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Characterization of functionally independent domains in the human ubiquitin conjugating enzyme UbcH2

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Abstract **UbcH2 encodes a human ubiquitin conjugating enzyme (E2) able to conjugate ubiquitin to histone H2A in an E3 independent manner in vitro, which indicates that UbcH2 directly interacts with its substrates. To identify parts of the enzyme that are capable of binding H2A, we expressed several deletion mutants of UbcH2 in *E. coli* and tested the ability of the affinity purified mutant proteins to ubiquitinate H2A in the presence of bacterial expressed E1 and ubiquitin. With this in vitro assay we identified a C-terminal part of UbcH2 to be important for the interaction with H2A. Transfer of this C-terminal domain to another human E2, which is unable to catalyze ubiquitination of histones, leads to a fully active hybrid human ubiquitin conjugating enzyme capable of H2A ubiquitination. These results demonstrate that UbcH2 consists of two functionally independent domains. A N-terminal core domain with ubiquitin conjugating activity, and a C-terminal domain which interacts with substrate proteins.**

Key words: Ubiquitin-conjugation; Histone ubiquitination

1. Introduction

Genetic studies in yeast revealed that ubiquitin conjugation is responsible for many different cellular functions, such as cell cycle progression and regulation [1,2], DNA repair, sporulation [3], heat shock and cadmium resistance [4,5] and peroxisome biogenesis [6].

Attachment of the highly conserved protein ubiquitin to other eukaryotic proteins targets them for selective degradation. Substrate specific ubiquitin conjugating enzymes (E2 enzymes) and accessory factors recognize specific signals on substrates and attach ubiquitin to specific lysine residues, which triggers their degradation by the proteolytic pathway (for reviews see [7–9]). Substrate specificity of the ubiquitin conjugating system is mediated by different E2 enzymes and accessory factors known as ubiquitin ligases (E3 enzymes).

A variety of in vivo substrates of the ubiquitin conjugating system have been identified, including histones [10], actin [11], cyclins [12], the tumor suppressor p53 [13,14], MATα2 transcription repressor [15], cell surface receptors [16–18], c-jun [19] and NFκB [20].

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Some ubiquitin conjugating enzymes are able to transfer ubiquitin to the model substrate histone in vitro without any accessory factors, such as E3s. Among these E2s are yeast RAD6/UBC2 [3] and CDC34/UBC3 [1]. We have recently isolated a human ubiquitin conjugating enzyme, UbcH2 and its putative yeast homologue UBC8 and demonstrated their E3 independent ability to ubiquitinate histone H2A in vitro [21]. However, the cellular functions and in vivo substrates of UBC8 and UbcH2 remain unclear, since ubc8 deletion mutants show no obvious phenotype [22]. In this communication we report the functional analysis of mutant UbcH2 proteins and human hybrid ubiquitin conjugating enzymes. Our data suggest that UbcH2 consists of two functionally independent domains, an N-terminal part with basic ubiquitin conjugation functions, and a C-terminal domain which interacts with substrates.

2. Experimental

2.1. Construction of expression plasmids

All constructs were cloned into the unique *Stu*I site of pMAL™-p (New England Biolabs). For expression as 6 × His tagged proteins the inserts were cut out from pMAL™-p with *Bam*HI and *Hin*dIII and ligated into the expression vector pQ 31 (Quiagene, CA). The following oligonucleotides were used in this study to construct the deletion mutants and hybrid clones. Parts of the oligonucleotides which were used to add restriction sites or termination codons are written in italics: O-1: ATGTCATCTCCCAGTCCG; O-2: *TAATTAAGCTTCA*AGAGCTCTCCGATGAGC (reverse complementary); O-3: *TAATTAAGCTTCA*CGTGGCGTATTTCTGG (reverse complementary); O-4: ATGAAGCTCATCGAGAGTAAACATG; O-5: *TAATTAAGCTT*CTACAACTCCATATCC (reverse complementary).

UbcH2 and UbcH2 deletion constructs: Oligonucleotides O-1 and O-2 were used in a standard PCR reaction to amplify *UbcH2* lacking 39 nucleotides at the 3′ end (*UbcH2Δ170*). The purified PCR fragment was rendered blunt ended at its 5′ end, digested with *Hin*dIII at its 3′ end and cloned into *Stu*I and *Hin*dIII digested pMAL™-p expression vector. The resulting clone was cut with *Bam*HI and *Hin*dIII and the insert was ligated to the expression plasmid pQ 31 cut with the same enzymes. Clones in pMAL™-p are expressed as fusion proteins with malE, whereas pQ 31 clones get an N-terminal 6 × His tag. Exactly the same strategy was followed to construct *UbcH2Δ151* (O-1 and O-3) and *Δ15-UbcH2* (O-4 and O-5). To get *UbcH2Δ128* we used the unique *Pst*I site in the coding sequence of *UbcH2* and cloned the fragment in *Stu*I/*Pst*I cut pMAL™-p. Subsequently a *Bam*HI/*Pst*I fragment was ligated in pQ 31.

Expression and construction of *SM1*, *SM1-C2* and *N2-SM1-C2* was similar to that of the other constructs. To express recombinant human ubiquitin activating enzyme in *E. coli*, the coding sequence of the human E1 [23] was amplified by the PCR reaction and cloned into the expression vector pQ 31.

2.2. Protein expression and purification

Protein expression and purification was performed according to manufacturer. Recombinant E1 and all E2 constructs were expressed as N-terminal 6 × His tagged proteins, and used in the assay after affinity purification on Ni-NTA resin (Quiagene, CA). Contaminating imidazol was removed by anion exchange chromatography on DEAE-cellulose: Proteins were bound to the column in buffer P1 (50 mM

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Tris-HCl pH 7.2, 0.2 mM DTT). After washing with 10 column-volumes buffer P1, proteins were eluted in 50 mM Tris-HCl pH 7.2, 0.2 mM DTT, 0.5 M KCl. E2s were also expressed as malE fusion proteins. Expression, purification and factor Xa cleavage was performed as described [21].

2.3. Thioester formation and histone ubiquitination assays

Ubiquitin conjugation was performed in a total volume of 25 μl for 30 min at 37°C. Reaction conditions were exactly as described [21], except that approximately 10 ng of recombinant human E1 was used to activate ubiquitin. Proteins were separated on 12.5% or 15% SDS-PAGE prior to their transfer to a nitrocellulose membrane. Detection of biotinylated ubiquitin by strepavidin-alkaline phosphatase conjugates was according to the procedure described in [21]. To detect thioesters between E2s and ubiquitin samples were not reduced prior to electrophoreses.

To assay histone ubiquitination 1 μg bovine histone H2A (Boehringer Mannheim) was added to the thioester reaction mixture followed by a 30 min incubation at 37°C. Samples were boiled in the presence of 100 mM DTT for 5 min prior to electrophoreses to break thiolester linkage between E2 and ubiquitin.

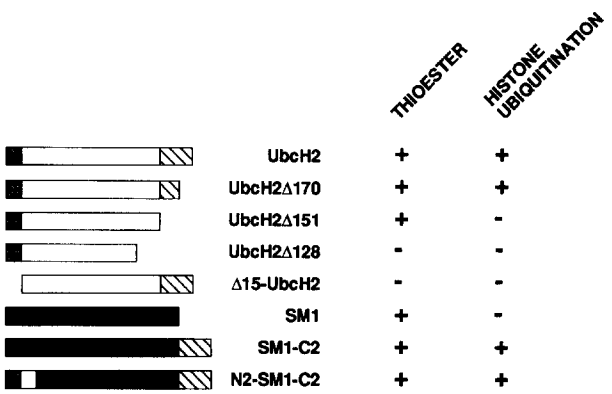

Fig. 1. Expression constructs. For cloning strategies see section 2. The constructs were analysed for thioester formation activity and histone ubiquitination. Detectable (+) or not detectable (−) activity of the particular constructs is indicated.

3. Results and discussion

We have recently isolated the human ubiquitin conjugating enzyme UbcH2 and demonstrated its ability to ubiquitinate histone H2A in vitro [21]. This reaction is independent from any accessory proteins such as ubiquitin ligases (E3s) and therefore requires direct interaction between the UbcH2 protein and the substrate molecules (H2A). Because UbcH2 shows high homology to all other ubiquitin conjugating enzymes in the core domain surrounding the active cystein, we predicted that the C-terminal and the N-terminal regions of the protein would be responsible for interaction with substrates.

To analyze the function of the N- and C-termini in substrate binding, we constructed several mutated UbcH2 proteins lacking either 15 N-terminal amino acids or different parts of the C-terminus (Fig. 1). Proteins were expressed in *E. coli* either as a fusion with the maltose binding protein or with an N-terminal 6 × His tag. Because the malE fusions turned out to be inactive, malE was removed by factor Xa cleavage [21]. In general, the 6 × His tag reduces the thioester formation activity compared to factor Xa cleaved malE-UbcH2 fusion protein, therefore higher amounts of the 6 × His-tagged proteins were used in the assays.

For ubiquitin activation we used bacterial expressed 6 × His tagged human ubiquitin activating enzyme (E1) purified on Ni-NTA resin. We found the recombinant E1 to be a very convenient source of human E1, because it is easy to purify in considerable amounts without contamination by E2 activities, which are often found in E1 preparations purified from human placenta.

Prior to examining H2A ubiquitination we checked the ability of the mutant proteins to accept activated ubiquitin from E1 by forming a thioester conjugate with ubiquitin.

Successive deletion of sequences from the C-terminal part of UbcH2 leads to proteins which are fully active in terms of thioester formation providing the deletion does not exceed amino acids 151 (Fig. 2a). Sequences between amino acid 128 and 151 are absolutely required for thioester formation (compare Fig. 2a, lanes 3 and 4). These 23 amino acids may be involved in the interaction with E1 and/or ubiquitin. Alternatively the conformation of UbcH2Δ128 could be changed so that the active cystein is hidden. However, deletion of the last 32 amino acids of UbcH2 (UbcH2Δ151) has no effect on thioester formation (Fig. 2a).

On the other hand, deletion of the first 15 N-terminal amino acids (Δ15-UbcH2) abolishes thioester formation (data not shown). This is surprising because a very similar truncation of the yeast homolog UBC8 does not affect formation of thioester bonds between UBC8 and ubiquitin [22].

We were interested in whether the C-terminal deletions of UbcH2 have any influence on histone ubiquitination. In our assay biotinylated ubiquitin is activated by recombinant human E1 and is transferred to bovine histone H2A by E2 enzymes. After Western blotting biotinylated ubiquitin is detected by avidin-alkaline phosphatase conjugates. Because samples were boiled in the presence of DTT prior to their separation on SDS-PAGE, which breaks thioester bonds between E1-ubiquitin and E2-ubiquitin, the stable H2A-ubiquitin conjugates can easily be detected.

We found that deletion of 13 C-terminal amino acids of UbcH2 (UbcH2Δ170) does not affect its ability to ubiquitinate H2A in vitro, while deletion of additional 19 amino acids (UbcH2Δ151) abolishes histone ubiquitination activity completely. Of course, UbcH2Δ128 which failed to form thioester adducts with ubiquitin, cannot catalyze ubiquitination of H2A (Fig. 2b). Taken together, these results suggest that the C-terminal region between amino acids 151 and 170 of UbcH2 is necessary for substrate binding. Interestingly, in vitro studies with yeast UBC2/RAD6 revealed that truncation of its C-terminus affects its ability to polyubiquitinate histones but does not affect thioester formation [24]. Moreover, it was shown that the C-terminus of UBC2/RAD6 is required for sporulation but not for the DNA repair functions of UBC2/RAD6 [25]. The human RAD6 homologue UbcH1 [26] which lacks comparable C-terminal extensions complements the repair defect of rad6 mutants but not the sporulation deficiency [27,28].

The question arises, whether the C-terminal part of UbcH2 is sufficient for interaction with H2A, or if there are other sequences within UbcH2 which are required for substrate binding. To address this question we constructed hybrid human ubiquitin conjugating enzymes (Fig. 1) and tested their ability to ubiquitinate histone H2A. The hybrid E2s consist of catalytic inactive N- and C-terminal parts of UbcH2 and an active enzy-

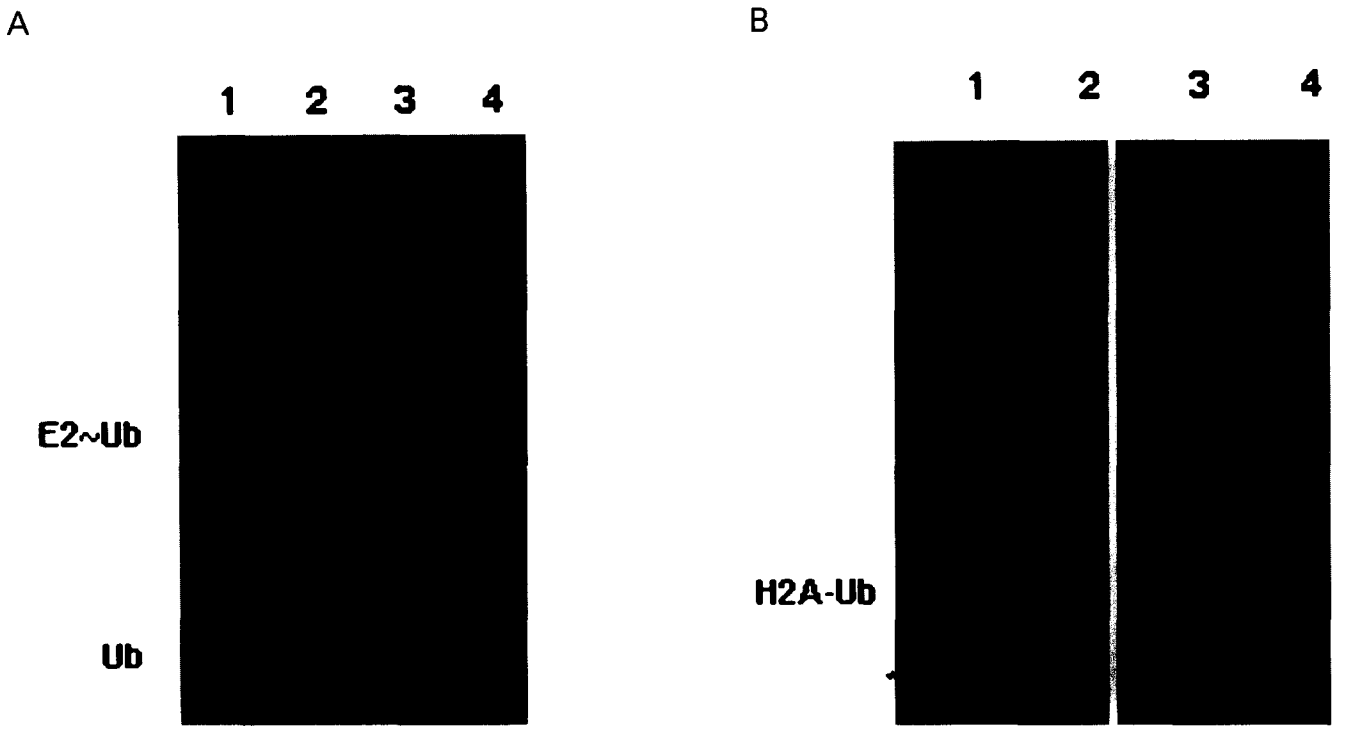

Fig. 2. Thioester formation and histone ubiquitination activities of UbcH2 deletion mutants. (1) UbcH2, (2) UbcH2Δ170, (3) UbcH2Δ151, (4) UbcH2Δ128. (a) Thioester formation of UbcH2 and deletion mutants: samples were not reduced prior to electrophoreses. Bands corresponding to the UbcH2-, UbcH2Δ170- and UbcH2Δ151-ubiquitin conjugates (E2~Ub) could not be detected when thioesters were broken by boiling the samples for 5 min in the presence of 100 mM DTT (data not shown). Because of the low amount of E1 used the thioester between E1 and ubiquitin was not detectable. E2~Ub indicates thioester conjugates and Ub indicates free biotinylated ubiquitin. (b) Histone ubiquitination: reactions contained 1 μg bovine histone H2A (Boehringer Mannheim). Samples were boiled in the presence of 100 mM DTT for 5 min to break thiolester linkage between E2 and ubiquitin. H2A-Ub marks ubiquitinated histone H2A. The star indicates a contaminating protein in the ubiquitin preparations used for biotinylation.

matic part, which is able to form thioester linkage with ubiquitin, but lacks histone ubiquitinating function. This enzymatic function was fulfilled by SM1, a human ubiquitin conjugating enzyme recently isolated in our lab [29]. The entire coding sequence of SM1 was fused to the C-terminal region of UbcH2 (amino acids 152 to 183) which resulted in the hybrid molecule SM1-C2 (C2 = C-terminus UbcH2) (Fig. 1). Analysis of the role of N-terminal sequences in UbcH2 in H2A ubiquitination was hampered by the loss of ubiquitin conjugation activity upon deletion of the 15 N-terminal residues. Therefore we constructed the hybrid molecule N2-SM1-C2 (N2 = N-terminus of UbcH2) to test the influence of UbcH2's N-terminus on H2A ubiquitination. In N2-SM1-C2 the 29 amino terminal residues of SM1-C2 were replaced by the first 30 amino acids of UbcH2 (Fig. 1).

Since SM1 and the two hybrid ubiquitin constructs (SM1-C2 and N2-SM1-C2) are able to form thioester bonds with ubiquitin in our in vitro assay (data not shown), we can compare their histone ubiquitination activities.

As shown in Fig. 3, SM1 itself is unable to catalyze H2A ubiquitination, which is consistent with previous results [30]. However, fusion of 32 C-terminal amino acids of UbcH2 to the entire coding sequence of SM1 (SM1-C2) alters the substrate recognition properties of SM1, such that it gains the ability to ubiquitinate H2A. On the other hand replacement of the N-terminal part of SM1 with the 30 first residues of UbcH2 has no influence on histone H2A ubiquitination. Although, comparison of lanes 2 and 3 in Fig. 3 might suggest slightly different activities of SM1-C2 and N2-SM1-C2, we attribute that to difficulties with quantitative reproducible histone transfer to nitrocellulose.

The results from the experiments with the hybrid constructs are consistent with the results with UbcH2 deletion mutants described above. Our data suggest that UbcH2 consists of two separated domains with independent functions. Domain I extends to a region around amino acid 150 and functions as a ubiquitin conjugating enzyme. Domain II consists of sequences starting around amino acid 150 mediates interaction with substrates. This substrate recognition function of domain II is similar to that performed by E3 enzymes in E3 dependent ubiquitination reactions. UbcH2 therefore seems to contain a combination of E2 and E3 enzymes in a single polypeptide chain with a N-terminal E2-domain and a C-terminal 'E3-like' part.

The two domain structure UbcH2 can be compared with the yeast ubiquitin conjugating enzyme UBC3/CDC34. In an elegant experiment the C-terminal (substrate binding) domain was fused to the catalytic domain of yeast UBC2/RAD6. Subsequently it was demonstrated that this hybrid molecule with ubiquitin conjugating activity resulting from the RAD6 portion and substrate recognition conferred by the CDC34 part is able to rescue cdc34 mutants [31,32].

Fig. 3. Histone ubiquitination abilities of UbcH2-SM1 hybrids. (1) SM1, (2) SM1-C2, (3) N2-SM1-C2. Ub: free biotinylated ubiquitin. H2A-Ub: ubiquitinated histone H2A. The star marks a contaminating protein in ubiquitin preparations used for biotinylation.

No phenotype has yet been described for the deletion of the yeast UbcH2 homologue UBC8. This makes us unable to test the UbcH2-SM1 hybrid ubiquitin conjugating enzymes in complementation experiments to demonstrate the biological relevance of the in vitro experiments described above. But our results can help to design experiments which use the C-terminal substrate binding part to screen for interacting proteins (substrates) or use it in dominant negative strategies in the search for the biological role of UbcH2.

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