Glycosylphosphatidylinositol (GPI)-anchored proteins are an important class of cell wall proteins in *Candida albicans* because of their localization and their function, even if more than half of them have no characterized homolog in the databases. In this study, we focused on the IFF protein family, investigating their exposure on the cell surface and the sequences that determine their subcellular localization. Protein localization and surface exposure were monitored by the addition of a V5 tag on all members of the family. The data obtained using the complete proteins showed for Iff3 (or -9), Iff5, Iff6, and Iff8 a covalent linkage to the β-1,6-glucan network but, remarkably, showed that Iff2/Hyr3 was linked through disulfide bridges or NaOH-labile bonds. However, since some proteins of the Iff family were undetectable, we designed chimeric constructions using the last 60 amino acids of these proteins to test the localization signal. These constructions showed a β-1,6-glucan linkage for Iff1/Rbr3, Iff2/Hyr3, Iff4 and Iff7/Hyr4 C-terminal–Iff5 fusion proteins, and a membrane localization for the Iff10/Flo9 C terminus–Iff5 fusion protein. Immunofluorescence analyses coupled to these cell fraction data confirmed the importance of the length of the central serine/threonine-rich region for cell surface exposure. Further analysis of the Iff2/Hyr3 linkage to the cell surface showed for the first time that a serine/threonine central region of a GPI-anchored protein may be responsible for the disulfide and the NaOH bonds to the glucan and glycoproteins network and may also override the signal of the proximal ω site region.

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*Candida albicans* is responsible for the majority of fungal infections in humans. Since the cell wall represents the first element of the fungal cell encountered by human cells during the colonization or the infection, major attention has focused on this compartment. By electron microscopy, two layers are distinguishable: a translucent layer closer to the plasma membrane that comprises chitin and β-1,3-glucan polymers, and an electron-dense exterior layer, mainly composed of fibrillar highly glycosylated proteins (33). The major class of these mannoproteins is represented by the glycosylphosphatidylinositol (GPI)-anchored proteins (40, 57). The second class consists of the Pir proteins (protein with internal repeats) bound by alkali-sensitive linkages to the β-1,3-glucan (11). Several *in silico* studies have identified about 100 GPI-modified proteins in the *C. albicans* genome (15, 19). Some of these proteins are retained in the plasma membrane and behave as GPI-anchored plasma membrane proteins, such as Exg2 (6). Others are released from the membrane and become covalently linked, mainly to the β-1,6-glucan in the cell wall through a remnant of the GPI anchor (34, 64). We have named these proteins GPI-anchored cell wall proteins hereafter in the text. Several studies have attempted to identify the signal governing the final localization of GPI-anchored proteins, and they pointed out the crucial role of basic amino acids upstream of the GPI anchor attachment site (the ω site) for plasma membrane retention (8, 24, 25, 29, 47).

The overall organization of a typical GPI-anchored protein can be divided into three parts: a hydrophobic signal sequence at the N terminus that allows entry into the secretory pathway; a central region that corresponds to the functional region and may contain N- or O-glycosylation sites; a second hydrophobic domain at the C terminus that triggers the GPI anchor addition. Typically, many cell wall GPI-anchored proteins are harboring between the functional region and the C terminus an additional serine- and threonine-rich region highly O-glycosylated and of variable size. Among these cell wall proteins are proteins with enzymatic functions, like the aspartyl proteases Sap9 and -10 (1, 54), the glucanosyltranferases Phr1 and -2 (7, 22), or the transglycosidase Chh11 (55), as well as proteins with adherence properties, like Als5 and Als1 (27, 31), Hwp1 (60), and Eap1 (41). Some GPI-modified proteins have been successfully tagged and localized. Examples include Pga59 tagged with green fluorescent protein (GFP) (50), Eap1 tagged with hemagglutinin (43), and Dfg5 tagged with the V5 epitope (59). However, to date only a quarter of the putative GPI-anchored proteins have been detected at the surface of the cells based on liquid chromatography-tandem mass spectrometry, leaving around 75 proteins with an unknown localization (9, 14, 46, 49, 58).

GPI-anchored proteins can be grouped into 21 different families, which include the two very large *ALs* (30) and *HYR/IFF* families (57). The latter family includes 12 members encoded by genes orthologous to the first protein characterized, Hyr1 (2). Reverse genetics studies of *HYR1* (45), *IFF4* (37), and *IFF11* (3) have not yet identified a common function for the corresponding proteins, despite the strong conservation of
their N-terminal domain. However, a role of Iff4 in adherence and interaction with host cells has been suggested (38). Recently, Luo and coworkers identified Hyr1 as a potential virulence factor (45); indeed, overexpression of HYR1 enhanced C. albicans resistance to phagocyte-mediated killing. Moreover, comparison of eight Candida genomes revealed that the IFF family was enriched in pathogenic species, as was the ALS family (5). To get a better understanding of the localization of the uncharacterized members of this family and to validate a putative role of these proteins in processes that rely on surface components, such as adherence or resistance to host phagocytic cells, we tagged nine members of the Iff family. Fractionation and immunofluorescence experiments were performed on the epitope-tagged strains. This study demonstrated that among the nine Iff proteins studied, at least five are distributed to the cell wall, where they are either covalently linked to the β-1,6-glucan (four of them) or are retained through disulfide bridges and/or alkali-sensitive linkages (for Iff2/Hyr3). Among the four remaining proteins, three contain a cell wall-targeting C-terminal signal and one harbors a plasma membrane-anchoring C-terminal signal. The results confirm that the plasma membrane or cell wall localization of these GPI-anchored proteins depends on the nature of the amino acid residues just upstream of the α site and also show for the first time that the serine/threonine-rich domain might interfere with the formation of a covalent linkage between a GPI moiety and the β-1,6-glucan, overriding the cell wall GPI-anchoring C-terminal signal. Moreover, we show that the surface exposure of the N-terminal functional domain is independent of the nature of the cell wall anchoring but strongly linked to the length of the serine/threonine-rich region.

MATERIALS AND METHODS

Strains. The C. albicans strain used for DNA amplification was BWPI7 (67), and CAI-4 was used for expression of the tagged copies of the Iff proteins (23). CAI-4 was grown either in liquid YPD (2% glucose, 2% Bacto peptone, 1% yeast extract) or synthetic medium (0.67% yeast nitrogen base with ammonium sulfate and without amino acid [Difco], 2% glucose, and 0.17% amino acid dropout mix) at 30°C. Following transformation, Ura− ammonium sulfate and without amino acid [Difco], 2% glucose, and 0.17% sequence under the control of the gLUC59, expressing an internal fusion between Pga59 and the luciferase coding expression/secretion vector was derived from the plasmid Cip10::ACT1p- a purified recombinant -1,6-glucanase (4) or the laminarinase from Sigma.

Construction of an expression vector. To epitope tag the Iff proteins, an expression/secretion vector was derived from the plasmid Cip10::ACT1p-gLUC59, expressing an internal fusion between Pga59 and the luciferase coding sequence under the control of the ACT1 promoter (21). For this purpose two primers were designed, PACTSP-3360R and PACTERMER-4228 (Table 1, primers 1 and 2), which allowed the replacement of the sequence between amino acid 19 of Pga59 and its stop codon by a multiple cloning site harboring, serine/threonine-rich region.

Cell lysis and preparation of cell wall, membrane, and soluble protein fractions. Candida albicans CAI-4 transformed cells were harvested by centrifugation, washed with 10 mM Tris-HCl containing 10 mM NaCl, and disrupted in ice after resuspension in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing antiproteases (Complete, EDTA-free; Roche) and glass beads in a Bead-Beater 24 (MP Biomedicals) four times for 20 s each with 5 min on ice between each round. Subsequently, the lysate was collected and further centrifuged at 1,000 × g for 10 min at 4°C to collect a low-speed supernatant (S1,000) and a low-speed pellet (C1,000).

To obtain a plasma membrane-enriched fraction, the low-speed supernatant was further centrifuged at 100,000 × g for 1 h at 4°C, and the high-speed supernatant corresponding to the soluble protein fraction was removed. For membrane protein solubilization, the high-speed pellet was resuspended in 50 mM Tris-HCl (pH 7.4) containing 2% SDS and antiproteases and then heated for 5 min at 95°C. After a 5-min centrifugation at 10,000 × g, the nonsolubilized material was discarded.

For cell wall protein extraction, the low-speed pellet was washed extensively with 1 M NaCl and the resulting cell walls were boiled twice in the presence of 50 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8), 2% SDS, and 40 mM β-mercaptoethanol (βME) to solubilize the noncovalently linked cell wall proteins and to remove any contaminant proteins from the cytosol and/or plasmalemma. SDS- and βME-extracted cell walls were extensively washed with H2O, resuspended in 20 mM Na-acetate (pH 4.5), and incubated for 3 h at 37°C either with a purified recombinant β-1,6-glucanase (4) or the laminarinase from Sigma. After a 5-min centrifugation at 10,000 × g, the insoluble material was discarded and the solubilized proteins were concentrated on a Microcon 50 membrane (Millipore) before Western blot analysis.

For βME extraction, NaCl- and Tris-washed cell walls from the low-speed pellet were resuspended in 50 mM Tris-HCl (pH 7.4) containing 2% βME and then incubated for 1 h at 37°C. After a 5-min centrifugation at 10,000 × g, the nonsolubilized material was discarded. In some experiments, the NaCl- and Tris-washed low-speed pellet was boiled in the presence of 2% SDS only before being washed again and extracted either with 2% βME for 1 h at 37°C or 30 mM NaOH for one night at 4°C (52). Released proteins were collected by a 5-min centrifugation at 10,000 × g and concentrated on a Microcon 50 membrane (Millipore) before Western blot analysis.

Western blot analysis. Secreted or extracted proteins were separated by SDS-polyacrylamide gel electrophoresis either on NuPage Novex Tris-ace- tate 3-8% or NuPage Novex bis-Tris 10% precast gels (Invitrogen) in NuPage Novex Tris-ace-tate SDS running buffer or NuPage Novex mor-pholinopropanesulfonic acid-SDS running buffer (Invitrogen) using the XCell Mini-Cell system from Invitrogen. The proteins were transferred onto a nitrocellulose membrane (Protran) for Western blot analysis. Following transfer, membranes were rinsed in phosphate-buffered saline (PBS) and blocked in PBST (PBS plus 0.1% Tween 20) and 2% skimmed milk from

specific primers. The forward primers contained an AvrII site, and the reverse primers contained a Sphl site (Table 1, primers 7 to 16). After cloning of the amplified fragments into the pGEMT-Easy vector, they were subcloned at the AvrII and Sphl sites of the pExp-V5 plasmid (see above) in frame with the V5 epitope fused to the Pga59 signal sequence at the 5′ end and with the stop codon at the 3′ end. All fusion junctions were sequenced. The same procedure was used to tag the DCW1 sequence (Table 1, primers 17 and 18). Construction of the internal deleted copy of Iff2/Hyr3 was performed by restriction using two SpeI sites which created an in-frame deletion from amino acid 425 to amino acid 934. To increase the length of the Iffs Ser/Thr-rich region by the Iff2/Hyr3 internal domain, two primers were designed (Table 1, primers 19 and 20) that allowed amplification of the Iff2/Hyr3 region from amino acid 402 to amino acid 932 and further cloning after PstI restriction at the unique NsiI site of the IF38 coding sequence, which encodes amino acids 394 and 395. Chimeric Iff proteins fused to Iff1/Iff3, Iff2/Hyr3, Iff4, Iff7/Hyr4, and Iff10/Flu9 C termini were constructed using the PstI unique site in the IF5 coding sequence 192 bp upstream of the stop codon. New forward primers (Table 1, primers 21 to 24 and 29) were designed for 5 former sequences in order to insert a PstI site. Products amplified with these primers and the previous reverse primers were cloned into pGEMT- Easy and sequenced, and PstI/Sphl-digested fragments were subcloned in the pExp-V5. An IF5 recombinant strain was restricted with the same enzymes.

Candida albicans transformation. Recombinant expression vectors were transformed into CAI-4 after linearization by Stul to allow the homologous recombination at the RPS10 locus (a neutral integrative locus) (53). Ura+ selected clones were screened by PCR for the correct integration of the construction using the following primers: RP10-1250 and PACTGLUC-4642 (Table 1, primers 5 and 6). All fusions constructed in this study are presented in Table 2.

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Difco for 1 h at room temperature. The membranes were then incubated overnight at 4°C in PBST containing a 1:5,000 dilution of either the monoclonal anti-V5 antibody (Invitrogen) or the monoclonal anti-GFP antibody (Clontech). After 3 washes in PBST, 1-h incubations in the presence of peroxidase-conjugated anti-mouse IgG antibodies (GE Healthcare) were performed. The membranes were washed three times before detection of the signal using the GE Healthcare enhanced chemiluminescence (ECL) detection system.

### TABLE 1. Primers used in this study

| Construction of the pExp-V5 vector | Primer name | Sequence |
|-----------------------------------|-------------|----------|
| 1 PACTSP-3360R | GCATGCATCTAGGGCACTGGTGGCTAAAGCCGGAACAGG | |
| 2 PACTTERM-4228 | AGCTGCCCTAAGTGAATGCTCTCAATCTTTT | |
| 3 V5Up | CACGGTTGGAATCCATTTGGAATTGGAAGTG | |
| 4 V5Down | AGCTGAGTGAAGTATGGAATTGGAAGTG | |
| 5 RP10-1250R | CAGATCTTATATGGAATTGGAAGTG | |
| 6 PGLUC-4652 | GGTGTGACCTTATATGGAATTGGAAGTG | |

### Cloning of the IFF coding sequences

| Primer purpose and no. | Primer name | Sequence |
|-----------------------|-------------|----------|
| 7 IFF2-64ClaAvr | CACCATGAGTCTCTAGGGTTACTACGTAGAGTAGTTG | |
| 8 IFF3-61Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 9 IFF5-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 10 IFF7-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 11 IFF9-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 12 IFF10-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 13 IFF11-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 14 IFF12-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 15 IFF13-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 16 IFF14-58Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |

### Cloning of the DCW1 coding sequence

| Primer purpose and no. | Primer name | Sequence |
|-----------------------|-------------|----------|
| 17 DCW1-55Avr | AGCCCTTACGATTGCATACCGAAAAACAGG | |
| 18 DCW1R-1356Sph | CGCATGCGCAAGAAAAAACAAAAACATTGAAG | |

### Cloning of the IFF2/HYR3 S/T domain

| Primer purpose and no. | Primer name | Sequence |
|-----------------------|-------------|----------|
| 19 IFF2-1202Pst | GTAAGTTTGCTGCAACATGGATCTAAC | |
| 20 IFF2R-2797Pst | GTAAGTTTGCTGCAACATGGATCTAAC | |

### Cloning of the IFF C-terminal coding sequences

| Primer purpose and no. | Primer name | Sequence |
|-----------------------|-------------|----------|
| 21 IFF1-4498Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 22 IFF4-4375Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 23 IFF5-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 24 IFF6-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 25 IFF7-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 26 IFF8-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 27 IFF9-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |
| 28 IFF10-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |

### Cloning of the IFF2 C-terminal coding sequence

| Primer purpose and no. | Primer name | Sequence |
|-----------------------|-------------|----------|
| 29 IFF2-3547Pst | CAGCTGACATTTCTCTCTTCGTC | |

### TABLE 2. Strains used in this study

| Strain | Parental strain | Genotype | Reference |
|--------|----------------|----------|-----------|
| CAI-4  | ura3Δ::imm434/ura3Δ::imm434 |          | 20        |
| BWP17  | CAI-4          | ura3Δ::imm434/ura3Δ::imm434 ARG4::HisG HIS1::HisG | 55        |
| VIF101 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2 | This study |
| VIF102 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF3 | This study |
| VIF103 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF5 | This study |
| VIF104 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF6 | This study |
| VIF105 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF8 | This study |
| VIF106 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9 | This study |
| VIF107 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF108 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF109 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF110 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF111 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF112 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF113 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF114 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
| VIF115 | CAI-4          | Same as CAI-4 but RPS10/psrl0apExpV5::IFF2/5/9/DCW1 | This study |
Immunofluorescence detection. Cells were fixed in 8-fold-diluted 37% formaldehyde for 40 min and after washes were placed on polylysine-coated slides, then blocked in PBS plus 0.5% bovine serum albumin (BSA) and incubated in the presence of monoclonal anti-V5–Cy3 conjugate antibody (4 mg/ml; Sigma) in PBS plus 0.5% BSA for 1 h in the dark. After washes in PBS, 4',6-diamidino-2-phenylindole was added (2 ng/ml, final concentration), and rinsed before the mounting step. For permeabilization, cells grown to an optical density of 1 to 2 were washed and resuspended in phosphate buffer (pH 7.5) at a 5% concentration before being fixed in 8-fold-diluted 37% formaldehyde and incubated for 40 min at room temperature. After 3 washes in phosphate buffer, cells were incubated for 20 min at 37°C in sorbitol buffer plus Zymolyase 100T (0.5 mg/ml, final concentration) plus 0.1% BME. Two volumes of cold sorbitol buffer were added before centrifugation and 2 washes in sorbitol buffer. Cells were resuspended in sorbitol buffer before proceeding with immunofluorescence. For proteinase K treatment, fixed cells were resuspended in 50 mM Tris-HCl (pH 7.4) buffer containing proteinase K (0.5 mg/ml, final concentration) and incubated for 2 h at 37°C. Cells were extensively washed before using them for immunofluorescence. Cells were examined by fluorescence microscopy (Olympus BX51) with 512-nm excitation and 565-nm emission filters and an Olympus 100× oil immersion objective and 10× oculars.

RESULTS

**IFF gene family sequence analysis.** (i) The **IFF** gene families evolved from a common ancestor. The **IFF** (YPF [Individual Protein File] Family E) gene family was described by Bates et al. for *C. albicans* and by Butler et al. for other fungi (3, 5), but no data on the phylogenetic evolution of this family were available in the literature. We used the first 360 amino acids of If2/Hyr3 as a probe in the sequence databases to collect the sequences of each member of the **IFF** families, and we collected 76 paralogs in 4 different genera (*Candida dubliniensis*, *Candida lusitaniae*, *Pichia stipitis*, *Candida parapsilosis*, *Candida tropicalis*, *Debaryomyces hansenii*, *Lodderomyces elongisporum*, and *Candida guilliermondii*). Butler et al. showed this family is present in fungi only and, interestingly, only in fungi from the CTG clade (a subphyllum of *Saccharomycotina* that is characterized by the translation of the CUG codon into serine instead of leucine). Thus, no paralog was identified in *Saccharomyces cerevisiae* or *Aspergillus fumigatus*. A phylogenetic analysis was performed (see Fig. S1 in the supplemental material for an illustration of the tree). It was striking to see that the paralogs are not distributed evenly between each species, but each species seems to have independently produced its own family. This would suggest a divergent evolution, where families have evolved independently in the different species from a common ancestor rather than evolving from an ancestral family, but keeping a tight pressure of selection on the N-terminal domain sequence.

(ii) **Modification of IFF1/RBR3 and IFF10/FLO9** **CGD annotation.** The *Candida albicans* genome available through the *Candida* Genome Database (http://www.candidagenome.org) provided most of the data used in this study. Although the **IFF** gene family has already been described, 2 discrepancies were remaining in the CGD database: (i) the *IFF1/Rbr3* N-terminal sequence did not seem annotated correctly, since the **IFF1/RBR3** CDS was only 1,419 bp long, while the open reading frame (ORF) on the genome sequence available on the site was 4,689 bp long. (ii) For **IFF10/FLO9**, ORF19,5404 and ORF19,5401 were obviously two parts of the same gene; indeed, in their publication, Bates et al. suggested a frameshift mutation to join the two ORFs. In order to address these two interrogations, sequences of both genes were obtained in the laboratory, and it was found that **IFF1/RBR3** is an ORF of 4,689 bp devoid of any stop codon. Concerning **IFF10/FLO9**, the CGD sequence of ORF19,5404 showed an insertion of 1 bp, creating an early stop codon 3 nucleotides later. The final If10/Flo9 polypeptide is thus 1,244 amino acids long. The new sequences and annotation suggestion for **IFF1** and **IFF10** were submitted to the CGD curators on the CGD website (www.candidagenome.org).

(iii) **Allelic variation in the **IFF1** gene.** During **IFF1** sequence analysis, we observed that **IFF1** amplification from *SC5314* genomic DNA showed two PCR fragments, suggesting a large difference in sizes between the two alleles. Cloning and sequencing of these two alleles confirmed the first observation, showing an additional sequence of 369 bp in the larger allele in addition to numerous sequence differences between alleles. This additional sequence is localized at the beginning of the Ser/Thr-rich domain. A BLAST analysis of this specific sequence identified numerous similarities in the *Candida* genome and in the NCBI sequence database, but only due to the high number of serine residues and the low complexity of the sequence (52% of serine residues and 12% of threonine residues) (12). Additionally, downstream of this insertion within the same Ser/Thr-rich domain there are 5 repeated sequences of 138 bp. Five more copies of shorter length (105 bp) and less-conserved repeats are found in the following 800 bp. The encoded sequences, although enriched in serine and threonine, show similarities to repeats present in several members of the **IFF** family (see below). In particular, the 13-amino-acid sequence NSDGSVSTESGIV can be also found as 2 to 21 repeats in proteins in numerous other fungi, such as *C. tropicalis*, *C. dubliniensis*, *Magnaporthe grisea*, and *Pichia guilliermondii*.

Intrigued by these two features we looked among the different *Candida albicans* clades in order to search for the presence of the insertion. Interestingly, only 5 out of 20 different clinical isolates showed the same pattern as the one identified in *SC5314*. A simple analysis based on the PCR product size showed a large heterogeneity between isolates. Some isolates contained 2 alleles of differing sizes, and other isolates only yielded a single band of variable molecular weight (Fig. 1; see also Table S2 in the supplemental material). A preliminary analysis of these variations was to determine whether they were due to either the absence of the 369-bp insertion or a change in the number of 138-bp repeats. PCR results showed heterogeneous data, where each combination of the former hypotheses occurred depending on the case studied (data not shown). We were not able to show that a specific pattern of PCR on **IFF1** gene (size and number of bands) can be associated with specific clades: the repartition within the clades appeared to be random. No function was associated with If11/Rbr3, so we did not have the tools to prove that these variations in size actually modified the functionality of the different If11/Rbr3 proteins.

Epitope tagging and Western blot analysis of If proteins. The overall organization of the 12 If proteins is presented in Fig. 2A. All If proteins begin with an N-terminal signal sequence, which indicates that they enter the secretory pathway (20). Except If11, all members of the **IFF** family are predicted to be GPI modified at their C termini, based on the Eisenhaber predictive site (19; http://mendel.imp.ac.at/gpi/fungi_server.html). Figure 2B presents the alignment of
the last 60 amino acids of the studied Iffs displayed by the Multalin server (http://multalin.toulouse.inra.fr/multalin/) (13) and the proposed \( [H]9275 \) sites based on Eisenhaber’s rules are boxed. In the case of Iff7/Hyr4, the prediction gives S (at position 1195), which is an unexpected result compared with the other members of the family. Between these two terminal regions, a Ser/Thr-rich domain of variable length was found which represents a putative highly O-glycosylated region. Indeed, using the NetOGlyc prediction site (http://www.cbs.dtu.dk/services/NetOGlyc/; a neural algorithm developed for prediction of mucin O-glycosylation sites in mammalian proteins [32]), all the Iff proteins displayed a high number of putatively O-glycosylated serine and threonine residues. Located at the 3’ end of the Ser/Thr-rich region, 10, 4, 2, 5, and 3 repetitions of an imperfect 35-amino-acid motif, including the NSDGSVSTESGIV sequence, is present in Iff1/Rbr3, Iff2/Hyr3, Iff3 (or Iff9), Iff5, and Iff7/Hyr4, respectively, as shown in Fig. 2A. Hyr1, Iff2/Hyr3, and Iff6 contain an additional glycine/asparagine-rich region upstream from the hydrophobic tail.

Our first aim was to tag and localize the uncharacterized Iff proteins. However, except for Hyr1 (2), the conditions permitting the expression of the \( IFF \) genes are not known. Preliminary experiments on transcriptional regulation of this family indicate that under usual growth conditions, \( IFF \) genes are poorly expressed (A. Cornu, unpublished data).

FIG. 1. \( IFF1/RBR3 \) allelic variation in 23 different clinical isolates representing 11 clades of \( C. \) albicans (isolated from different countries in different body niches), based on 0.8% agarose gel electrophoresis of the PCR product obtained from genomic DNA extracted after 24 h of culture in YPD. Lanes: Mk, 1-kb Plus marker (Invitrogen); lane 1, DAY286. Lanes 2 to 24 are clinical isolates; 2, M15; 3, M21; 4, M26; 10, H14; 11, Bougn11; 12, Bougn14; 13, M47; 14, M61; 15, APRURC3; 16, Bougn6; 17, Bougn7; 18, Bougn9; 19, Bougn10; 20, Bougn15; 21, F-E; 22, F-J; 23, DPC63; 24, BCHURS6.

FIG. 2. (A) Schematic representation of the Iff protein family domain organization with, from left to right, the signal peptide sequence (white box), the IFF family conserved domain (dashed box), the Ser/Thr-rich region (gray box) containing the imperfect repeats of the 35-amino-acid motif TEFTTTWV3TNSDGVSTEGVISESFSFTITIT (black circles), and the GPI-anchor signal (black box). The Gly/Asp-rich regions of Iff2, Iff6, and Hyr1 are symbolized by a dotted box. (B) Alignment of the Iff protein family GPI anchor addition C-terminal sequences, determined using the Multalin server. Shown in bold are the \( \omega-4 \) and \( \omega-5 \) I or L; underlining shows the \( \omega-1 \) K; the putative \( \omega \) sites for each protein are surrounded by black frames.
The first step thus consisted of developing a convenient plasmid with constitutive, easily detectable expression and epitope tagging of *C. albicans* proteins. The presence of both N- and C-terminal targeting signals for endoplasmic reticulum localization and addition of a GPI anchor prevented cloning of the epitope at either end. A plasmid containing the *ACT1* promoter and the Pga59 coding sequence (21) were modified to introduce the V5 epitope, which has successfully been used in *C. albicans* (3, 59), in frame with the last predicted Pga59 signal peptide residue and unique restriction sites to allow cloning of any coding sequence (see Materials and Methods). Targeting of the tested proteins to the secretory pathway thus depended on the Pga59 signal peptide, and all the mature proteins started with the V5 epitope. Since localizations of Hyr1 and Iff11 were previously published (3, 45) and Iff3 and Iff9 coding sequences were not distinguishable, only nine *IFF* coding sequences were cloned in this expression vector.

As a first approach to determine if these nine Iff family members are cell wall proteins, we localized the epitope-tagged proteins by a fractionation experiment followed by Western blot analyses. Selected clones transformed by each tagged proteins by a fractionation experiment followed by sequences were cloned in this expression vector. Surprisingly, only four epitope-tagged Iffs were detected in this cell subfraction: as shown in Fig. 3A, a signal was present in the β-1,6-glucanase-solubilized protein fraction for the V5-fused Iff3 (or Iff9), Iff5, Iff6, and Iff8 proteins (lanes 3). Recovery of the proteins was dependent on the presence of the β-1,6-glucanase enzyme, since no signal was observed when the cell walls were incubated only in the buffer solution (compare lanes 3 and 2). This specificity indicates that these Iffs are true cell wall-localized GPI-modified proteins, as expected considering their ω-proximal sequences. The molecular weights of the detected proteins, while proportional, were higher than the calculated ones. This is in accordance with the presence of high numbers of putatively O-glycosylated serine and threonine residues in these proteins (Fig. 2A). Endoglycosidase H treatment, performed to eliminate sugars of the N-glycosylation type, did not drastically change the apparent molecular weight of the tagged proteins (data not shown), confirming that the difference was mainly due to O-glycosylation.

In contrast, Iff2/Hyr3 was detected only in the SDS- and βME-solubilized cell wall protein fraction and not in the β-1,6-glucanase-treated fraction (Fig. 3B, left panel, lanes 1 and 2), although protein release was observed (Fig. 3B, right panel, compare lane 4 to lane 3). To gain insight into the Iff2/Hyr3 localization, we prepared a new fraction enriched in plasma membrane proteins according to the methods of Lu and collaborators (44). Indeed, some GPI-modified proteins were shown to be plasma membrane anchored (6), and SDS-extracted cell wall fractions might contain such proteins. Two proteins, a previously characterized GFP-tagged plasma membrane protein, Sur7 (66), and a putative plasma membrane-GPI-anchored protein, Dcw1 (39), were included in this experiment as controls. As shown in Fig. 3C, while Sur7 and Dcw1 were present in the SDS-solubilized fraction from a high-speed pellet as expected for plasma membrane-localized proteins (lanes 2, first and second panels), unexpectedly Iff2/Hyr3 was totally absent from this fraction (lane 2, third panel), indicating that Iff2/Hyr3 is not plasma membrane anchored but is targeted to the cell wall, where it is retained through a different linkage than a covalent bond between the GPI moiety and the β-1,6-glucan. Sur7 and Dcw1 were also detected in the SDS- and βME-solubilized fraction from a low-speed pellet (lanes 3, first and second panels), thus confirming that this fraction contained plasma membrane components.

In order to identify the nature of the Iff2/Hyr3 cell wall linkage, an extensively washed low-speed pellet was submitted to reducing agent treatment. Indeed, some cell wall proteins are connected by disulfide bridges to covalently linked proteins (51). Incubating this fraction in the presence of 2% βME allowed the efficient release of Iff2/Hyr3 from the cell wall (Fig. 3D, compare lane 2 to lane 1), thus indicating that Iff2/Hyr3 retention in the cell wall involves at least disulfide linkages with proteins. Comparable results were obtained when the cell wall was boiled in 2% SDS and thoroughly washed before the βME treatment (data not shown), suggesting the existence of disulfide bridges with glycoproteins covalently linked to the cell wall. On the other hand, Dcw1 was not released by simple βME treatment but needed hot SDS extraction to be freed (data not shown). Since at least one Pir protein was shown to be linked to a cell wall component through disulfide bridges (51) and most members of this Pir family are connected to the cell wall through alkali-sensitive bonds (36), an extensively washed SDS-extracted cell wall fraction was also treated with NaOH. As shown in Fig. 3D, lane 3, a large amount of Iff2/Hyr3 was extracted by such a mild alkali treatment. Moreover, successive extractions with βME and then NaOH still released Iff2/Hyr3 (Fig. 3D, lane 5), thus confirming that Iff2/Hyr3 is retained in the cell wall both through covalent bonds with the β-1,3-glucan as well as through disulfide linkages. However, laminarinase digestion of the cell wall did not release any Iff2/Hyr3 (Fig. 3B, lanes 3 and 6), and we can hypothesize that the size of β-1,3-glucan-linked highly O-mannosylated protein prevented from entering in the gel.

Analysis of culture supernatant samples and all intracellular fractions did not reveal any signal for Iff1/Rbr3, Iff4, Iff7/Hyr4, and Iff10/Flo9 (data not shown), which were thus not localized using this approach.

**Immunofluorescence analysis of V5-fused Iff2/Hyr3, Iff3 (or Iff9), Iff5, Iff6, Iff8, and Dcw1 proteins.** To determine whether these proteins are exposed on the *Candida albicans* cell surface, an immunofluorescence experiment was performed on fixed but intact cells expressing the epitope-tagged Iff proteins. The four V5-fused Iff2/Hyr3, Iff3 (or Iff9), Iff5, and Iff6 proteins were detected on the cell surface by direct addition of an anti-V5–Cy3–coupled antibody without any cell permeabilization (Fig. 4A). The fluorophore displayed a similar strong peripheral staining for these four proteins, as already described for GPI-anchored proteins (47, 50). Only Iff8 showed a weak peripheral signal, suggesting that the V5 epitope was not accessible to the antibody from the surface. This may be in
accordance with the smaller length of the Iff8 protein, which contains a 340-amino-acid Ser/Thr-rich region (Fig. 2A). The localization of the V5-tagged Dcw1 protein devoid of any Ser/Thr-rich region was studied by the same approach. The immunofluorescence labeling performed on intact cells gave no signal (Fig. 4A), suggesting that for a membrane-bound GPI-anchored protein the cell wall represents a strong barrier to the antibody.

A classical immunofluorescence experiment was thus performed with cell wall permeabilization by Zymolyase treatment before incubation with the anti-V5–Cy3–coupled antibody. As shown in Fig. 4B, the permeabilization led to punctuate labeling on the cell surface for the two V5-tagged proteins, confirming that the antibody cannot diffuse through the network of β-glucans in the absence of any Zymolyase treatment and thus cannot reach either Iff8 within the glucan network or Dcw1 at the plasma membrane.

Since Iff2/Hyr3 was exposed to the cell surface, immunofluorescence was performed on fixed cells, which were further incubated in buffer containing proteinase K before labeling. As illustrated in Fig. 4C, the incubation in the presence of the proteinase led to the complete disappear-

**FIG. 3.** Cellular localization of the Iff2, Iff3 (or -9), Iff5, Iff6, and Iff8 proteins. (A) Western blot analysis of SDS- and βME-solubilized proteins from the cell wall fraction (C1,000) (lanes 1) and of extracted proteins after incubation of the resulting cell walls either in Na-acetate (NaAc) buffer alone (lanes 2) or in NaAc buffer plus 2 U of β-1,6-glucanase (lanes 3) from the V5 epitope-tagged Iff3, Iff5, Iff6, and Iff8 recombinant strains. Samples were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted with monoclonal anti-V5 antibody. (B) Analysis of SDS- and βME-solubilized proteins from the cell wall fraction (lane 1) and of extracted proteins after incubation of the resulting cell walls either in NaAc buffer alone (lane 4), in NaAc buffer plus 2 U of β-1,6-glucanase (lanes 2 and 5), or in NaAc buffer plus 0.1 U of laminaranase (lanes 3 and 6) from the V5 epitope-tagged Iff2 strain. (Left panel) Western blotting with monoclonal anti-V5 antibody; (right panel) SDS-PAGE and EZblue staining (Sigma-Aldrich). (C) Western blot analysis of the low-speed supernatant (S1,000) (lanes 1), of the SDS-solubilized proteins from the high-speed pellet (C1,000) (lanes 2), and of the SDS- and βME-solubilized proteins from the cell wall fraction (C1,000) (lanes 3) from the GFP-tagged Sur7 strain (first panel), the V5-tagged Dcw1 strain (second panel), and the V5-Iff2 strain (third panel). Samples were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted either with monoclonal anti-GFP antibody (first panel) or monoclonal anti-V5 antibody (second and third panels). (D) Western blotting with monoclonal anti-V5 antibody of the released proteins either after incubation of the NaCl- and Tris-washed low-speed pellet (C1,000) in 50 mM Tris (pH 7.4) buffer alone (lane 1) or in Tris buffer plus 2% βME (lane 2) for 1 h at 37°C or after incubation of the SDS-extracted cell wall fraction in 30 mM NaOH for 20 h at 4°C (lane 3) or after incubation of the SDS- and βME-extracted cell wall fraction in H2O (lane 4) or in 30 mM NaOH for 20 h at 4°C (lane 5) from the V5 epitope-tagged Iff2 strain.
Role of the Ser/Thr-rich region in Iff2/Hyr3. To test if the Ser/Thr-rich region was responsible for the surface exposure of the Iff2/Hyr3 N-terminal domain, a truncated version of Iff2/Hyr3 was constructed that lacked 510 amino acids from the central Ser/Thr-rich region. Western blot analysis of cell wall protein fractions isolated from cells expressing the Iff2/Hyr3 \( S/T \) protein showed that the shortened protein was now detected in the \( \beta-1,6 \)-glucanase-solubilized fraction, although a signal at a lower molecular weight was also present in the SDS-plus \( \beta \)ME-extracted fraction (Fig. 5A, lanes 3 and 4). Since an equivalent signal was observed in a concentrated supernatant sample (data not shown), it might correspond to degraded forms. So, one of the consequences of the Iff2/Hyr3 shortening is the modification of its cell wall linkage: deletion of the Ser/Thr-rich region is associated with the formation of a covalent bond between Iff2/Hyr3 and the \( \beta-1,6 \)-glucan, thus arguing for the GPI modification of Iff2/Hyr3. Direct immunofluorescence labeling on the same cells did not give any staining, as shown in Fig. 5B (upper panel), confirming the direct relationship between the length of the internal Ser/Thr-rich region and the protrusion at the cell surface (26). As shown in Fig. 5B (lower panel), the permeabilization of the cell wall before treatment with the anti-V5–Cy3–coupled antibody allowed the detection of the Iff2\( \Delta S/T \) tagged-protein on the cell surface.

To further confirm our hypothesis, a complementary experiment to increase the Iff8 internal domain length was performed. A 530-amino-acid region corresponding to the Ser/Thr-rich domain of Iff2/Hyr3 was inserted into Iff8 between amino acids 394 and 395 (Fig. 6A), and localization and surface exposure of the chimeric protein were studied further. As presented in Fig. 6B, C, and D, this extension not only rendered the Iff8 and Iff2 Ser/Thr rich protein accessible from the surface to the anti-V5 antibody, as illustrated by the immunofluorescence image obtained on nonpermeabilized cells (Fig. 6B), but also modified the cell wall anchoring of Iff8. Indeed, experiment confirmed that the Iff2/Hyr3 molecules protrude at the cell surface.

FIG. 4. In vivo localization of Iff2, Iff3 (Iff9), Iff5, Iff6, Iff8, and Dcw1 proteins. (A) Fixed transformed cells were directly treated with anti-V5–Cy3–coupled antibody, and immunofluorescence was observed using a microscope. (B) Cells were permeabilized prior to addition of anti-V5–Cy3–coupled antibody. (C) Fixed cells were incubated in the presence of proteinase K for 2 h at 37°C prior to immunofluorescence detection.
the Iff8 extended protein now fractionated in the SDS-plus 
βME-extracted cell wall protein fraction (Fig. 6C, lane 3), like 
Iff5-Iff10C was detected only in the SDS- and 
βME-extracted protein fraction (lane 1). When a similar construction 
was performed with the last 67 amino acids of Iff2/Hyr3, the 
Iff5-Iff2C protein was detected in the β-1,6-glucanase-solubilized 
protein fraction (Fig. 7A, last panel, lane 3). In order 
to confirm that the chimeric Iff5-Iff10C protein was anchored 
in the plasma membrane, the low-speed supernatant was further 
centrifuged and membrane proteins were SDS solubilized. 
As shown in Fig. 7B, a significant amount of the hybrid protein 
was detected in this new fraction (compare lanes 2 and 3). 
These results thus indicate that the 60 last amino acids of 
Iff1/Rbr3, Iff2/Hyr3, Iff4, and Iff7/Hyr4 act as cell wall localization 
signals, while the Iff10/Flo9 65 C-terminal amino acid 
residues are sufficient to ensure plasma membrane anchoring 
of the hybrid Iff5-Iff10C protein. Moreover, this experiment 
confirms again that the Iff2/Hyr3 C-terminal sequence targets 
Iff2/Hyr3 to the cell wall.

**DISCUSSION**

The *Candida albicans IFF* family comprises 12 different 
genes encoding proteins which all display a highly conserved 
360-amino-acid N-terminal domain. Among these 12 members, 
11 are predicted GPI-anchored proteins, thus forming the 
largest family of the dominant class of fungal cell wall proteins 
(40). A search for similar domains in other organisms produced 
significant hits only in fungi from the CTG clade. Further 
analysis of the protein sequences showed enrichment for 
gene duplications in this particular family and, more specifically, 
in all the pathogenic species of the clade. Numerous repeats and high mutation rates have also been described in 
the majority of these proteins (5). Variation in size at least was 
confirmed in *C. albicans* for *IFF1*, but our knowledge on the 
function of the family hampered any functional analysis in 
relation to these size changes. These observations suggest 
that the Iff2/Hyr3 Ser/Thr-rich region prevents the formation of 
PDI moiety/β-1,6-glucan association and contributes to both disulfide bridges 
and β-1,3-glucan covalent bonds.

Expression and localization of chimeric *IFF5-IFF1C, -IFF4C, -IFF7C, -IFF10C, and -IFF2C* proteins. Since Iff1/Rbr3, Iff4, Iff7/ 
Hyr4, and Iff10/Flo9 were undetectable using the previous approach with the different fractionation methods, we decided to 
construct chimeric proteins using the recombinant V5-fused 
Iff5 protein. The last 64 amino acids of Iff5 were replaced by 
the last 63, 68, 66, and 65 amino acid residues of Iff1/Rbr3, Iff4, 
Iff7/Hyr4, and Iff10/Flo9, respectively. Indeed, previous studies 
based on chimeric proteins showed that the last 47 amino acids 
of the cell wall-localized Hwp1 were sufficient to localize GFP to the cell wall, while the last 66 amino acids of the plasma 
membrane-anchored Ecm33 targeted GFP to the plasma membrane (47). Western blot analysis of SDS- and βME-
extracted proteins and β-1,6-glucanase-solubilized proteins 
from cell walls showed that Iff5-Iff1C and Iff5-Iff4C chimeras 
were exclusively covalently bound to the β-1,6-glucans in the 
cell wall (Fig. 7A, lanes 3) as with Iff5, Iff5-Iff7C was also mainly 
found in this fraction, although a weak signal was present 
in the noncovalently linked fraction (compare lanes 1 and 
3) and Iff5-Iff10C was detected only in the SDS- and 
βME-extracted protein fraction (lane 1). When a similar construction 
was performed with the last 67 amino acids of Iff2/Hyr3, the 
Iff5-Iff2C protein was detected in the β-1,6-glucanase-solubilized 
protein fraction (Fig. 7A, last panel, lane 3). In order 
to confirm that the chimeric Iff5-Iff10C protein was anchored 
in the plasma membrane, the low-speed supernatant was further 
centrifuged and membrane proteins were SDS solubilized. 
As shown in Fig. 7B, a significant amount of the hybrid protein 
was detected in this new fraction (compare lanes 2 and 3). 
These results thus indicate that the 60 last amino acids of 
Iff1/Rbr3, Iff2/Hyr3, Iff4, and Iff7/Hyr4 act as cell wall localization 
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residues are sufficient to ensure plasma membrane anchoring 
of the hybrid Iff5-Iff10C protein. Moreover, this experiment 
confirms again that the Iff2/Hyr3 C-terminal sequence targets 
Iff2/Hyr3 to the cell wall.
reported by Verstrepen and coworkers (65). Comparison with the other fungi also showed that except for C. albicans and C. guilliermondii, where only one member of the family is secreted, in all the other species there are at least as many putative GPI-anchored proteins as putative secreted proteins (5). Although the pressure of selection on the N-terminal domain was strong enough within each species to keep the sequences highly similar, it seems that the functionality does not necessarily need cell wall localization but only surface exposure or secretion.

With this interrogation on the specific localization of each member of the IFF family and considering the limited data we had so far, we undertook a global study of their localization. For this purpose, a vector was constructed in which (i) the expression of the IFF coding sequences was controlled by the ACT1 promoter, (ii) the entry in the secretory pathway was governed by the Pga59 peptide signal sequence (21), and (iii) the V5 epitope was cloned just downstream from the predicted signal peptidase cleavage site and upstream from the first predicted amino acid of the mature form of each Iff. Using this strategy, five tagged proteins were detected: Iff2/Hyr3, Iff3 (or -9), Iff5, Iff6, and Iff8, with a clear distribution to a particular fraction, and very little to no contamination of any of the other fractions (supernatant or intracellular) was observed. These data indicate that the constitutive expression did not lead to saturation of the secretory pathway and to precursor accumulation in intermediate compartments and that the observed localizations must reflect the true localizations in a wild-type background. Mao and coworkers also used in their GPI-anchored protein localization studies a strong TEF2 promoter and did not observe any discrepancies between the labeling obtained with these constructions and the pattern observed with the HWPI promoter under hyphal growth induction (47).

The length of the Ser/Thr-rich domain greatly participates in the surface exposure. Immunofluorescence microscopy performed on intact cells indicated that Iff2/Hyr3, Iff3 (or -9), Iff5, and Iff6 are surface exposed, while Iff8 is embedded in the glucan polymers. The predicted lengths of these proteins suggested that the size had a direct impact on the surface presentation, as has already been reported for Eap1 in C. albicans (42) and Epa1 in C. glabrata (26), with the large central domain acting as a shaft, exposing the N terminus above the different layers of the cell wall. Indeed, Iff8 has the smallest Ser/Thr-rich domain, 340 amino acids, compared to the 580 amino acids of Iff2/Hyr3; therefore, we concluded that the minimal size of this region needed to allow protrusion of the N-terminal domain at the cell surface was much longer than in C. glabrata. Indeed, the question of the minimal size of this rod was already addressed by Frieman and coworkers with Epa1 in C. glabrata (26); they showed that with a size of fewer than 100 amino acids the protein was nonfunctional and that 170 amino acids was the optimal size for surface exposure. The differences between our results might simply be protein dependent or organism dependent, since C. glabrata and C. albicans do not exhibit the same cell wall composition, which can influence the cell wall thickness (16). Hence, since these five Iffs are cell wall localized (see below), surface exposure appears not to be related to the cell wall distribution but rather tightly correlated to the length of the highly O-glycosylated Ser/Thr-rich region.

Chimeric constructions with Iff proteins complete the localization of all members of the family. Using the first strategy, four remaining Iffs, Iff1/Rbr3, Iff4, Iff7/Hyr4, and Iff10/Flo9, were not detectable either by Western blot analysis or by immunofluorescence. To overcome this absence of data we decided to decipher the signal harbored by the site-proximal amino acids by using the last 60 amino acids of each of the four
proteins, through the construction of chimeric proteins by replacement of the last 64 amino acids of Iff5. The Iff10/Flo9 C-terminal signal was shown to be able to localize the hybrid protein to the plasma membrane, while Iff1/Rbr3 C-term, Iff4 C-term, and Iff7/Hyr4 C-term as well as Iff2/Hyr3 C-term led to cell wall distribution of the Iff5-fused proteins. There is indeed strong experimental evidence showing the importance of the amino acids just upstream of the site of GPI-anchored proteins for their final localization (47). By a mutational approach, Frieman and Cormack showed that the presence of two basic amino acid residues at position -1 or -2 act as a plasma membrane retention signal in S. cerevisiae (24), while Hamada and coworkers pointed out the role of an isoleucine, a valine, or a leucine at position -3 to -5 for cell wall targeting (28). Mao and coworkers confirmed these rules in C. albicans, as they reported that KK or FE residues at positions -1 and -2 targeted, respectively, a reporter protein to the plasma membrane or to the cell wall, while a loss of I at position -5 increased plasma membrane retention (47). Considering the site-proximal amino acids predicted in Fig. 2B, our observations were expected; indeed, Iff1/Rbr3, Iff2/Hyr3, Iff3 (or -9), Iff4, Iff5, Iff6, and Iff8 C termini lack a lysine at positions -1 and -2 and display an isoleucine or a leucine at -4 or -5. In contrast, Iff10/Flo9 contains a lysine at position -1. According to its ambiguous C terminus (Fig. 2B), Iff7/Hyr4 localized the Iff5 chimera mainly in the β-1,6-glucanase-extracted cell wall fraction but also in the SDS-solubilized one. These results thus confirmed previous data showing that the residues surrounding the site determine the distribution of GPI-anchored proteins either to the cell wall or to the plasma membrane.

Iff2/Hyr3 is tethered to the cell wall components both through disulfide and alkali-labile bonds. Epitope tagging of Iff2/Hyr3, Iff3 (or -9), Iff5, Iff6, and Iff8 showed cell surface exposure of all but Iff8. Among these five Iffs, Iff2/Hyr3 was the only protein that was partially released from the cell wall fraction by treatment with reducing agent. Thus, unlike Iff3 (or -9), Iff5, Iff6, and Iff8, which were shown to be covalently linked to the β-1,6-glucan in the cell wall, likely through a GPI moiety like Hry1 (45), Iff2/Hyr3 was retained in the cell wall at least through disulfide linkages (Fig. 8). To date, Iff2/Hyr3 is the first characterized GPI-modified protein that is connected to the cell wall in part through disulfide bonds. Indeed, proteins previously described as reducing agent extractable do not display all the characteristics of GPI-modified cell wall proteins, like Scw1, described by Castillo and coworkers (9), or Pir 4, identified by Moukadiri et al. (51). Interestingly, on a more broad level of analysis, this study gave us a better insight in a poorly studied fraction of the cell wall: the fraction extracted by treatment of the cell wall with SDS, EDTA, and β-mercaptoethanol (SEB) at 100°C. This step of washing was designed to remove all the noncovalently linked cell wall proteins. In many studies this fraction is then discarded with no further analysis. Although plasma membrane protein extraction needs a specific step of purification, it was a general assumption in the community studying the GPI-anchored proteins that the subgroup of GPI-anchored proteins that were membrane bound, like Gas1, were in a large proportion trapped in this fraction, because many of these proteins also interacted with the cell wall components (acting as cell wall remodeling or biosynthesis enzymes). Consequently, the understanding was that such strong treatment of the cell wall would remove all noncovalently linked proteins: among them the well-known Bgl2 or Pra1 but also the membrane bound GPI-anchored proteins (11). To our knowledge only one study tried to define the proteins present in this fraction in C. albicans, but no cell wall proteins were identified, mostly because two-dimensional gel analysis is inadequate for glycoprotein analysis (56), and even for S. cerevisiae, there are very few data available on the Scw protein family, a group of proteins extracted with similar methods (52). Here the analysis of Dcw1

![FIG. 8. Global representation of the predicted distribution of the Iff proteins (freely adapted from reference 17 with permission of the publisher).](image)
and Iff10/Flo9 localization confirmed the general belief that the two proteins are extracted in high quantity by this SEB treatment at 100°C. In addition we showed that Dcw1 as well as Iff10/Flo9 are also detected in the membrane fraction prepared with the specific fractionation method (Fig. 3C and 7B). Hence, we confirmed that the membrane-bound GPI-anchored proteins are extracted from the cell wall by SEB treatment.

A second cell wall protein retained through disulfide bridges is Pir4 of S. cerevisiae (51). Members of the Pir family are highly O-glycosylated, usually Kex2 substrates, and contain one or more internal repeated sequences (63) as well as several cysteine residues, but no GPI anchor. Characterization of the cell wall linkage of Pir proteins indicated that they are mostly linked through alkali-sensitive bonds to the β-1,3-glucan (10, 35, 52). Surprisingly, when we treated the Iff2/Hyr3 cell wall fraction with NaOH, we were able to release a large amount of the protein (Fig. 3D). Thus, Iff2/Hyr3, although belonging to the GPI-anchored cell wall protein class, behaves like Pir4 in S. cerevisiae with a fraction of the protein retained in the cell wall through disulfide bridges and a second fraction directly connected to β-1,3-glucan through an alkali-labile linkage. Deletions as well as hybrid protein construction brought evidence that the unique internal repetitive sequence (DVIS QIGDGOVQATSAAA) of Pir4 is responsible for its alkali-sensitive linkage to the β-1,3-glucan (10, 61). A more recent study described this covalent linkage as an ester linkage between the γ-carboxyl groups of glutamic acids derived from the glutamine residues contained in this motif and the hydroxyl groups of glucoses of the β-1,3-glucan polymer (18). Iff2/Hyr3 displays no homology with the C. albicans Pir1 (48), but its sequence contains many glutamine and cysteine residues that might be involved in the two different linkages that connect it to the cell wall.

The Ser/Thr-rich domain of Iff2/Hyr3 alone is responsible for this double linkage to the cell wall. We brought the evidence that this unexpected double cell wall linkage through both disulfide bridges and alkali-sensitive bonds depend on the Iff2/Hyr3 Ser/Thr-rich region through the study of modified Iff2/Hyr3 and Iff8 proteins. First, a shortened Iff2/Hyr3 protein after deletion of this Ser/Thr-rich domain was able to make covalent linkages to the β-1,6-glucan, likely through its GPI moiety; second, an extended Iff8 protein created by the addition of the same domain from Iff2/Hyr3 was connected to the cell wall through disulfide bonds and NaOH-labile linkages (Fig. 8). The putatively highly O-glycosylated Iff2/Hyr3 Ser/Thr-rich region was thus shown to not only favor the formation of both covalent linkages with the β-1,3-glucan polymer and disulfide bridges but also prevent the formation of a covalent linkage between the remnant of the GPI anchor and the β-1,6-glucan. Such an involvement of a Ser/Thr-rich region has not yet been reported. Moreover, the Iff3, Iff5, and Iff6 Ser/Thr-rich regions do not have the same influence. Again, on a broader point of view considering all the GPI-anchored proteins and the mechanisms of localization, the particular example of Iff2/Hyr3 brings several questions. What is the effect of this Ser/Thr-rich region in the link with the β-1,6-glucan? One simple explanation would be that the size of the highly glycosylated domain hampers the creation of the covalent link by, for instance, perturbing the access to the target molecule in the protein. Are there other GPI-anchored proteins behaving similarly? One can consider that many other cell wall GPI-anchored proteins also have disulfide bonds with other proteins, but the SEB treatments before the digestion hides their existence. We can hypothesize that a part of GPI-anchored proteins cleaved from the plasma membrane is retained in the cell wall network by such means. Specific analysis of such a fraction of the cell wall is the logical next step to address this question. Are there specific partner proteins? Several putative GPI-anchored proteins that display tandem repeats containing cysteine residues have been reported (Cx, Cx18-22 YTTYCPL [50]); these proteins might be good candidates for such a disulfide bond and thus deserve specific attention in future studies. Whether this interaction is only important for localization or has some functional importance has not yet been determined, but this question would need the identification of at least 2 partners.

Conclusion. In light of these results Iff2/Hyr3 localization, it is more difficult to extrapolate on the exact linkage of Iff1/Rbr3, Iff4, and Iff7/Hyr4 with the cell wall based on the data obtained using chimeric proteins with only 60 amino acids of their C-terminal domain. One can now argue that the entire protein might be anchored to the cell wall differently than expected and as observed for the fusions involving their C terminus (as for Iff2/Hyr3). Nonetheless, this study clearly demonstrated that Iff proteins are true GPI-anchored proteins that distribute either in the cell wall (most of them) or in the plasma membrane, as expected for Iff10/Flo9. Independently of this localization, we think that the vast majority (except for Iff8) protrude at the cell surface, since their Ser/Thr-rich regions are long enough to allow them to cross the cell wall layers. Altogether, these data suggest that even if much effort has been focused on understanding the different signals orientating the GPI-anchored proteins either to the membrane or to the cell wall, the cell has found ways to bring any domain to the very surface of the cell. Consequently, it seems crucial in all studies of GPI-anchored proteins to experimentally report the exposure of the target protein rather than predict it through sequence analysis, which can be misleading. Finally, it is very likely that as with Ecm33 (62), correct localization influences the protein functionality, which in the case of Iff proteins would be the surface exposure of the N-terminal domain. Cell surface exposure is compatible with a function of members of the Iff family in host cell interactions and/or a role in adherence, the initial step of colonization. Preliminary results suggest variations in the number of repeats in Iff1/Rbr3 within the different clades, which is another way to introduce variation not only to the length of the protein but also to its structure, thus increasing the adaptability of C. albicans. All this might reflect an adaptation of the C. albicans cell surface to the environment, a subject that needs to be pursued in future studies.

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