Role of UEV-1A, a homologue of the tumor suppressor protein TSG101, in protection from DNA damage

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Abstract The open reading frame YGL087c in the budding yeast *Saccharomyces cerevisiae* genome encodes a polypeptide highly similar to the human UEV (ubiquitin-conjugating *E2* enzyme variant) proteins, which have been proposed to belong to a family of putative dominant negative ubiquitin regulators. Deletion of the YGL087c open reading frame yields viable cells which are sensitive to UV irradiation or methyl methanesulfonate, but not to hydroxyurea. This phenotype is reminiscent of that of *rad* mutants and suggests that the YGL087c-encoded protein functions in a process related to tolerance to DNA damage. We also show that the mutant phenotype is fully complemented by expression of the human UEV-1A cDNA and we propose that UEV-1 proteins could also have a role in protecting higher eukaryotic cells from DNA damaging agents.

Key words: Ubiquitination; DNA repair; *Saccharomyces cerevisiae*

1. Introduction

The newly described proteins known as UEV (ubiquitin-conjugating *E2* enzyme variant [1]) are defined as proteins similar in sequence and structure to the *E2* ubiquitin-conjugating enzymes, but devoid of their enzymatic activity, since they lack a critical Cys residue essential for the conjugation and transfer of ubiquitin to substrates [1]. On the basis of these structural features, it has been proposed that UEV and related proteins, which include the product of the tumor suppressor gene *TSG101* [2,3], could be novel regulators of protein ubiquitination, possibly in the form of dominant negative variants of *E2* enzymes [1,4,5]. Although the precise molecular mechanisms have not been elucidated, UEV proteins exert specific functions in mammalian cells, including cell cycle regulation [1] and transcriptional control [6]. Further interest in this novel class of proteins has been stimulated by the implication of TSG101 in human neoplasia [3,7,8].

UEV proteins are highly conserved in phylogeny, being present in all eukaryotic organisms. The YGL087c open reading frame of the budding yeast *Saccharomyces cerevisiae* is located on chromosome VII [9], encodes a protein with 38% identity to human UEV-1A [1], and maintains the same structural features of this class of proteins. This significant degree of conservation allows to predict that some of the functions performed by UEV proteins could be maintained between distant organisms. Here, we describe the first steps in the analysis of the function of the UEV homologue in *S. cerevisiae*, by means of disruption of the YGL087c gene and complementation with cDNA corresponding to human UEV-1A. We postulate that the product of the yeast gene YGL087c is a homologue of human UEV-1A [1], and functions to protect yeast cells from DNA damage. We further propose that this function is maintained in higher organisms.

2. Materials and methods

2.1. Growth of Escherichia coli and yeast strains

E. coli cells (strain NM522) were grown in LB medium (containing 50 μg/ml ampicillin, when needed for plasmid selection) at 37°C. Yeast cells were grown at 28°C on YPD medium or, when indicated, on SD medium [10]. Yeast strains used in this work derive from wild-type strain BMA64-1A (*mat a ura3-1 trp1-Δ2 leu2-3,112 his3-11 ade1-1 can1-100*), an haploid derivative of strain W303.

2.2. Recombinant DNA techniques, generation of mutant strains and plasmids construction

E. coli cells were transformed using standard calcium chloride techniques. Yeast cells were transformed by a modification of the lithium acetate method [11]. Restriction reactions, DNA ligations and other standard recombinant techniques were carried out essentially as described [12].

Deletion of the entire open reading frame of YGL087c was accomplished by the short flanking homology replacement technique [13]. The heterologous marker kanMX4, that contains the kan^r gene of transposon *Tn*903, was amplified by PCR from plasmid pFA6-KanMX4 using oligonucleotides S1-087c (5′-GGAATGCTGCAAATACTGTTTAGGAAAAAGTAGATAA*CTA*CGTACGCTGCAGGTCGA-3′) and S2-087c (5′- TATATGCAACGTAGAAGAAAGCAGCGTTTACACAAAA*ATG*ATCGATGAATTCGAGCTCG-3′). These oligonucleotides were designed to precisely replace the entire ORF of YGL087c. Underlined sequences correspond to genomic YGL087c sequence. The stop codon and the Met initiating triplet are indicated in italics. Three μg of DNA were used to transform BMA64-1A cells and positives were selected from YPD plates containing 200 μg/ml of geneticin (G418). The correct integration of the marker in the genome was assessed by PCR using oligonucleotides A1-087c (5′-GCGAATTCGTAACACGTACAGTATC-3′), and A4-087c (5′-GCGGATCCAAGAAAGGAGAGGACC-3′), corresponding to regions upstream and downstream, respectively, in the chromosomal sequence. Underlined sequences correspond to *Eco*RI and *Bam*HI sites added to facilitate cloning. These oligonucleotides were combined with oligonucleotides 5′-CACGTCAAGACTGTCAAGGA-3′, and 5′-GTTAAGTGCGCAGAAAGTAA-3′, that hybridize within the marker.

A 1.2 kbp fragment of genomic DNA containing the YGL087c ORF and flanking regions was amplified by PCR from wild-type cells using oligonucleotides A1-087c and A4-087c, digested with *Eco*RI and

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Abbreviations: HU, hydroxyurea; MMS, methyl methanesulfonate; ORF, open reading frame; PCR, polymerase chain reaction; UV, ultraviolet light

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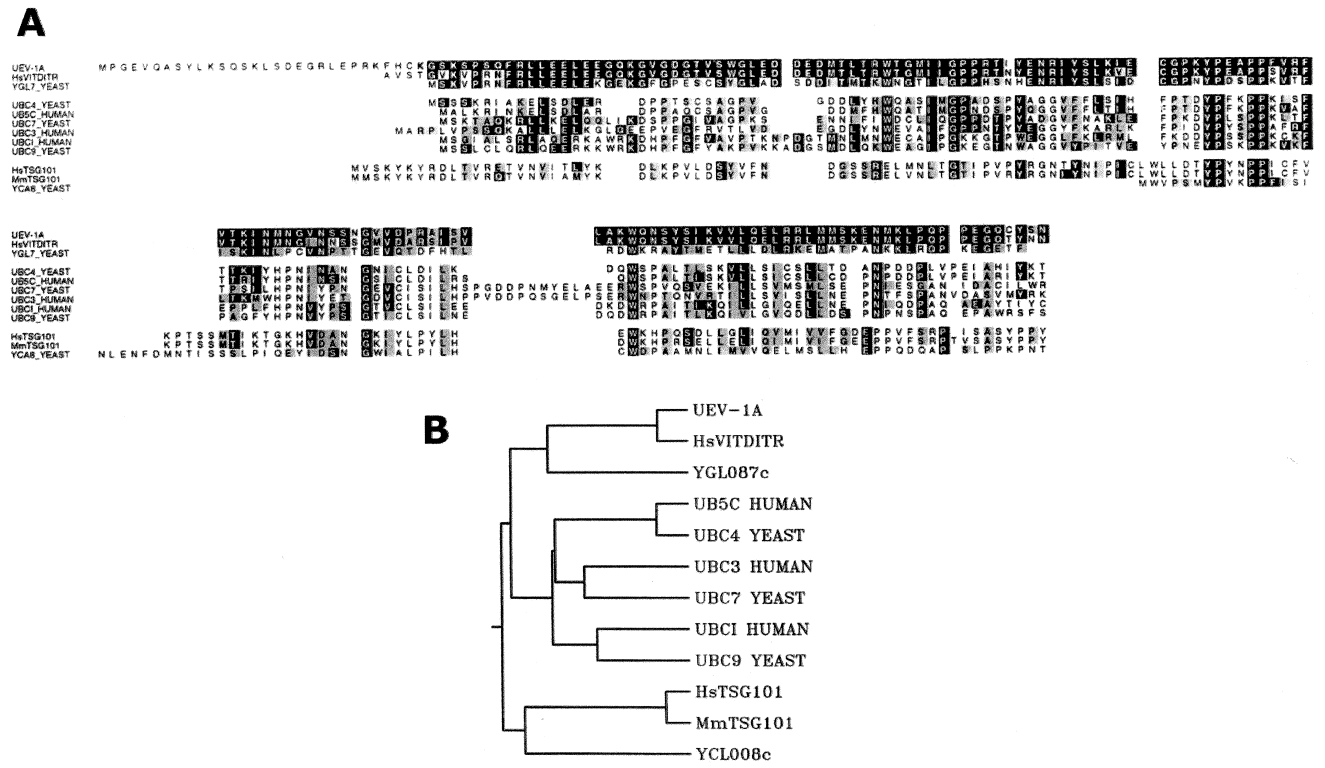

Fig. 1. A: Alignment of human UEV-1A/CROC-1A and its related human and yeast proteins with ubiquitin-conjugating enzymes (UBC) and TSG101-related proteins. The carboxyl-terminal domains of the UBC and TSG101 proteins are not represented in the alignment. B: Dendogram of phylogenetic relationships corresponding to the sequences aligned in A. The mean distances between the three subfamilies of proteins are: UEV/UBC, 0.77 (±0.03); TSG101/UBC, 0.79 (±0.04); UEV/TSG101, 0.85 (±0.04). Alignments and dendograms were generated with ClustalW and PHILIP packages.

*Bam*HI and cloned into the low-copy, centromeric plasmid pRS416 [14], previously linearized with the same enzymes. The human UEV-1A ORF was expressed in yeast cells by cloning it, downstream of the *GAL1* promoter, into plasmid pYES 2.0 (Invitrogen). To this end, the entire coding sequence of UEV-1A was amplified by reverse transcriptase-PCR using total RNA from the HT-29 cell line and oligonucleotides 5′-*ATG*CCAGGAGAGGTTCAAGCGTCTT-3′ and 5′-*TTA*ATTGCTGTAACACTGTCCTTC-3′. Sequences in italics correspond to the initiating Met and stop codons, respectively. Amplification was carried out using Vent DNA polymerase (New England Biolabs). An additional 15 min treatment with *Taq* DNA polymerase was performed to create overhanging ends, and the amplified DNA was cloned into the pGEM-T vector (Promega) and sequenced (both strands). The construct was digested with *Sac*I and *Sph*I and cloned in these same sites of pYES 2.0 to yield plasmid pYES/UEV1-A.

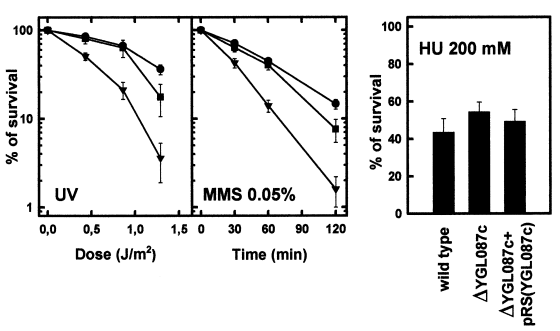

Fig. 2. Deletion of the yeast gene YGL087c, a putative dominant negative ubiquitin regulator, results in hypersensitivity to DNA damaging agents. The complete YGL087c ORF was precisely deleted and replaced by the kanMX4 module as described in the haploid *S. cerevisiae* strain BMA64-1A. Wild-type cells (●), deletion mutant (▼) (both carrying the centromeric plasmid pRS416), or mutants carrying a wild-type copy of the YGL087c gene in pRS416 (■), were grown in SD medium lacking uracil (plus 2% glucose) until an optical density of 0.8–1.2. UV irradiation, and MMS and HU treatments were as described in Section 2. Results are expressed as percentages referred to untreated cells and are means ± S.E.M.

2.3. Survival determination after UV irradiation, MMS and HU treatments

For all treatments, exponential cells were grown on SD medium lacking uracil to a density of 1–1.5. For UV irradiation, cells were diluted in the same medium and a volume of 200 μl was plated. Plates were irradiated at the indicated doses in a Stratalinker UV crosslinker (Stratagene) and incubated at 30°C for 48 to 72 h in the dark. Colonies were counted and the percentage of survival determined by comparison with the unirradiated control plates.

For MMS treatment, cultures were incubated for the indicated times in the presence or absence of 0.05% MMS at 30°C. Following the incubation, cells were plated on SD lacking uracil plates, incubated at 30°C, and colonies counted after 48–72 h. The percentage of survival was determined by comparison with untreated control cells. HU treatment was similar to MMS treatment but cells were incubated in the presence or absence of 0.2 M HU for 3 h.

3. Results and discussion

A new class of proteins that could function as regulators of protein ubiquitination has been recently identified. Based on sequence alignments and molecular modeling analysis, it has been proposed that UEV-1/CROC-1 [1,6] and the protein encoded by the human tumor suppressor gene *TSG101* [2] are inactive variants of the *E2* ubiquitin-conjugating enzymes

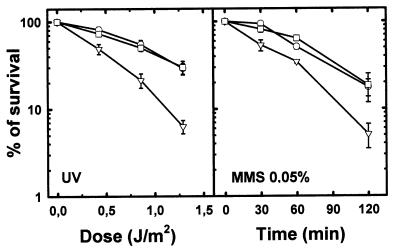

Fig. 3. Expression of human UEV-1A cDNA complements the defect of a YGL087c-deficient strain. Wild-type BMA64-1A yeast cells were transformed with plasmid pYES2 (○). YGL087c deletion mutants were transformed with either pYES 2.0 (▽) or pYES/UEV-1A (□). Cells were grown on SD medium (lacking uracil) plus 2% of glucose until an optical density of 1, harvested, washed once with SD medium (lacking uracil) plus 2% galactose and grown on this medium for 5 h to initiate expression of the UEV-1A cDNA from the *GAL1* promoter. Cells were then treated and survival assessed as indicated above, except that cells were plated in SD plates (lacking uracil) containing 2% galactose and growth was scored after 3 days.

[1,4,5]. Although their predicted structures are very similar, a more detailed analysis shows that UEV-1A and the product of *TSG101* belong to distinct subfamilies of proteins, which are phylogenetically equidistant from the catalytically active *E2* proteins (Fig. 1).

The study of the biological role of ORF YGL087c was carried out by generating deletion mutants. Replacement of the entire coding region by the kanamycin marker yielded viable cells, indicating that YGL087c is not an essential gene. Mutant cells could not be distinguished from wild-type cells when a number of growth conditions were tested (different carbon sources, temperatures, and exposure to salt, caffeine or calcofluor). However, mutant cells showed a clear increase in their sensitivity to UV irradiation. The sensitivity to UV of mutant cells was reduced when a copy of the gene was placed in the centromeric plasmid pRS416 (Fig. 2). Mutant cells were also highly sensitive to the DNA damaging agent MMS, but they did not display a significant difference in sensitivity, as compared to wild-type cells, when they were exposed to HU for 3 h (Fig. 2). This difference in behavior is of biological significance, since UV and MMS negatively affect cell growth through alterations in the structure of DNA (although by different mechanisms), while HU acts as an inhibitor of ribonucleotide reductase, thus preventing DNA synthesis and progression through the S phase of the cell cycle (see [15] and references therein).

The fact that human and yeast UEV proteins have common structural features allows to predict that some of their functions will be conserved. Thus, we sought to test the possibility that human UEV-1A could function in a *S. cerevisiae* background. Yeast mutant cells lacking YGL087c were transformed with the plasmid pYES/UEV-1A, which permits expression of the human cDNA after shift of the carbon source from glucose to galactose. It can be seen that the sensitivity to both UV and MMS of mutant cells expressing human UEV-1A reverted to wild-type levels (Fig. 3).

In summary, the phenotype observed in *S. cerevisiae* cells lacking YGL087c is reminiscent of that of *rad* mutants [16], and our data indicate that UEV proteins function in processes related to tolerance to DNA damage. The observed role in DNA repair/tolerance of YGL087c, a potential regulator of ubiquitination, suggests that it could interact, directly or indirectly, with active ubiquitinating enzymes that promote DNA repair. For instance, the *E2* ubiquitin-conjugating enzymes Rad6 [17] and Ubc9 [18,19] and a mammalian *E1* ubiquitin-activating enzyme [20], play critical roles in DNA damage repair/tolerance. It is conceivable that YGL087c exerts its protective effects against DNA damage through physical or functional interactions with one or more of such proteins. Alternatively, this protein could be involved in a separate pathway regulating DNA damage repair/tolerance in *S. cerevisiae*.

The complementation by human UEV-1A of the *S. cerevisiae* YGL087c mutant phenotype indicates that UEV proteins from distant organisms have similar functions. Therefore, we propose that human UEV-1A could also have a role in protecting higher eukaryotic cells from DNA damaging agents. Expression of UEV-1A in human cells is cell cycle regulated, and its unregulated expression from a constitutive promoter leads to the inhibition of the mitotic kinase Cdk1 and arrest in the G_2-M phase of the cell division cycle [1]. Such observations provide an indirect support for the involvement of UEV-1A in the regulation of a G_2-M checkpoint, similar to that activated in response to DNA damage [16].

While so far the gene for UEV-1 has not been implicated in human disease, structural alterations or abnormal expression of *TSG101* have been reported in breast cancer [3,7,8]. Our findings provide the first evidence linking this new class of proteins to protection from DNA damage, and might establish the basis for unraveling novel mechanisms of human tumorigenesis. A possible scenario would be one in which loss of function of UEV-1 could lead to an increased rate of mutagenesis, therefore contributing to the establishment, maintenance and/or progression of the neoplastic phenotype.

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