inefficient in E6-AP-dependent ubiquitination as full-length UbcH6. UbcH6 and UbcM3 are identical at the amino acid sequence level, except for two amino acid exchanges in the N-terminal extension (22, 26) and thus can be considered functionally equivalent. For cloning reasons, a cDNA encoding UbcM3 was used for the following rather than a cDNA encoding UbcH6.

To determine the region(s) of UbcH5 that is responsible for the observed difference to UbcM3, initially two cDNAs encoding two different chimeric E2s between UbcH5 and UbcM3 were generated and cloned into the bacterial pET expression system. The respective cDNAs were termed 353 and 355 (the nomenclature reflects the relative contribution of the UBC domain of UbcH5 and UbcM3 to the UBC domain of the respective chimeric E2; Fig. 1B) and consist of the N-terminal 62 amino acids of UbcH5 fused to the C-terminal 85 amino acids of UbcM3 and of the UbcH5 amino acids of UbcM3 fused to the C-terminal 85 amino acids of UbcH5, respectively. The chimeric E2s were expressed in bacteria and tested for their ability to interact with E6-AP.

An essential intermediate step in E6-AP-dependent ubiquitination is the formation of a thioester complex between E6-AP and ubiquitin in the presence of E1 and UbcH5 or UbcH7 (19, 22). As shown in Fig. 2, 355 was able to transfer ubiquitin to E6-AP with an efficiency similar to UbcH5, whereas 533 was almost inactive under the conditions used. It should be noted that similar amounts of active E2s were used as determined by their ability to form thioester complexes with ubiquitin in the presence of E1 (data not shown). Taken together, this clearly shows that the difference between UbcM3 and UbcH5 with respect to their ability to interact with E6-AP maps to the C-terminal 85 amino acids of UbcH5.

**A Region Encompassing the Catalytic Site Cysteine Residue Is Critical for Interaction of UbcH5 with E6-AP and RSP5**—To further delineate the region of UbcH5 that is critical for functional interaction with E6-AP, additional chimeric E2s were constructed (Fig. 1B). Because some of the chimeric E2s were expressed at rather low levels, if at all, in the pET expression system (not shown), making it difficult to assess the amount of active E2, the E2s were expressed as GST fusion proteins. The fusion proteins were concentrated by affinity chromatography and then the GST part was removed by thrombin cleavage to ensure that the ability of the respective E2s to interact with E6-AP was not influenced by their fusion to GST. This showed that a chimeric E2 (termed “35”), consisting of the C-terminal 70 amino acids of UbcH5 was as active as 355 in E6-AP/ubiquitin thioester assays, whereas a chimeric E2 containing the C-terminal 40 amino acids of UbcH5 (335) was significantly less active. This indicates that a region of UbcH5 comprising amino acids 78–107 is critical for interaction with E6-AP. Indeed, the chimeric E2 353 in which the corresponding region of UbcM3 was replaced by amino acids 78–107 of UbcH5
Amino acid sequence comparison of UbcH5 with UbcM3 (Fig. 1A) reveals that amino acid region 78–107 of UbcH5 contains six amino acid exchanges to the corresponding region of UbcM3. The most significant difference would appear to be the presence of a serine residue at position 83 of UbcH5, whereas UbcM3 contains a valine residue at the corresponding position. Therefore, point mutants of 35, 335, and UbcM3 (or UbcH1, respectively). The sequence of the N-terminal extension of UbcM3 (46 amino acids in size) is only partially shown, with 40 amino acid residues abbreviated as X40. Arrows indicate the sites that were used to generate chimeric E2s between UbcH5 and UbcM3 or UbcH1, respectively. The sequence of the N-terminal extension of UbcM3, which corresponds to the initial methionine of UbcH5, was defined as position 1 in those chimeric E2s that contain parts of UbcM3 at the N terminus. It should be noted, however, that these chimeric E2s also contain the extra N-terminal 46 amino acids of UbcM3 as indicated in A. The nomenclature reflects the relative contribution of the UBC domain of UbcH5, UbcM3, or UbcH1 to the UBC domain of the respective chimeric E2. The various chimeric E2s were generated in bacteria and tested for their interaction with E6-AP or RSP5, as assessed by their ability to load E6-AP or RSP5 with ubiquitin in the presence of E1 (see “Experimental Procedures”). +, transfers ubiquitin to E6-AP and RSP5 with an efficiency similar to UbcH5; +/-, significantly less efficient (more than 3-fold reduced) in transferring ubiquitin to E6-AP and RSP5 than UbcH5; −, thioester complex formation of E6-AP and RSP5 with ubiquitin was not observed under the conditions used.

was nearly as active as 355 in transferring ubiquitin to E6-AP.

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Taken together, the data presented above indicate that the structural conformation of the region surrounding the catalytic site cysteine residue is important for UbcH5 to functionally interact with E6-AP. Furthermore, the amino acid sequence differences in this region are mainly responsible for the observed difference between UbcH5 and UbcM3 with respect to their ability to interact with E6-AP. This notion is further supported by the finding that only those chimeric E2s that were able to load E6-AP with ubiquitin were able to support E6-AP-dependent ubiquitination of an artificial substrate protein (see Fig. 5 and Fig. 1B).
The yeast hect domain protein RSP5, which except for the hect domain is not related to E6-AP at the amino acid sequence level, was previously shown to form thioester complexes with ubiquitin in the presence of both UbcH5 and UbcM3 but with significantly less efficiency in the presence of UbcM3 (16, 22). Therefore, ubiquitin thioester assays with RSP5 were performed using the various chimeric E2s. As shown in Fig. 3B, the same chimeric E2s that could interact with E6-AP were able to load RSP5 with ubiquitin, whereas those that did not interact with E6-AP also did not interact with RSP5. This indicates that similar amino acid sequences or structures of UbcH5 and UbcM3 are required for its interaction with both E6-AP and RSP5.

**A Phenylnalanine Residue Conserved among All Members of the UBC4/UBC5 Subfamily of E2s Is Essential for Interaction with E6-AP and RSP5**—Replacement of the N-terminal 62 amino acids of UbcH5 with the corresponding region from UbcM3 resulted in a chimeric E2 that was capable of transferring ubiquitin to both E6-AP and RSP5 (355; Figs. 2 and 3). This shows that this region is not responsible for the observed difference between UbcH5 and UbcM3. Because UbcH5 and UbcM3 belong to the same subfamily of E2s, however, the possibility that the N-terminal 62 amino acids of UbcH5 contain a determinant(s) that is critical for UBC4/UBC5 subfamily members to interact with hect domain proteins cannot be excluded. This possibility was addressed by testing whether or not the N-terminal region of UbcH5 can be functionally replaced by the corresponding region of an unrelated E2. A chimeric protein was constructed consisting of the N-terminal 65-amino acid residues of UbcH1, the human homolog of S. cerevisiae UBC2RAD6 (28), and the C-terminal 85 amino acids of UbcH5 (155; Fig. 1). UbcH1 was used because it had previously been shown that UbcH1 or its Arabidopsis thaliana homolog UBC1 can neither transfer ubiquitin to E6-AP nor RSP5 nor support E6-AP-dependent ubiquitination (9, 11). Ubiquitin thioester assays showed that, although 155 itself can form thioester complexes with ubiquitin with an efficiency similar to that of UbcH5 (data not shown), it was not able to transfer ubiquitin to either E6-AP or RSP5 (Fig. 4). Similarly, 155 was inactive in E6-AP-dependent ubiquitination (Fig. 5). Thus, the N-terminal 62 amino acids of UbcH5, as well as the corresponding region of UbcM3, contain a determinant that is required for interaction with hect domain family members.

Alignment of various UBCs (not shown) reveals that a phenylalanine residue at position 62 of UbcH5 (Fig. 1A) is conserved among the members of the UBC4/UBC5 subfamily but is not present in any of the other known E2s. For instance, UbcH1 contains an asparagine residue at the corresponding position (Fig. 1A). Therefore, this asparagine residue was substituted by phenylalanine in both UbcH1 (1-N65F) and 155 (155-N65F). Functional analysis of these mutants revealed that 155-N65F interacted with E6-AP with an efficiency similar to UbcH5 in both ubiquitin thioester assays (Fig. 4A) and ubiquitination assays (Fig. 5), whereas 1-N65F was as inactive as UbcH1. The results obtained for RSP5 were slightly different in that 155-N65F was able to transfer ubiquitin to RSP5 but with an approximately 2-fold reduced efficiency compared with UbcH5 (Fig. 4A). Taken together, these results indicate that a phenylalanine residue at position 62 is crucial for the ability of UbcH5 to interact with E6-AP and RSP5.

*S. cerevisiae* UBC4 and UBC5 are involved in stress-related functions (24, 29). Yeast strains carrying mutations in either one of these genes are viable and lead to only moderate phenotypes, whereas ubc4 ubc5 double mutants grow slowly and are inviable at elevated temperatures (24). It has previously been reported that members of the UBC4/UBC5 subfamily of E2s, including *Drosophila* UbcD1, can rescue the deficiencies of yeast *ubc4 ubc5* mutants (30, 31). Furthermore, the genome of *S. cerevisiae* does not encode a functional homolog of E6-AP but, in addition to RSP5, encodes four additional hect domain proteins (4). Therefore, the various chimeric E2s were tested for their ability to complement the *ubc4 ubc5* phenotype. In yeast, however, the chimeric E2s 35, 335, and 155 and their respective point mutated forms were expressed at vastly different levels. Thus, it was not possible to assess whether the ability of the various chimeric E2s to interact with E6-AP and RSP5 *in vitro* correlates with their ability to complement the phenotype of *ubc4 ubc5* mutants *in vivo*.

**DISCUSSION**

It is assumed that the specificity of ubiquitin-dependent degradation is mediated at least in part by the E2 and E3 enzymes of the ubiquitin conjugation system (1, 2, 4, 5). Furthermore, it appears that distinct E3s interact only with certain E2s, suggesting that a specific interplay between E2s and E3s contributes to the specificity and selectivity of protein ubiquitination. Members of the hect domain family of putative E3s have not been shown to specifically interact *in vitro* with members of the UBC4/UBC5 subfamily of E2s including human UbcH5 (9–11, 22). The mutational analysis of UbcH5 presented in this study shows that the amino acid composition of a region encompassing the thioester-forming cysteine residue (amino acid residues 78–107) and a phenylalanine residue at position 62 are essential determinants for its ability to
functionally interact with hect domain proteins. The presence of a phenylalanine residue at the respective position is evolutionally conserved among the members of the UBC4/UBC5 subfamily but is not present in any of the other known E2s. This supports the notion that the ability of UbcH5 and related proteins to interact with hect domain proteins is important for their physiological function.

The three-dimensional structure of several class I E2s, including *S. cerevisiae* UBC4 and *A. thaliana* UBC1, the putative homolog of UbcH1 and *S. cerevisiae* UBC2RAD6, has been determined (32, 33). This revealed that the overall structure of the UBC domain is conserved and contains four α-helices and a four-stranded antiparallel β-sheet. The putative catalytic site cysteine residue is located in a long extended stretch between the fourth β-strand and the second α-helix. As breakpoints to generate chimeric E2s between UbcH5 and UbcM3 or UbcH1,
the amino acid residues at position 62/63, at position 77/78, and at position 107/108 (numbering according to the UbcH5 sequence; Fig. 1) were used. Amino acid residues 62/63 are located in a loop connecting the third and the fourth strand of the β-sheet, whereas amino acid residues 77/78 are located in the stretch between the fourth β-strand and the second α-helix. Such chimeric E2s were expressed in bacteria to appreciable levels and were active in thioester complex formation with ubiquitin. This demonstrates that the fusion between different E2s at these positions does not grossly interfere with proper folding of the resulting chimeric E2s.

Amino acid residues 107/108 are located within the second α-helix and thus fusion of the respective parts of different E2s at this site may disturb the overall structure of some of the resulting chimeric E2s. This was clearly not the case for the chimeric E2 353, because it was nearly as active as UbcH5 in transferring ubiquitin to both E6-AP and RSP5, respectively. This shows that the difference between UbcH5 and UbcM3, with respect to their ability to interact with hect domain proteins, is mainly determined by the catalytic site cysteine residue at position 85 of UbcH5. In particular, the presence of a serine residue at position 83 seems to be critical because a mutant form of the chimeric E2 35 bearing a valine at this position, the corresponding amino acid residue of UbcM3, interacts with E6-AP and RSP5 as inefficiently as UbcM3. Conversely, substitution of this valine residue in UbcM3 with serine significantly enhances its ability to interact with E6-AP and RSP5. It is notable that the presence of a bulky hydrophobic residue at position 83 appears to be a peculiarity of UbcM3 and E2s that are highly similar to UbcM3 (26), apparently not being found in other members of the UBC4/UBC5 subfamily as well as in unrelated E2s. Because the cellular functions of UbcM3 and its related E2s are unknown, the significance of this difference is presently unclear. The data obtained with the chimeric E2 353, however, do not exclude the possibility that the C-terminal 40 amino acids of UbcH5/UbcM3 are critically involved in the interaction with E6-AP and RSP5. To address this possibility a chimeric E2 analogous to 353 was generated by replacing the C-terminal UbcM3 part by the corresponding region of UbcH1. However, this chimeric E2 termed 351 was not expressed in bacteria to significant levels. A reasonable explanation for this is that fusion of the C-terminus of UbcH1 to the respective part of UbcH5 results in a structural distortion of the resulting chimeric protein. In any

![Fig. 5. Effectiveness of chimeric E2s in E6-AP-dependent ubiquitination.](image-url)
case, because of the lack of detectable expression, it cannot be excluded that the C-terminal 40 amino acids of UbcH5/UbM3 are critically involved in the interaction with E6-AP and RSP5.

Based on the results obtained with the chimera 155 and its mutant form 155-N65F, it appears that the N-terminal 61 amino acids of UbcH5 are not critical for its ability to interact with heat domain proteins, as indicated by the fact that this region can be replaced by the corresponding region of a functionally unrelated E2. Although this does not exclude the possibility that the N-terminal region of E2s is directly involved in contacting heat domain proteins, it suggests that this region is required for properties common to all E2s, e.g., interaction with E1 and/or proper folding. The distinct property of UbcH5 to functionally interact with heat domain proteins, however, is determined by its C-terminal 87 amino acid residues. Furthermore, the presence of a phenylalanine residue at position 62 of UbcH5 seems to be of particular importance. Interestingly, all known members of the UBC4/UBC5 subfamily, including UbM3, contain a phenylalanine residue at the corresponding position, and this seems to be unique to members of this subfamily. The results presented strongly indicate that the presence of a phenylalanine residue at this position is necessary for the ability of an E2 to interact with E6-AP and RSP5. However, it is not sufficient as demonstrated by the results obtained with 1-N65F, a mutant form of UbcH1 in which an asparagine residue at the respective position was replaced by phenylalanine.

An essential intermediate step in E6-AP-dependent ubiquitination is the transfer of ubiquitin from UbcH5 to E6-AP resulting in thioester complex formation of E6-AP with ubiquitin (19). Thus, it is not surprising that the region of UbcH5 surrounding the putative ubiquitin-donating cysteine residue at position 85 is critical for the ability of UbcH5 to functionally interact with E6-AP and RSP5 and presumably with heat domain proteins in general. SCF complexes also have E3 activity (14, 15) but, unlike heat domain proteins, SCF complexes do not appear to form thioester complexes with ubiquitin but instead function as docking proteins by stably binding both substrate proteins and their cognate E2. UBC3/CDC34, UBC3/CDC34 is a class II enzyme containing a C-terminal extension of approximately 70 amino acids in addition to the UBC domain (34). Consistent with the notion that in 3C-dependent ubiquitination the final transfer of ubiquitin to substrates is probably directly catalyzed by UBC3/CDC34, the interaction of SCF complexes with UBC3/CDC34 is not mediated by the UBC domain. Rather, it was shown that the C-terminal extension of UBC3/CDC34 is necessary and sufficient to mediate its interaction with SCF complexes (18). Thus, it appears that, according to their mode of action, different E3 proteins recognize different regions on their cognate E2s. Interestingly, it was recently reported that UBC4 is involved in UBR1-dependent ubiquitination of certain proteins (35). UBR1 was originally isolated as an E3 that, in conjunction with UBC2/RAD6, mediates the recognition of substrates according to the N-end rule (8, 36, 37).

Although UBR1 does not appear to be related to heat domain proteins at the amino acid sequence level, it has been reported that UBR1 can form thioester complexes with ubiquitin (5). It will be interesting to see whether this property of UBR1 requires the activity of UBC4/UBC5 and whether the regions of UBC4/UBC5 that are required for interaction with heat domain proteins are also involved in the interaction with UBR1.

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