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Authors
Miranda, Isabel
Silva-Dias, Ana
Rocha, Rita
et al.

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Candida albicans CUG Mistranslation Is a Mechanism To Create Cell Surface Variation

Isabel Miranda, Ana Silva-Dias, Rita Rocha, Rita Teixeira-Santos, Carolina Coelho, Teresa Gonçalves, Manuel A. S. Santos, Cidália Pina-Vaz, Norma V. Solis, Scott G. Filler, Acácio G. Rodrigues

Department of Microbiology, Faculty of Medicine, University of Porto, Porto, Portugal; Cardiovascular Research and Development Unit, Faculty of Medicine, University of Porto, Porto, Portugal; Center for Neurosciences and Cell Biology, University of Coimbra and Faculty of Medicine, Coimbra, Portugal; RNA Biology Laboratory, Department of Biology and Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal; Department of Microbiology, Hospital São João, Porto, Portugal; Department of Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California, USA; David Geffen School of Medicine at UCLA, Los Angeles, California, USA; Burn Unit and Department of Plastic and Reconstructive Surgery, Hospital São João, Porto, Portugal

ABSTRACT In the human fungal pathogen Candida albicans, the CUG codon is translated 97% of the time as serine and 3% of the time as leucine, which potentially originates an array of proteins resulting from the translation of a single gene. Genes encoding cell surface proteins are enriched in CUG codons; thus, CUG mistranslation may influence the interactions of the organism with the host. To investigate this, we compared a C. albicans strain that misincorporates 28% of leucine at CUGs with a wild-type parental strain. The first strain displayed increased adherence to inert and host molecules. In addition, it was less susceptible to phagocytosis by murine macrophages, probably due to reduced exposure of cell surface β-glucans. To prove that these phenotypes occurred due to serine/leucine exchange, the C. albicans adhesin and invasin ALS3 was expressed in Saccharomyces cerevisiae in its two natural isoforms (Als3p-Leu and Als3p-Ser). The cells with heterologous expression of Als3p-Leu showed increased adherence to host substrates and flocculation. We propose that CUG mistranslation has been maintained during the evolution of C. albicans due to its potential to generate cell surface variability, which significantly alters fungus-host interactions.

IMPORTANCE The translation of genetic information into proteins is a highly accurate cellular process. In the human fungal pathogen Candida albicans, a unique mistranslation event involving the CUG codon occurs. The CUG codon is mainly translated as serine but can also be translated as leucine. Leucine and serine are two biochemically distinct amino acids, hydrophobic and hydrophilic, respectively. The increased rate of leucine incorporation at CUG decoding triggers C. albicans virulence attributes, such as morphogenesis, phenotypic switching, and adhesion. Here, we show that CUG mistranslation masks the fungal cell wall molecule β-glucan that is normally recognized by the host immune system, delaying its response. Furthermore, we demonstrate that two different proteins of the adhesin Als3 generated by CUG mistranslation confer increased hydrophobicity and adhesion ability on yeast cells. Thus, CUG mistranslation functions as a mechanism to create protein diversity with differential activities, constituting an advantage for a mainly asexual microorganism. This could explain its preservation during evolution.

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Address correspondence to Isabel Miranda, imiranda@med.up.pt.

Many pathogenic microorganisms switch surface antigens through constant variation of cell surface molecules. This process enables microbes to occupy diverse niches within the host and reduces microbe recognition by the immune system. Various mechanisms of antigenic switching have been described in both commensal and pathogenic organisms, including Plasmodium falciparum, Trypanosoma brucei, Pneumocystis carinii, and Candida glabrata (1–9).

Antigenic switching can also influence microbial adhesion, an important virulence attribute that prevents a microorganism from being removed from a favorable location, thus providing the ability to infect specific niches within the host (10). The C. albicans ALS (agglutinin-like sequence) gene family encodes adhesins that bind to various substrates. Some of these Als proteins also mediate biofilm formation, host cell invasion, and iron acquisition (11–13). Some ALS genes are differentially expressed by yeast versus hyphae. However, unlike the Saccharomyces cerevisiae FLO and C. glabrata EPA genes, which also specify adhesins, C. albicans ALS genes are not located near the telomeres and are not subject to subtelomeric silencing. Interestingly, the coding sequences of ALS genes contain a high number of CUG codons (14, 15). This codon is translated as leucine in most organisms. However, in C. albicans and other Candida species, the CUG codon is dually translated as serine (95 to 97% of the time) and leucine (3 to 5% of the time) (15). This atypical CUG translation is mediated by a Ser-tRNA<sub>CAG</sub> that, in contrast to other tRNAs, is recognized by two aminoacyl-tRNA synthetases, seryl- and leucyl-tRNA synthetases (16). Because serine is hydrophilic, whereas leucine is hydrophobic, the
variable incorporation of these two amino acids into a protein has the potential to generate a family of proteins with altered structure and function (17). The first successful manipulation of genetic code ambiguity in *C. albicans* resulted in the increase of virulence factor expression, in particular phenotypic switching, morphogenesis, and adhesion (18). However, the adhesion phenotype was not quantitatively measured, and it was not clear whether adhesion resulted from the high rates of spontaneous transitions of white to opaque and yeast to hyphal forms.

We hypothesized that Ser/Leu incorporation variability in cell surface proteins of *C. albicans* may influence its adherence to various substrates as well as its interactions with host immune effector cells. To investigate this hypothesis, we used a highly CUG-mistranslating strain of *C. albicans* that has increased incorporation of leucine at CUG codons (18). We found that CUG mistranslation increased adherence to host substrates, by changing cell surface hydrophobicity. Furthermore, the diversity in the cell wall proteome created by CUG mistranslation altered the cell surface exposure of 1,3-β-glucan and reduced *C. albicans* phagocytosis by macrophages. These results suggest that protein diversity caused by variable translation of the CUG codon has important functional consequences for the interactions of *C. albicans* with the host.

**RESULTS**

**Forced CUG mistranslation increases adhesion and cell surface hydrophobicity.** To test whether variable serine/leucine incorporation at CUG sites potentiates adhesion and hydrophobicity, we compared yeast forms of a wild-type *C. albicans* strain (pUA12) with those of a mutant strain that has a high level of CUG mistranslation (pUA15), displaying 28% leucine incorporation rather than the usual 3% leucine incorporation in the wild-type *C. albicans* (15, 18). We used a quantitative flow cytometry assay to characterize the adhesion of the two *C. albicans* strains to polystyrene and the representative host ligands fibronectin, vitronectin, and gelatin (collagen) (19). In this assay, fluorescent polystyrene beads uncoated or coated with various substrates were incubated with nonfluorescent *C. albicans* yeasts (Fig. 1B). The number of organisms with at least one bead attached (Fig. 1A and B; see also Fig. S1) was quantified by flow cytometry (Fig. 1C; see also Fig. S1 in the supplemental material). MFI is directly proportional to the number of beads attached to each organism (Fig. 1C; see also Fig. S1). The majority of wild-type organisms did not bind to any beads (Fig. 1A and B; see also Fig. S1). A small percentage of these cells were attached to a single bead, and even fewer cells were attached to two or more beads (Fig. S1). In contrast, a high percentage of cells of the highly CUG-mistranslating strain bound to at least one bead, and many of them bound to multiple beads (Fig. 1A to C; see also Fig. S1). Consistent with these results, the highly CUG-mistranslating cells were more adherent than wild-type cells to all tested abiotic and biotic substrates (Fig. 1A and C). While the greatest difference in adherence was found for polystyrene (more than a 4-fold increase), the increase of CUG mistranslation also
enhanced C. albicans adherence to biotic substrates: 1.5-fold to fibronectin, 2.6-fold to vitronectin, and 2.25-fold to gelatin (Fig. 1A). Collectively, our results clearly indicate that enhancing the leucine incorporation at CUG sites results in increased adherence to a variety of substrates. The increased adherence of the highly CUG-mistranslating strain could also be due to the altered surface expression or function of adhesins proteins. To test whether the level of CUG mistranslation influenced surface expression of adhesins, we analyzed Als1 and Als3, which mediate adherence to a variety of host substrates (20). The level of surface expression of Als1 and Als3 proteins was quantified by flow cytometry using a polyclonal antibody that recognizes both Als proteins (21). No difference in Als protein expression was found between the wild-type and highly CUG-mistranslating strains (see Fig. S2), suggesting that the increased adherence of the CUG-mistranslating strain is likely due to altered function of adhesins rather than increased surface expression of these adhesins.

Cell surface hydrophobicity is known to be important for the adherence of C. albicans (22). Because leucine is hydrophobic and serine is polar, it is likely that the increased leucine content in the cell surface proteins of the highly CUG-mistranslating strain enhances the hydrophobicity of this strain. Cell surface hydrophobicity was quantified using a classical approach based upon microbial adherence to hydrocarbons (23). Cells of the highly CUG-mistranslating strain were significantly more hydrophobic than those of the wild-type strain (Fig. 1D). These results suggest that serine/leucine exchange in CUG translation contributes significantly to the promotion of cell surface hydrophobicity.

The CUG-encoded residues of Als proteins are predicted to be surface exposed. If serine/leucine switching in CUG decoding alters C. albicans adherence, where are CUG codons located in cell wall-coding genes? To gain insight into this topic, we analyzed the ALS gene family, which is composed of 8 genes. Als proteins are composed of four domains: a C-terminal domain, containing a site for covalent attachment of the glycosylphosphatidylinositol (GPI) anchor and rich in serine/threonine amino acid sequences; tandem repeats of highly conserved 108-bp units; an amyloid-forming region; and an N-terminal domain which contains the substrate-binding region (24). The coding sequences of ALS genes contain multiple CUG codons, and their mistranslation increases the number of proteins synthesized from each mRNA transcript by a factor of $2^n$ (Fig. 2A), depending on the arbitrary serine/leucine incorporation at CUG positions. Based on the number of CUG codons and their localization, ALS genes can be divided into two groups: ALS1 to ALS4 and ALS5 to ALS9 (Fig. 2A and B). Members of the ALS1 to -4 group contain 2 to 5 CUG codons, located mainly at the 5′ end of the mRNA sequence that codes for the N-terminal region (Fig. 2B). Members of the ALS5 to -9 group possess 5 to 18 CUG codons, which are predominantly located in the 3′ end of the mRNA, corresponding to the C terminus of the protein. Since the crystal structure of the Als9 N-terminal domain was recently solved and the residue identity is 41 to 84% among Als sequences (25), we did a multiple protein sequence alignment in order to predict the location of CUG-encoded residues in this domain. The solvent accessibility of each residue was scored (Fig. 2B). The three-dimensional structure of the Als9 N-terminal domain (Protein Data Bank [PDB] code 2Y7N) showed that serine/leucine residues encoded by CUG codons are totally or partially exposed to the solvent (Fig. 2C), where both residues can be accommodated with no protein folding disruption. However, the aliphatic side chain of leucine lacks the chemical reactivity of the serine hydroxyl group, which can act as a hydrogen donor. Hence, the variable incorporation of leucine or serine in the N-terminal domain should create functional and structural diversity at the Als9 molecular surface, which is exposed in the cell wall. The Als9 crystal structure does not include residues 379 and 433 (encoded by CUG codons); however, the multiple sequence alignment demonstrated that these residues are mostly encoded by CUG codons among members of the ALS9 gene family, suggesting that they have an important role in Als9 function (Fig. 2B).

S. cerevisiae expressing Als3p-Leu has increased adherence. We used a heterologous expression approach to investigate the functional effects of incorporating serine versus leucine at residues 379 and 433 in Als3p, a key C. albicans adhesin. Two different isoforms of C. albicans ALS3 were expressed in the normally non-adherent S. cerevisiae. One was the wild-type allele, which, when expressed in S. cerevisiae, resulted in the production of Als3p containing leucine at residues 379 and 433 (Als3p-Leu). In the other allele, the 2 CUG codons were mutated to UCG, so that the resulting protein contained serine at these positions (Als3p-Ser) (Fig. 3A). When grown in liquid medium, S. cerevisiae expressing Als3p-Leu showed greater flocculation than did the strain expressing Als3p-Ser (Fig. 3B), indicating that the variable translation of the CUG codon significantly influences cell-cell adhesion. Substitution of leucine for serine in Als3p also altered its capacity to bind to different substrates. S. cerevisiae cells expressing Als3p-Leu had significantly greater adherence to polyethylene, fibronectin, vitronectin, and gelatin than did cells expressing Als3p-Ser, as determined both by the number of cells with beads attached and by the mean fluorescence intensity (Fig. 3C). Again, these phenotypes were not due to differences in the amount of Als3p expressed on the fungal cell surface (see Fig. S3 in the supplemental material). Overall, our findings that the Als3p-Ser and Als3p-Leu isoforms exhibit markedly different adherence properties indicate that CUG mistranslation has significant functional consequences.

CUG mistranslation reduces susceptibility to macrophage phagocytosis. Next, we investigated the influence of CUG mistranslation on the phagocytosis of C. albicans by macrophages. RAW 264.7 macrophages were challenged with the wild-type and highly CUG-mistranslating C. albicans strains (pUA12 and pUA15, respectively). The CUG-mistranslating cells were more resistant than the wild-type strain to macrophage phagocytosis, as indicated by a reduced number of phagocytosed yeasts and fewer phagocytosed yeasts per macrophage (Fig. 4A to C). This resistance to phagocytosis was most evident after 1.5 h of incubation, and it decreased by 3 h. The reduced phagocytosis of the highly CUG-mistranslating cells could be due to the reduced surface expression of cell wall components that are recognized by macrophage pattern recognition receptors. One of these receptors is dectin-1, which recognizes β-glucans exposed on the fungal surface and triggers the phagocytosis of C. albicans (26–29). We hypothesized that the reduced phagocytosis of the highly CUG-mistranslating strain was due to reduced cell surface exposure of β-glucans. To test this hypothesis, we assessed the level of surface-exposed β-glucan on wild-type C. albicans and the highly CUG-mistranslating strain with an anti-β-1,3-glucan monoclonal antibody using flow cytometry (30). We found that the highly CUG-mistranslating strain had 2- to 3-fold less exposure of β-glucans on its surface than did wild-type cells (Fig. 4D and E). This reduced exposure was seen in viable cells as well as heat-killed cells.
Collectively, these data suggest that increased CUG mistranslation results in decreased β-glucan exposure on the fungal surface, which in turn reduces susceptibility to macrophage phagocytosis.

**DISCUSSION**

*C. albicans* has evolved multiple mechanisms to alter its surface characteristics. These mechanisms include white-opaque switching and yeast-to-hypha transition (31). Our data demonstrate that CUG mistranslation through the variable incorporation of polar serine or hydrophobic leucine residues is another mechanism for expanding the structural and functional diversity of cell surface proteins (Fig. 5). Genes encoding these proteins are particularly enriched in CUG codons (58% of cell wall proteome), suggesting that the protein diversity generated by CUG mistranslation may play an important role in the interactions of *C. albicans* with host cells (17). We found that increasing the rate of CUG mistranslation enhanced the adherence of *C. albicans* to polystyrene and host constituents. This increase in adherence is mediated by at least two mechanisms. First, CUG mistranslation enhances cell surface hydrophobicity, which is known to play a role in *C. albicans* adherence to both plastic and host cells (32). Second, CUG mistranslation increases the substrate avidity of adhesins such as the Als proteins. Indeed, expression of Als3p-Leu in *S. cerevisiae* conferred significantly greater adherence to some substrates than did the expression of Als3p-Ser.

The highly conserved location of CUG codons in the regions of ALS1 to ALS4 that encode the substrate-binding domain suggests that CUG mistranslation likely influences the adhesive function of other Als proteins besides Als3p. Furthermore, *C. albicans* possesses additional adhesins such as Eap1 that are not members of the Als protein family (33). The EAP1 gene also contains 2 CUG codons. Thus, CUG mistranslation might enhance the structural diversity and modulate the adherence function of other proteins, as well. This functional variability may broaden the range of host substrates to which *C. albicans* can bind by changing the ligand interaction region. Recently, it has been reported that Als proteins

**FIG 2** Number of CUG codons and their localization in the 5′ domain (N-terminal) coding sequence of the ALS gene family. (A) Coding sequences of ALS1, ALS2, ALS3, ALS4, ALS5, ALS6, ALS7, and ALS9 were retrieved from the Candida Genome Database. The number of CUG codons (n) present in each ORF and the number of theoretical proteins (2^n) resulting from its translation, depending on probabilistic serine-versus-leucine incorporation during CUG translation. (B) Multiple sequence alignment of N-terminal domains of Als proteins in which the CUG-encoded serines are highlighted in green. Strictly conserved residues among Als proteins are indicated in red letters against a white background, while conserved residues are represented by white letters boxed in red. Secondary elements from the *C. albicans* ALS9 crystal sequence structure (PDB code 2Y7N) are displayed above the alignment. The solvent accessibility for each residue is indicated by a bar at the bottom of the sequence (blue, accessible; cyan, intermediate; white, buried; red, no correspondence between ALS9 crystal structure and protein sequence). (C) Distribution of CUG positions (red residue; ALS9 protein numbering) on the molecular surface of the ALS N-terminal domain (expecting residues 379 and 433).
can coalesce into amyloid-like aggregates that result in the formation of adhesive nanodomains and increased cell-cell adherence (24, 34, 35). The CUG codons in ALS1 to ALS4 are located near the regions of these genes that are predicted to encode amyloid-forming domains. Therefore, it is also possible that CUG mis-translation might modulate the amyloid profile of Als proteins.

Even though the highly CUG-mistranslating strain of C. albicans had increased adherence to host constituents, we found that it was phagocytosed poorly by macrophages. Macrophages possess multiple receptors that recognize various components of the C. albicans cell wall (36). CR3 and dectin-1 both recognize /H9252-glucans exposed on the surface of C. albicans, and both of these receptors mediate the phagocytosis of yeast-phase organisms (26, 29, 36). We found that the highly CUG-mistranslating strain of C. albicans had lower surface exposure of /H9252-glucans than did the wild-type strain. This reduced /H9252-glucan exposure may contribute to the reduced macrophage phagocytosis of the highly CUG-mistranslating strain. Although Ser and Asp are the residues for O-glycosylation and 59% of the open reading frames (ORFs) associated with cell wall organization and biogenesis are CUG-containing ORFs (17), N-linked and O-linked mannan appear not to be affected by CUG mistranslation. In contrast, the mannan outer layer is effectively masking /H9252-glucans in the CUG-mistranslating strain comparably to the wild type. A defect in N- and O-glycosylation would imply an increase in C. albicans uptake by macrophages (37, 38). The lower phagocytosis rate observed in the CUG-mistranslating strain may also be related to a depletion of phosphomannan cell wall, as happens in the pmr1 mutant (38). Interestingly, the PMR1 gene has a single CUG codon encoding a strictly conserved serine (17). The impact on Pmr1p functionality of the replacement of a serine by a leucine resulting from CUG decoding is unknown; however, if this serine-to-leucine switching implies a reduction in Pmr1p activity, a defect in phosphomannan cell wall may occur. It was notable that the highly CUG-mistranslating strain had the same susceptibility to the /H9252-1,3-glucan synthase inhibitor caspofungin as did the wild-type strain. This result suggests that the total amounts of /H9252-1,3-glucan in the cell walls of the two strains might be similar (39).

It was previously demonstrated that the level of leucine misincorporation at CUG codons is variable and depends upon external conditions, namely, temperature and pH (15). In addition, a disparity in activity and stability between two protein isoforms of the C. albicans seryl-tRNA synthetase (SerRS) was found. When the only CUG codon in SerRS is translated as leucine, the activity of this enzyme is increased, thereby raising the percentage of tRNACAG that is serylated (17). Besides SerRS and through structural studies, authors suggest that other proteins associated with the pathogenesis process may display functional changes resulting from serine/leucine incorporation at CUG sites (17).

Although they are relatively uncommon, similar mistranslation events are known to occur in other biological systems and enhance resistance to stress. For example, when mammalian cells are subjected to oxidative stress, the population of tRNAs misacylated with methionine increases remarkably (40). The presence of
methionine in some proteins protects them from damage caused by reactive oxygen species. Similarly, the mistranslation of the UGA stop codon as selenocysteine protects neurons from oxidative stress (41) and is required for the normal function of macrophages (42). However, an important difference between mammalian and C. albicans cells is that approximately 20 selenoproteins have been described in mammalian cells (43), whereas 3,998 C. albicans genes contain at least one CUG codon.

Organisms able to generate statistical proteins are thought to be in the origin of life (44). Their improved adaptability is granted by the formation of protein variants, resulting from the same coding sequence, which possess enhanced activity but reduced specificity (45). Selective growth advantages conferred by ambiguous codes, similar to the natural CUG mistranslation system but artificially originated, have been demonstrated in S. cerevisiae (46), Escherichia coli (45), and Acinetobacter baylyi (47).

C. albicans has lived as a commensal with mammalian hosts for millions of years. Prerequisites for a successful commensal organism include the capacity to adhere to host constituents and to avoid being cleared by the host immune response. Thus, it is likely that CUG mistranslation may constitute an advantage by conferring increased capability of cells to adhere to a broad range of host substrates while minimizing interactions with immune effector cells. CUG mistranslation has the capacity not only to provide greater diversity in the cell surface proteins of C. albicans but also to expand the repertoire of metabolic responses of this organism (Fig. 5). Thus, CUG mistranslation likely expands the host microchines in which C. albicans can flourish.

MATERIALS AND METHODS

Strains, growth conditions, and cell culture conditions. The two C. albicans strains, wild type (pUA12) and highly CUG mistranslating (pUA15) (18), were grown in minimal medium lacking uridine (MM-uri) (0.67% [wt/vol] yeast nitrogen base without amino acids, 2% [wt/vol]
glucose, and 100 μg/ml of the required amino acids). For hydrophobicity and adherence assays, yeast cells were grown overnight at 30°C in a shaking incubator. For use in the phagocytosis experiments, the yeast cells were grown on MM-uri agar at 30°C for 48 h.

*A. coli* strain DH5α was used as a host to carry out ALS3 CUG codon mutagenesis (described below), and it was grown in LB with 100 μg/ml ampicillin.

*S. cerevisiae* strain S150-2B (leu2 his3 trp1 ura3) was used for heterologous expression of *C. albicans* ALS3-Leu and ALS3-Ser genes (20). The Als3p-expressing clones were grown overnight at 30°C in minimal medium supplemented with appropriate amino acids.

The RAW 264.7 murine macrophage cell line was obtained from Sigma-Aldrich (catalog no. ECACC 91062702) and maintained in RPMI 1640 medium (Sigma), containing 10% heat-inactivated fetal bovine serum and 100 μg/ml of the required amino acids. For hydrophobicity and adherence assays, yeast cells were grown on MM-uri agar at 30°C for 48 h.

The cell surface hydrophobicity determination. The cell surface hydrophobicity of the two strains of *C. albicans* was analyzed as described previously (23). Yeast cells were harvested at stationary growth phase and washed twice with PBS, pH 7.0. Then, yeasts were resuspended to an optical density at 600 nm (OD600) of 0.4 to 0.5 in PBS (A0). Next, 3 ml of the yeast suspension was added to acid-washed spectrophotometer glass vials and overlaid with 0.4 ml of n-hexadecane. After vigorous vortexing, the phases were allowed to separate for 10 min at 30°C and the OD600 of the aqueous phase was measured (A1). The percentage of hydrophobicity was calculated as follows: hydrophobicity (%) = [1 – (A1/A0)] × 100. All assays were performed in triplicate.

Sequence analysis. The amino acid sequences of the N termini of *C. albicans* Als proteins were retrieved from the Candida Genome Database (http://www.candidagenome.org/). The position of CUG-encoded residues in the corresponding nucleotide coding sequence (bp 1 to 1299) was determined using CodonPlot (48). Multiple sequence alignments of the N-terminal domains of Als1 to Als9 were carried out using the Clust-
**Supplemental Material**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00285-13/-/DCSupplemental.

Figure S1, TIF file, 1.7 MB.
Figure S2, TIF file, 0.8 MB.
Figure S3, TIF file, 0.9 MB.

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