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ORIGINAL PAPER

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Three-dimensional structure of the catalytic domain of the yeast β-(1,3)-glucan transferase Gas1: a molecular modeling investigation

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Abstract The three-dimensional (3D) structure of the catalytic domain of Gas1p, a protein belonging to the only family of β-(1,3)-glucan transferases so far identified in yeasts and some pathogenic fungi (family GH-72), has been predicted by combining results derived from threading methods, multiple sequence alignments and secondary-structure predictions. The 3D model has allowed the identification of several residues that are predicted to play a crucial role in structural integrity, substrate recognition and catalysis. In particular, the model of the catalytic domain can be useful for designing site-directed mutagenesis experiments and for developing inhibitors of Gas1p enzymatic activity.

Keywords Protein structure prediction · Computational methods · TIM barrel · Glycosidase

Introduction

Glycolipid anchored surface protein (Gas1p) of *Saccharomyces cerevisiae* is an exocellular glycoprotein endowed with β-(1,3)-glucan transferase activity [1, 2]. This enzyme catalyzes the splitting of an internal β-(1,3)-glycosidic linkage in a donor glucan followed by the transfer of the new reducing end to the nonreducing end of an acceptor glucan, with the formation of another β-(1,3)-glycosidic bond in which the anomeric configuration of the linkage is conserved (retaining enzyme). The Gas1p plays a crucial role in the correct incorporation of glucan molecules in the cell wall [1, 2], which, in fungal pathogens, is involved in interactions with host cells. This feature and the absence of analogous activities in mammalian cells make this enzyme an interesting molecular target for developing new antifungal drugs [3].

The identification of Gas1p homologues in yeast species, fungi and also in several human fungal pathogens has led to the definition of a new family of glycosyl hydrolases (family GH-72) (http://afmb.cnrs~mrs.fr/~cazy/CAZY/index.html). Sequence analysis of proteins belonging to family GH-72 revealed a modular organization. In particular, Gas1p has three different domains: an amino terminal catalytic (C) domain of about 300 residues, a cysteine-rich region of about 100 residues (Cys-box), the functional role of which is presently unknown, and a carboxy-terminal serine-rich region of variable length (Ser-box), which is the site for O-mannosylation and is not essential for catalytic activity [2, 4].

Hydrophobic cluster analysis led to the conclusion that the sequence of the C-domain of GH-72 proteins is compatible with a $(\beta/\alpha)_8$ barrel fold, even though it does not share significant sequence similarity to structurally characterized proteins [5]. This observation allowed the GH-72 members to be inserted in the so-called GH-A clan [6], which contains glycoside-hydrolase families characterized by the same global fold of the C-domain.

Other molecular details have been unraveled recently. Site-directed mutagenesis experiments have demonstrated the crucial role of E161 and E262 for the catalytic activity of Gas1p. In addition, it has been shown that C74 is necessary for the correct fold of the protein, whereas C103 and C265 are dispensable [7]. In spite of this evidence, the detailed three-dimensional (3D) structure of the C-domain of Gas1p is still unknown, hindering the full rationalization of experimental data and the design of targeted mutagenesis studies.

Several approaches to predict protein structures from sequences are now available [8–10]. The most reliable computational approach to predict the 3D structure of a protein is homology modeling, which, however, can only be safely used if at least one protein characterized by a significant (>25% sequence identity) similarity to the

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target protein has a known 3D structure [11]. When dealing with remote homologues (< 25% sequence identity), the protein alignment and the subsequent construction of the 3D model are more problematic. In such cases, it has been shown that reliable results may sometimes still be obtained using fold-recognition (threading) methods. Notably, fold-recognition approaches often combine information obtained from many sources [9, 12, 13]. Along these lines, we have combined threading methods, sequence alignments, secondary-structure predictions and biochemical information to predict the 3D structure of the C-domain of Gas1p. The results disclose some key molecular characteristics of the C-domain of Gas1p and identify residues that play an important role in substrate recognition, maintenance of structural integrity and catalysis. Moreover, a map of the putative disulfide bridges of the entire protein is proposed. The 3D model is expected to be a useful tool for designing site-directed mutagenesis experiments and some possible inhibitors for Gas1p enzymatic activity.

Methods

Homologues of Gas1p were searched in the nonredundant database of protein sequences at NCBI, using both Blast and PSI-Blast [14, 15]. Multiple sequence alignments, as well as phylograms, were generated with Clustal W [16], using the Blosum scoring matrix. The gap insertion and extension penalties were set to 10 and 0.05, respectively. In order to highlight conserved regions, the alignment from Clustal W was submitted to ESPript [17].

Secondary structure was predicted by means of JPRED [18] and PSI-PRED [19]. Consensus secondary structures were obtained from comparison among PSI-PRED and individual JPRED results using a 75% stringency threshold.

Six threading methods (sequence-structure fitness) were used to detect remote similarities with proteins of known 3D structure: 3D-PSSM [20], mGen-THREADER [21], 123D+ [22], Fugue [23], Topits [24], SAM-T02 [25] and FFAS03 [26]. Only matches characterized by a high confidence level were used as templates to predict the structure of the C-domain of Gas1p. In particular, only proteins characterized by a confidence level higher than 70% or classified as CERTAIN/HIGH were taken from 3D-PSSM/TOPITS and MGen-THREADER, respectively. When the 123D+ and Fugue servers were used, only matches with a *z*-score equal or higher than 4 and 3.5, respectively, were considered, as suggested by Alexandrov et al. and Shi et al. [22, 23]. Accordingly, only FFAS03 matches characterized by a score lower than −9.5 were selected, as indicated by Rychlewsky et al. [26]. The SAM-T02 already shows only templates characterized by high reliability.

The 3D models were built using the Jackal protein-modeling software package (http://trantor.bioc.columbia.edu/~xiang/jackal). In particular, the alignments between targets and templates were submitted to the subprogram Nest, which generates a 3D model on the basis of a given alignment, carries out geometry optimization in torsional space to remove clashes between atoms, and finally optimizes the loop regions that are characterized by the presence of gaps in the alignment. In particular, the prediction of loop regions was carried out as followed by Honig and coworkers [27].

As the final step, the models were submitted to molecular-mechanics optimization using the CVFF forcefield [28]. In particular, only the geometry of the protein side-chains was optimized initially (1,000 steps using the steepest-descent algorithm followed by 10,000 steps using the conjugate-gradient algorithm). Then, the optimization was restarted restraining only the α-carbons of the peptide chain. The quality of the final models was evaluated using the Whatif suite of programs [29].

Analysis of the models was carried out using Insight II tools (Accelrys, San Diego, CA, USA) and VMD [30].

Fingerprints for family GH-72 were derived according to the following procedure: (1) amino-acid sequences from the family GH-72 were submitted to PRATT [31], to generate possible patterns common to all probe sequences; (2) if necessary, the PRATT patterns containing functionally important residues were refined manually, using as reference the multi-sequence alignment of the family members; (3) to verify that the patterns selected identify only members of family GH-72, they were submitted to PHI-Blast, a tool developed to evaluate the significance of a specific pattern within a protein [32].

Results and discussions

With the aim of retrieving proteins sharing significant sequence similarity to Gas1p, its protein sequence was submitted to Blast, searching the nonredundant database at NCBI (http://www.ncbi.nlm.nih.gov/). The scan of the database resulted in 40 proteins characterized by high-sequence similarity (*E*-value lower than e^{-27}) to Gas1p. This protein set can be considered an "up to date" representation of family GH-72 [33]. However, it should be noted that among the 40 proteins, 11 correspond to fragments or hypothetical proteins and consequently were not analyzed further.

In order to disclose high-similarity regions, the sequence portions spanning the C-domains were aligned as described in Methods (Fig. 1). The alignment, which is consistent with previously reported data [7], allowed residues that are strictly conserved in all GH-72 members to be highlighted. In particular, 30 residues out of 314 (9.5%) are identical in all C-domains. Among the strictly conserved residues there are seven glycine residues (G159, G197, G243, G264, G290, G291, G304 and Gas1p numbering), six tyrosine residues (Y92, Y113, Y198, Y231, Y294 and Y303), two arginine residues

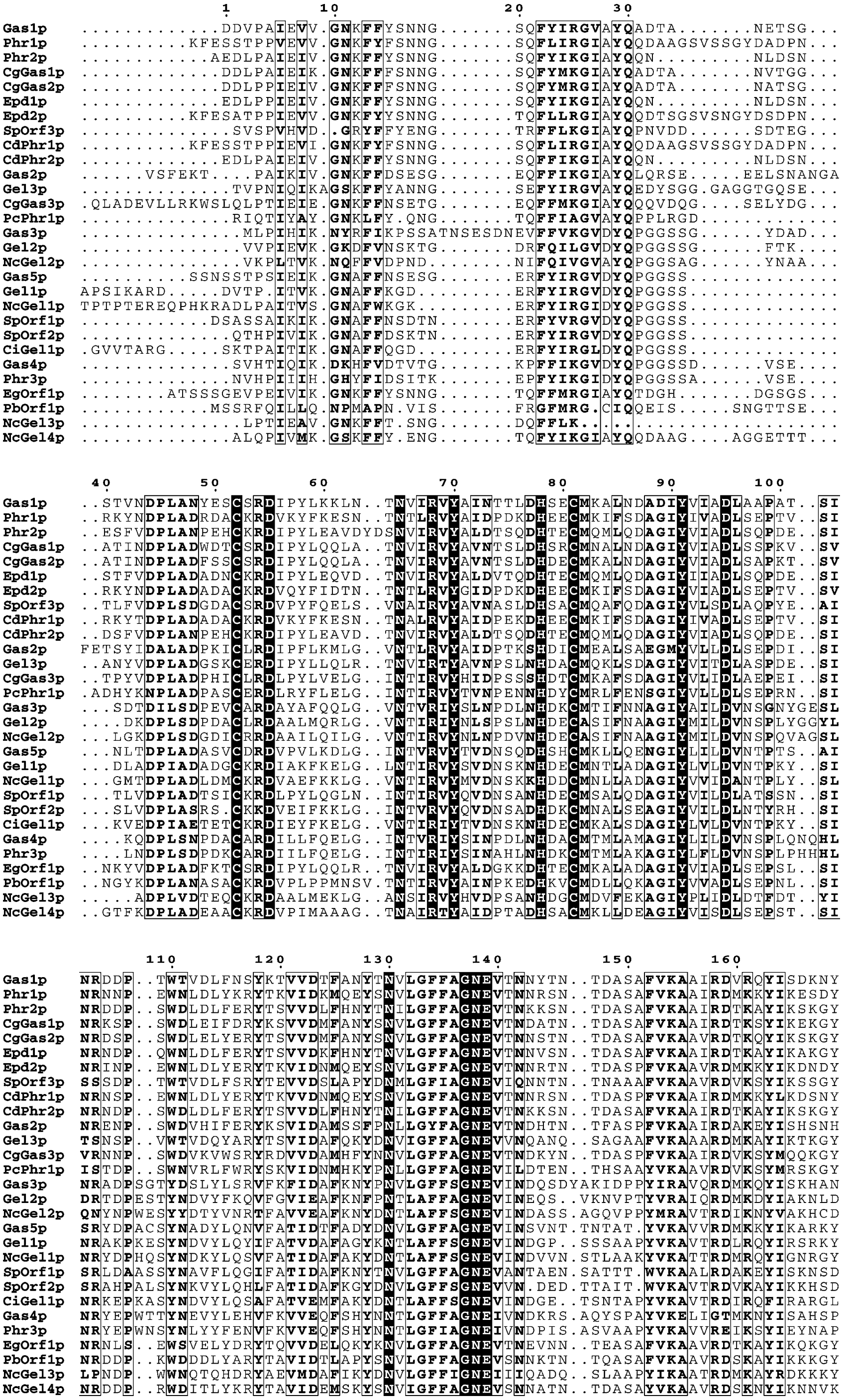
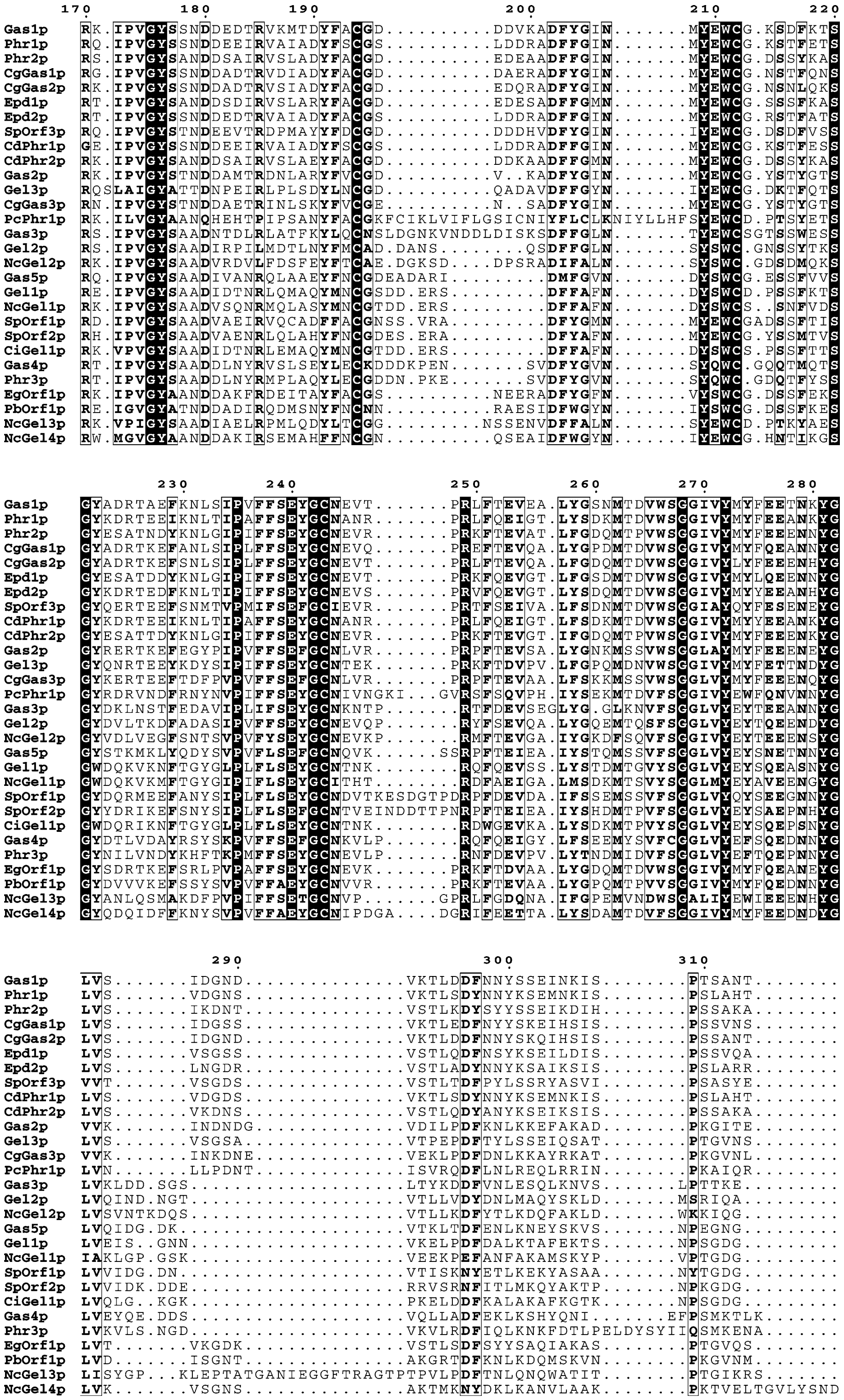


Fig. 1 Multiple sequence alignment of the portions spanning the C-domain of proteins belonging to family GH-72 of the GH-A clan.The sequences of the C-domain of Gas1p (D23-T336) and of other members of Family GH-72 are presented. The identical residues (in the *black boxes*), similar residues (*bold*) and regions with consecutive similar residues (*white boxes*) are indicated. In order to improve the alignment, the N-terminal signal peptides were not included. Therefore, the numbering of the sequences starts from the putative or experimentally determined amino acid of the mature proteins. Thus, the D22 is D1 and E161 and E262 of Gas1p correspond to E139 and E240 in this figure. (Sequences from *Saccharomyces cerevisiae* are: Gas1p (SwissProt code P22146), Gas2p (Q06135), Gas3p (Q03655), Gas4p (Q08271), Gas5p (Q08193); from *C. albicans*: Phr1p (P43076), Phr2p (O13318), Phr3p (Q9P8R2); from *A. fumigatus*: Gel1p (O74687), Gel2p (Q9P8U4), Gel3p (Q9P8U3); from *C. glabrata*: CgGas1p (Q8X0Z7), CgGas2p (Q8X0Z6), CgGas3p (Q8X0Z5); from *P. carinii*: PcPhr1p (Q9UVL7); from *C. maltosa*: Epd1p (P56092), Epd2p (O74137); from *S. pombe:* SpORF1p (O13692), SpORF2p (Q9Y7Y7), SpORF3p (Q9P378); from *C. dubliniensis*: CdPhr1p (Q9HG19), CdPhr2p (Q9HG18); from *N. crassa*: NcGel1p (Q8X0X4), NcGel2p (Q8X094), NcGel3p (Q873D1), NcGel4p (Q872H7); from *C. immitis*: CiGel1p (Q8X1E8); from *P. brasiliensis*: PbOrf1p (Q7Z8M3), from *E. gossypii*: EgOrf1p (GenPep code AAS51046.1)). The alignment is truncated since the C-terminal region of the C-domain (327–381) of NcGel4p presents an extra tail, which is not aligned to the other sequences

Fig. 1 (contd.)



(R90 and R271) and five cysteine residues (C74, C103, C216, C234 and C265), which might be involved in intra- or inter-domain disulfide bridges. Moreover, there are three residues (G48, Y51 and Q52) that are only missing in the NcGel3p sequence (Fig. 1).

Analysis of the alignment among the C-domains revealed that proteins from different species, but characterized by the same modular architecture, are more closely related evolutionarily than proteins belonging to the same organism but featuring a different modular organization (Figs. 2 and 3), suggesting that the appearance of different modular organizations preceded speciation. Interestingly, members of the family characterized by the presence of the Cys-box domain have

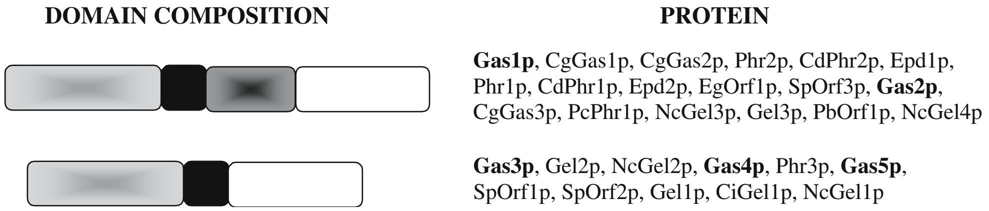

DOMAIN COMPOSITION
PROTEIN
Gas1p, CgGas1p, CgGas2p, Phr2p, CdPhr2p, Epd1p, Phr1p, CdPhr1p, Epd2p, EgOrf1p, SpOrf3p, **Gas2p**, CgGas3p, PcPhr1p, NcGel3p, Gel3p, PbOrf1p, NcGel4p
Gas3p, Gel2p, NcGel2p, **Gas4p**, Phr3p, **Gas5p**, SpOrf1p, SpOrf2p, Gel1p, CiGel1p, NcGel1p

Fig. 2 The domain composition of family GH-72 members, obtained by similarity with Gas members from *S. cerevisiae*. The C-domain, the pro-rich motif, the Cys-box, the Ser-box (or an *aspecific box*) are indicated in *light gray*,*black*, *dark gray* and *white*, respectively. The members of the family from *S. cerevisiae* are indicated in *bold* [48]

the LP [T, I] PP pro-rich motif, the only exceptions being Gas2p (LPETP) and NcGel3p (TPTPV) (not shown). In contrast, members in which the Cys-box is missing show more variability in the pro-rich motif, usually conserving only the LPXXP motif (which is missing in Gas4p and Phr3p).

The alignment also allowed us to define two fingerprints, which include the two catalytic residues E161 and E262, and are strictly specific for the family GH-72 of glycoside hydrolases:

– N-x(1,3)-[L, I]-[G, A]-[F, Y]-x-G-N-E-[I, V]
– P-x-[F, I]-x-[S,A]-E-[Y, F, T]-G-C

In fact, the two patterns correctly identified all proteins belonging to family GH-72 in the nonredundant database of NCBI, with no hits corresponding to false positive protein sequences (see Methods).

As expected, the Blast search did not reveal any homologous proteins with known 3D structure and this ruled out the possibility of using standard homology-modeling approaches. With the aim of finding possible remote homologues to Gas1p, its sequence was also submitted to PSI-Blast, obtaining statistically significant similarity to the LacZ domain of *β*-galactosidase (belonging to family GH-2 of the GH-A clan), in agreement with the previous observations made for other family GH-72 members [34], and also to the cellulase domain of glycosyl hydrolase (belonging to family GH-5 of the GH-A clan). To search for other possible remote homologues of Gas1p, we submitted the amino-acid sequences of the C-domain of Gas1p to six threading servers that use different methods to find suitable templates and generate the corresponding alignments: 3D-PSSM, mGen-THREADER, TOPITS, 123D+, Fugue, SAM-T02 and FFAS03. It should be noted that all hits found by TOPITS were characterized by low *z*-scores and therefore not analyzed further (not shown). Protein scaffolds retrieved by the majority of servers came from three families (GH-2, GH-5 and GH-17) belonging to the GH-A clan (Table 1), confirming and extending the results obtained by PSI-Blast, and strongly suggesting remote evolutionary relationship (and common fold) with family GH-72 members. Consequently, these proteins could be used as scaffolds to

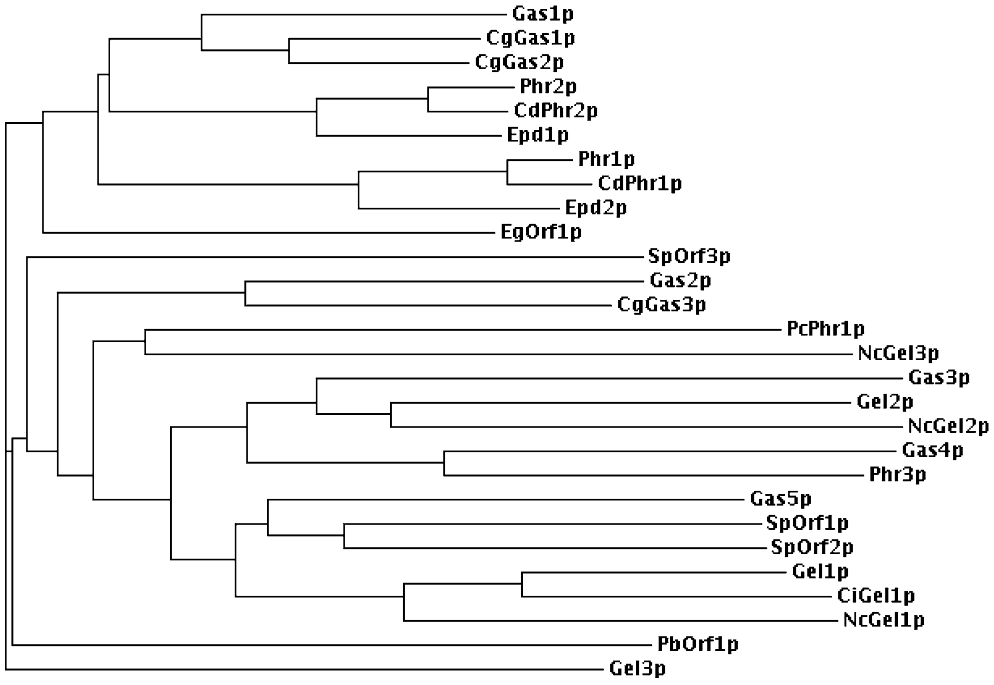


Fig. 3 The phylogram tree obtained by Clustal W. The labels for the sequences are the same of Fig. 1

Table 1 Template structures obtained by the threading servers using as probe the amino-acid sequence of the C-domain of Gas1p

Classification	Template (PDB code)				
	3D-PSSM	MG-THREADER	123D+	Fugue	FFAS03
GH-A 5	**7A3H**, **1BQC**, 1ECE, **1QN_**, 1GHS, **1EGZ**, 1EDG, 1G0C, 1GZJ	**7A3H**, **1BQC**, 1ECE, 1EDG, 1GZJ	**7A3H**, **1BQC**, 1ECE, **1QN_**, **1EGZ**, 1EDG	7A3H, 1BQC, 1ECE, 1QN_, 1EGZ, 1GZJ, 1CZ1	**7A3H**, **1BQC**, **1QN_**, **1EGZ**, 1EDG, 1G0C, 1GZJ, 1CZ1, 1LF1, 1CEC, 1H4P, 1NOF
GH-A 2	1BHG, BGAL	1BHG, BGAL	1BHG, BGAL	1BHG, BGAL	1BHG, BGAL
GH-A 17	n.f.	1AQ0	1AQ0	n.f.	1AQ0,1GHS
GH-A 42	n.f.	n.f.	n.f.	1KWG	1KWG
GH-A 10	n.f	n.f.	n.f.	1CLX, 1XYZ, 1BG4, 1TAX	1CLX, 1XYZ, 1BG4, 1TAX. 1E0X, 1US2, 1N82, 1NQ6,1ISY, 1HIZ, 1UQY
GH-A 26	n.f.	n.f.	n.f.	1J9Y	n.f.
GH-A 39	n.f.	n.f.	n.f.	n.f.	1PX8
GH-A 53	n.f.	n.f.	n.f.	n.f.	1HJQ, 1HJS, 1FHL
GH-A 1	n.f.	n.f.	n.f.	n.f.	1NP2, 1BGG, 10D0, 1QOX, 1PBG, 1E1E, 1GNX, 1CBG, 1MYR
GH-A 51	n.f.	n.f.	n.f.	n.f.	1PZ2
GH-A 30	n.f.	n.f.	n.f.	n.f.	1OGS
$(\beta/\alpha)_8$	n.f.	n.f.	n.f.	1K6W	1UG6, 1QVB
OTHER	n.f.	n.f.	1GCA	n.f.	n.f.

Note that for some proteins more than one structure is deposited in the Protein data bank. In particular, 7A3H stands for 7A3H, 1A3H and 1E5J; 1QN_ stands for 1QNR, 1QNO and 1QNS; BGAL stands for 1F49, 1F4A, 1DPO, 1JZ8, 1JZ7 and 1BGL. In *bold* the templates for which structural alignment were considered for the 3D-model generation are indicated. n.f. stands for not found

predict the 3D structure of the C-domain of Gas1p. However, it should be noted that the very low sequence similarity between Gas1p and these proteins (less than 15% identity) is expected to make room for errors due to local misalignment, which eventually can affect the quality of the 3D model. In particular, it is well known that alignments to the same scaffold produced by different threading methods can be affected by local errors, making the derivation of a good structural model a nontrivial task [35]. In fact, even though many threading servers converge on the same scaffolds (Table 1), corresponding alignments can be quite different (see below).

With the aim of selecting the most reliable sequence-structure alignments, we started from the observation that some amino acids, such as N160, E161 and E262 (Gas1p numbering) are strictly conserved in the GH-A clan [6] and therefore were expected to be aligned with the corresponding residues of the templates. In fact, the glutamic acid residue corresponding to E240 in Gas1p was always misaligned by Fugue and SAM-T02 (see Supporting information). It should also be noted that the corresponding alignments obtained with Clustal W [16] or T-Coffee [36] were affected by similar problems, even when using different scoring matrices and gap penalties (not shown), confirming the non-applicability of classical homology modeling approaches.

The surviving alignments were pruned further considering results from secondary-structure predictions. Indeed, we are aware that scores from some threading methods (mGen-THREADER, 3D-PSSM and 123D+) already take into account secondary-structure prediction results. However, the prediction of the $(\beta/\alpha)_8$ fold for the C-domain of these proteins is so well grounded [5] that secondary-structure prediction data are expected to be more easily evaluated than other parameters entering the scoring function of the threading methods, such as the solvation potential. In fact, due to the multidomain architecture of Gas1p and congeners, the evaluation of solvation potential might be partially misleading. If some interactions among the different domains take place, hydrophobic residues could be exposed on the surface of the C-domain, thus producing low values of the solvatation potential. Therefore, with the aim of evaluating the different alignments in light of secondary-structure predictions, we submitted the sequences of all C-domains of family GH-72 members to the JPRED [18] and PSI-PRED [19] servers, obtaining a consensus prediction according to the procedure outlined in Methods (Fig. 4). As expected, the general $(\beta/\alpha)_8$ architecture was predicted with high confidence, in agreement with previous proposals [5]. However, some irregularities are predicted to characterize the C-domain of Gas1p and congeners. In particular, $\beta1$ is preceded by two extra β-strands, $\beta5$ is very short, $\alpha7$ might be missing or very short and one extra β-strand is present before $\beta8$. In fact, slightly irregular $(\beta/\alpha)_8$ folds are quite common [37, 38] and also characterize some members of the GH-A clan for which the 3D structure has been solved by X-ray diffraction [39, 40].

In light of these results, alignments where the secondary-structure elements of the templates were badly aligned or largely different from those predicted for the

```
                             β1                             α1
Pred:      EEEE  EEEE     EEEEEEEE                    HHHHHHHHHHHH
GAS1: DDVPAIEVVGNKFFYSNNGSQFYIRGVAYQADTANETSGSTVNDPLANYESCSRDIPYLK

              β2             α2        β3                    α3
Pred: H    EEEEEE       HHHHHHHHH   EEEEE                HHHHHHHHH
GAS1: KLNTNVIRVYAINTTLDHSECMKALNDADIYVIADLAAPATSINRDDPTWTVDLFNSYKT

                  β4                        α4               β5
Pred: HHHHH     EEEEE   EE          HHHHHHHHHHHHHHHHH       EEEE
GAS1: VVDTFANYTNVLGFFAGNEVTNNYTNTDASAFVKAAIRDVRQYISDKNYRKIPVGYSSND

             α5               β6                    α6         β7
Pred:  HHHHHHHHHHHH         EEEE  EEE          HHHHHHHHH     EEEE
GAS1: DEDTRVKMTDYFACGDDDVKADFYGINMYEWCGKSDFKTSGYADRTAEFKNLSIPVFFSE

                  α7                 β8                          α8
Pred:            HHH              EEEEEEEE     EEEEE          HHHH
GAS1: YGCNEVTPRLFTEVEALYGSNMTDVWSGGIVYMYFEETNKYGLVSIDGNDVKTLDDFNNY


Pred: HHHHH
GAS1: SSEINKISPTSANT
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1AQ0 aligned to Gas1p (3D-PSSM)

```
           β1                          α1
        EEEEEEEE                      HHHHH
       20                    40
Gas1p DDVPIEVVGNKFFYSNNGSQFYTIRGVAYQADTAN
1AQ0  -------------------IGVCYGMSANNLPAAS
      120                        140
                          EEE           HH
                          β1            α1
```

1ECE aligned to Gas1p (G. THREADER)

```
              α5                β6
         HHHHHHHHHHHHH         EEEE  E
     180                200
Gas1p NDDEDTRVKMTDYFACGDDDVKADFYGINMY---
1ECE  YWWGGNLQGAGQYPV-VLNVPNRLVYSAHDYATS
             220                   240
                             EEEEEE
                                β6
```

Fig. 4 Secondary-structure prediction for Gas1p. The secondary structure of the regions predicted with high confidence are *highlighted*. The catalytic glutamic residues are underscored. Examples of alignments that have been discarded because secondary-structure elements are badly aligned are shown

C-domain were discarded. Note that in some cases the misalignment was not due to failure of the threading method but simply due to the structural features of the template, which did not fit properly to the predicted secondary structure of the C-domain. As an example, the analysis of the alignment between Gas1p and 1AQ0 reveals that the first β-strand (β1) in the template is too short and that the two extra β-strands preceding β1 are missing (Fig. 4). Similarly, α5 is missing in the templates 1ECE, 1EDG, 1BHG and 1BGL, whereas its presence was predicted with high confidence for Gas1p and congeners (Fig. 4). Moreover, the extra β-strands at the *N*-terminal are missing in 1CZ1 and 1GZJ (data not shown).

According to this analysis, only four templates survived the pruning procedure. Remarkably, they all belong to the GH-5 family even if they share low-sequence similarity. This suggests a closer evolutionary relationship between the GH-72 and GH-5 families. However, it is known that GH-5 family members can be characterized by significantly different sequences, an observation that led to the definition of different subfamilies [41]. In fact, the four templates belong to three different subfamilies. In particular, 1EGZ [42] and 7A3H [43] belong to the subfamily 5-2 and are characterized by endo-1,4-glucanase activity, whereas 1QNS [44] and 1BQC [45], which have been classified in the subfamilies 5-7 and 5-8, respectively, are both characterized by β-mannanase activity.

The alignments among the four template proteins and Gas1p, as obtained by the different threading methods, are extremely similar for the location of the amino acids conserved in the GH-72 family (see Supporting information), indicating that, starting from a specific template, similar 3D models are obtained even considering alignments from different fold-recognition servers. The structures of the C-domain of Gas1p, obtained using the alignments produced by 3D-PSSM, and using the four templates from family GH-5, are shown in Figs. 5, 6 and 7.

Considering the general structural features of the C-domain in the four templates, and consequently in Gas1p model, the $-NH_2$ and $-COOH$ termini of the domain are located at the bottom of the barrel (with

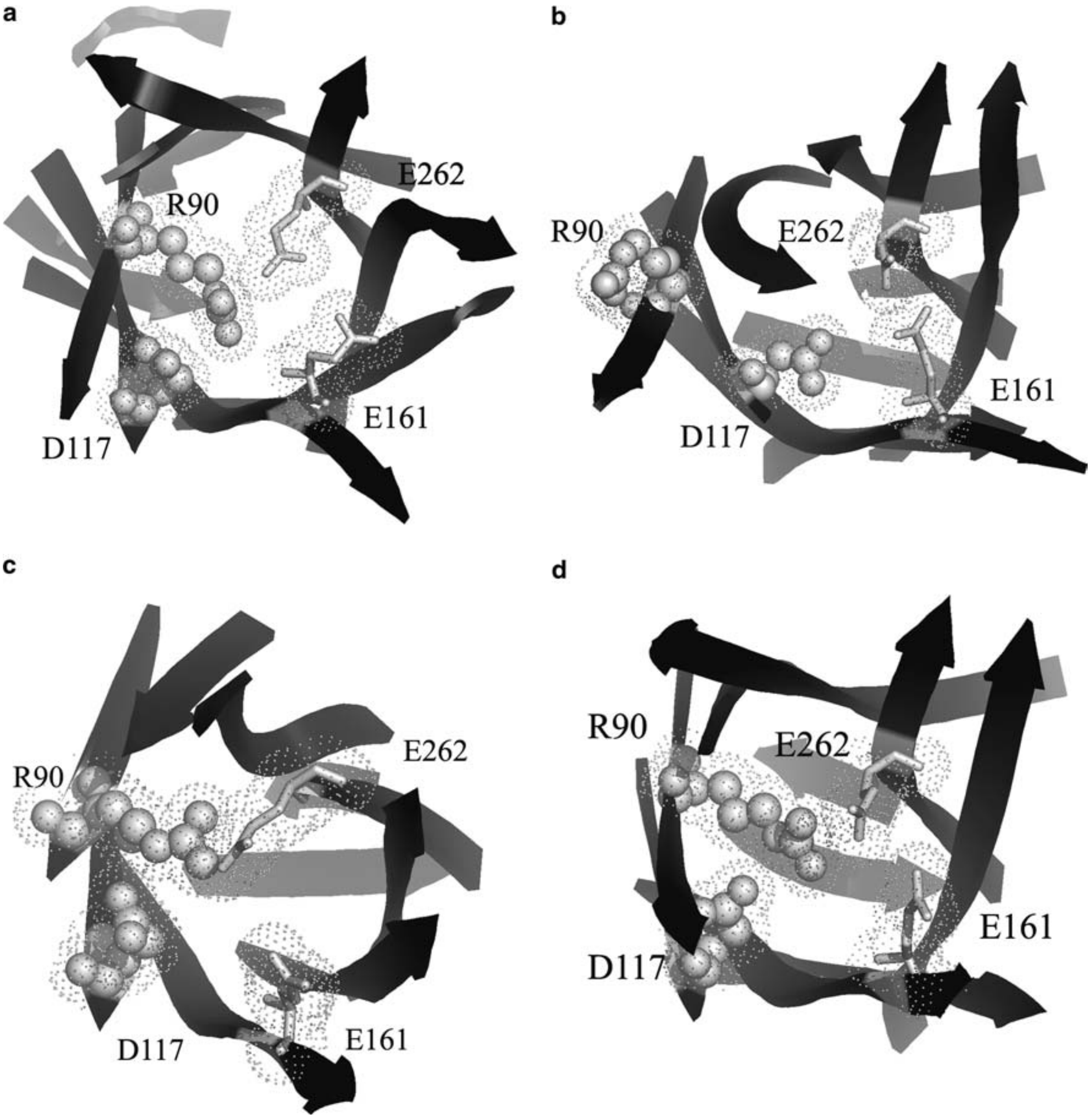

Fig. 5 Three-dimensional models of the Gas1p C-domain as predicted using as templates 1QNS (**a**), 1EGZ (**b**), 1BQC (**c**) and 7A3H (**d**). For the sake of clarity only the β-strands forming the barrel and the side chains of R90, D117, E161 and E262 have been explicitly shown

respect to the catalytic glutamic residues). In particular, the two short N-terminal β-strands preceding $\beta1$ reduce the accessibility to the bottom of the barrel, as also observed in other members of the GH-A clan [39, 42, 44, 45].

As discussed above, there are several residues that are strictly conserved in GH-72 family members. Residues corresponding to R90, G264 and Y231 (Gas1p numbering) are strictly conserved both in families GH-72 and GH-5. In particular, the functional role of the amino acids corresponding to R90 and Y231 has already been investigated in members of family GH-5. In the retaining cellulase Cel5A from *Bacillus agaradhaerens*, which corresponds to the template 7A3H, it has been argued that the hydrogen bond formed between the arginine residue and the catalytic nucleophile E228 is crucial to maintaining the proper orientation and protonation state of the nucleophile in the glycosylation step [42, 43]. The Y231 residue has been shown to be important for substrate recognition and orientation/activation of the nucleophilic glutamic catalytic residue [43, 46]. In both cases, a similar role in Gas1p and the other members of family GH-72 can be predicted confidently on the basis of the 3D models (Figs. 5, 7). The functional relevance in the GH-5 family of the glycine residue corresponding to G264 has never been investigated. Our structural analysis shows that this glycine residue is located near the two catalytic glutamic acid residues, suggesting a role in substrate recognition in both families (Fig. 7). Among the glycine residues conserved only in C-domains belonging to the family GH-72, G243 and G304 are solvent exposed and located at

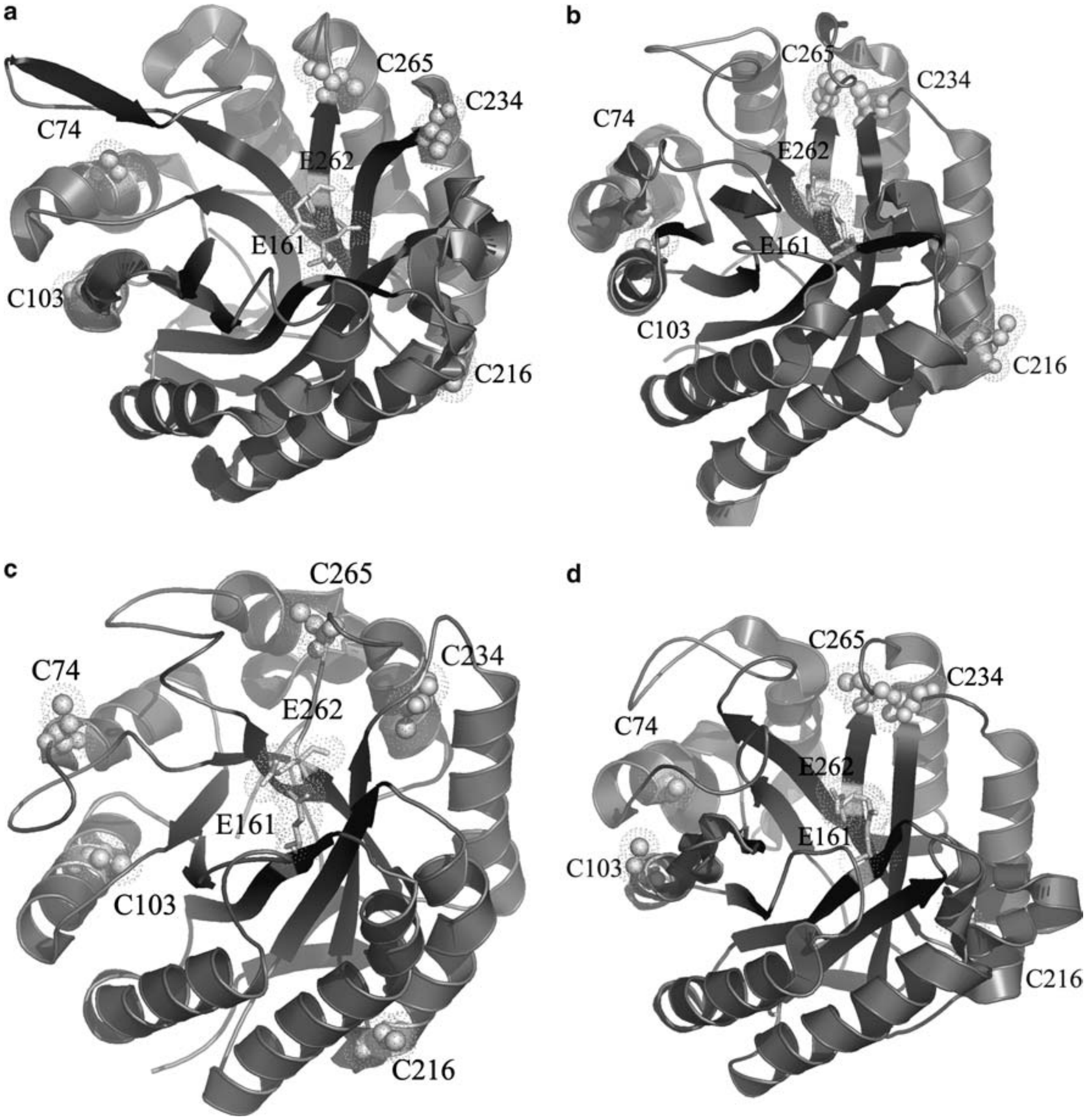

Fig. 6 Three-dimensional models of the Gas1p C-domain as predicted using as templates 1QNS (**a**), 1EGZ (**b**), 1BQC (**c**) and 7A3H (**d**). For the sake of clarity only the side chains of E161 and E262 and cysteine residues have been explicitly shown

the beginning of $\alpha6$ and in the loop following $\beta8$, respectively. All other glycine residues are buried inside the barrel and are predicted to play a structural role.

The residues F44 and Y113, which are strictly conserved in family GH-72 members, are buried in the protein core and form a hydrophobic cluster with F35 or Y37, which are conserved in many members of the family (not shown).

The side chain of R247, which is strongly conserved or substituted by a lysine residue in the family, points toward a negatively charged region formed by the peptide segment DDED (residues 202-205). The first aspartate residue (D202) is conserved in a large number of family GH-72 members and it might form a salt bridge with R247 (not shown).

The D117, which is conserved in GH-72 and GH-5 members (not shown), is placed at the end of $\beta3$ and its side chain can interact with Arg90 (Fig. 5), possibly forming a network of electrostatic interactions also involving the nucleophilic glutamate residue (E262). This is analogous to the corresponding role proposed in the cellulase Cel5A (7A3H) from *B. agaradhaerens* [43].

The analysis of the spatial location of cysteine residues is particularly important for predicting the possible formation of disulfide bridges. The Gas1p contains 14 cysteine residues: five residues are located in the C-domain, eight in the Cys-box and one in the linker region located between the C-domain and the Cys-box. Ten cysteine residues out of 14 are involved in intra-domain

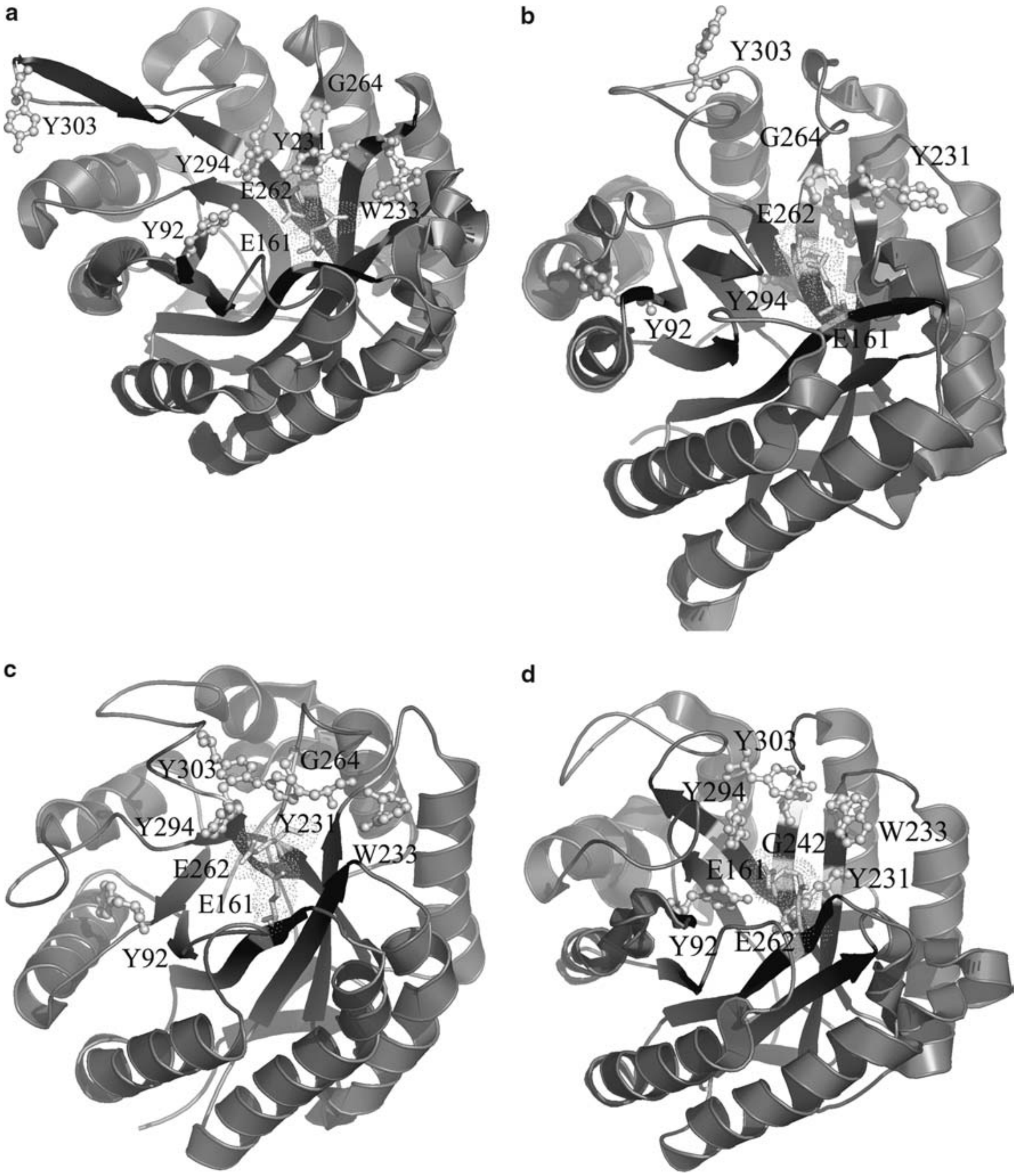

Fig. 7 Three-dimensional models of the Gas1p C-domain as predicted using as templates 1QNS (**a**), 1EGZ (**b**), 1BQC (**c**) and 7A3H (**d**). For the sake of clarity only the side chains of E161 and E262 as well as glycine, tryptophan, tyrosine residues predicted to be involved in substrate recognition have been explicitly shown

disulfide bonds in the native state of the protein [7]. Moreover, on the basis of the high similarity between the Cys-box of Gas1p and the corresponding domain in plants, it has been predicted that three disulfide bridges are formed in this domain [47]. These observations suggest that the other two disulfide bridges could involve cysteine residues in the C-domain (Fig. 6). The analysis of our 3D models of Gas1p suggests that a disulfide bridge can be formed between C234 and C265, which are located in loop regions in the proximity of the catalytic site between β6-α6 and β7-α7, respectively. The C74 and C103 are localized on α1 and α2, respectively, and their distance suggests that the formation of a disulfide bridge is possible only upon some rearrangement of the protein backbone. Finally, C216, which is located in the loop connecting α5 and β6, is far from the other cysteine residues and mainly solvent exposed. Remarkably, a cysteine residue (C348), which is conserved in the GH-72 family, is present in the sequence portion linking the C-domain and the Cys-box. This linker region is predicted to assume a coil conformation according to secondary-structure predictions carried out with the JPRED and PSI-PRED servers. To investigate the possibility that C348 might be involved in a disulfide bridge with a cysteine residue of the C-domain, we linked a peptide spanning the amino-acid sequence of the linker (KSYSATTSDVAC) to the carboxy-terminal end of the C-domain, evaluating, by computer-aided graphical analysis (not shown), the possibility that C348 could interact with C74, C103 and C216. It turned out that C216 is too far from the C-terminal end of the C-domain to allow the formation of a disulfide bridge with C348. On the other hand, C348 can interact with C74 and C103, suggesting possible formation of a disulfide bridge. In light of our modeling results and the experimental observation that C74 is crucial for proper folding and maturation of Gas1p (while mutation of C103 and C265 have only slight effects) it can be inferred that the two disulfide bridges involving residues of the C-domain should be C234–C265 and either C74–C348 or C103–C348. It should also be noted that the phenotype observed following C74 mutation might be due to its involvement in a transient disulfide bridge formed during the folding process [7]. Site-directed mutagenesis of C348 is predicted to be a crucial experiment to distinguish among these possibilities.

The spatial localization of tyrosine and tryptophan residues is particularly relevant because these residues are often involved in substrate recognition in GH-A members [39, 43, 44]. Most of the tyrosine residues conserved in Gas1p and congeners are located in the β-strands forming the barrel (Fig. 7). The Y294 and Y303 are predicted to be localized at the gate of the barrel, implying a role in substrate recognition. Remarkably, Y294 corresponds to a Trp residue that is conserved in family GH-5 members and is known to be involved in substrate recognition [42–45], suggesting that this position might be important for tuning substrate selection. Also, Y92 can interact with substrates, even if its spatial location is less conserved in the different models. On the other hand, Y51, Y113 and Y198, which are located in $\beta1$, $\beta3$ and $\beta5$, respectively, are deeply buried in the barrel and are predicted to play a structural role. Finally, W233 is placed on the top of the barrel and can be involved in substrate recognition.

In conclusion, the merging of biochemical data with results from threading methods, multiple sequence alignments and secondary-structure predictions has allowed to predict the 3D-structure of the C-domain of Gas1p, in spite of its low-sequence similarity to structurally characterized proteins. The inferred structural properties of the C-domain have been used to generate a working hypothesis about the structural and functional role of key residues. The model could also be relevant for designing specific inhibitors of Gas1p and therefore new antifungal agents. In addition, it opens the possibility for targeted mutagenesis experiments.

Supporting information

The sequence alignments among templates and Gas1p and *xyz* coordinates for Gas1p models.

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