Identification of the catalytic residues of the first family of β(1–3)glucanosyltransferases identified in fungi

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A new family of glycosylphosphatidylinositol-anchored β(1–3)glucanosyltransferases (Gelp), recently identified and characterized in the filamentous fungus *Aspergillus fumigatus*, showed functional similarity to the Gas/Phr/Epd protein families, which are involved in yeast morphogenesis. Sequence comparisons and hydrophobic cluster analysis (HCA) showed that all the Gas/Phr/Epd/Gel proteins belong to a new family of glycosylhydrolases, family 72. We confirmed by site-directed mutagenesis and biochemical analysis that the two conserved glutamate residues (the putative catalytic residues of this family, as determined by HCA) are involved in the active site of this family of glycosylhydrolases.

Key words: *Aspergillus fumigatus*, GEL protein family, GPI-anchored protein.

INTRODUCTION

The opportunistic filamentous fungus *Aspergillus fumigatus* has been associated with a wide spectrum of diseases in humans [1]. Even with the best anti-fungal agents currently available, the mortality rate is very high. The fungal cell wall is a structure that is both essential for the fungus and absent from the mammalian host, and consequently presents an attractive target for new anti-fungals [2]. The cell wall of *A. fumigatus* is a complex structure composed mainly of polysaccharides, β(1–3)glucans being the most abundant [3,4]. In a search for enzymic activity involved in the modification of β(1–3)glucans in the periplasmic space, a new β(1–3)glucanosyltransferase (Gel1p) has been identified [5]. This enzyme is able to cleave internally a β(1–3)glucan molecule, and transfers the newly generated reducing end to the non-reducing end of another β(1–3)glucan molecule. The generation of a new β(1–3)linkage between the acceptor and the donor molecule results in the elongation of β(1–3)glucan. The predicted amino acid sequence of Gel1p presented significant similarity to several yeast glycosylphosphatidylinositol (GPI)-anchored proteins [6], including Gas1p of *Saccharomyces cerevisiae* [7,8], Phr1p and Phr2p of *Candida albicans* [9,10] and Epd1p of *C. maltosa* [11] (which are involved in yeast morphogenesis), and also Epd2p in *C. maltosa* and 013692, P78785 in *Schizosaccharomyces pombe*, found in databases, but of unknown function. Gas1p, Phr1p and Phr2p were shown to display the same β(1–3)glucanosyltransferase activity as that of Gel1p [6].

Hydrophobic cluster analysis (HCA) [12] has proved to be useful in the classification of glycoside hydrolases and transglycosidases, and also in the identification of the putative amino acids in the catalytic sites of carbohydrate-modifying enzymes [13,14]. HCA, applied to this family of β(1–3)glucanosyltransferases, has shown that Gas/Phr/Gel/Epd proteins are all clustered in a distinct family of glycoside hydrolases (where Gas, Phr, Gel and Epd are acronyms for **g**lycophospholipid-**a**nchored **s**urface, **pH**-**r**egulated, **g**lucan-**e**longating glucanosyltransferase and **e**ssential for **p**seudohyphal **d**evelopment, respectively). Using Gel1p as a representative member of this family, we present here evidence, obtained by site-directed mutagenesis and biochemical analysis, that the two glutamate residues identified by HCA are involved in the catalytic site.

MATERIALS AND METHODS

HCA

The HCA method [12] is based primarily on the fundamental rules underlying the folding of globular proteins (i.e. the partition between a hydrophobic core and a hydrophilic surface). This method uses a two-dimensional plot of the protein sequence obtained after duplication of an unrolled cylinder, in which the amino acid residues follow an α-helical pattern. In this representation, the clusters of contiguous hydrophobic residues (Val, Ile, Leu, Phe, Met, Tyr, Trp) have been demonstrated to significantly correspond to the internal sides of regular secondary-structure elements in globular proteins [15]. HCA is also a sensitive method for amino-acid-sequence comparison, because

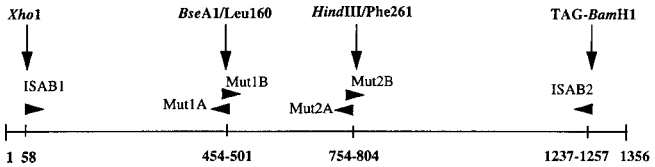

Figure 1 Construction of the two mutated Gel1pMut$_{Glu-160}$ and Gel1pMut$_{Glu-261}$ proteins

The fragments ISAB1–Mut1A and Mut1B–ISAB2 were amplified independently, resulting in the incorporation of a *Bse*AI site and a Leu-160 residue in place of Glu-160. The fragments ISAB1–Mut2A and Mut2B–ISAB2 were also amplified independently; these incorporated a *Hin*dIII site and a Phe-261 residue in place of Glu-261. The horizontal line represents the *GEL1* gene.

Abbreviations used: GPI, glycosylphosphatidylinositol; HCA, hydrophobic cluster analysis; HPAEC, high-performance anion-exchange chromatography.

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The nucleotide sequence data reported in this paper for Gelp have been submitted to the EMBL, Genbank® DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers AF072700 for *GEL1*, AF208039 for *GEL2* and AF208040 for *GEL3*.

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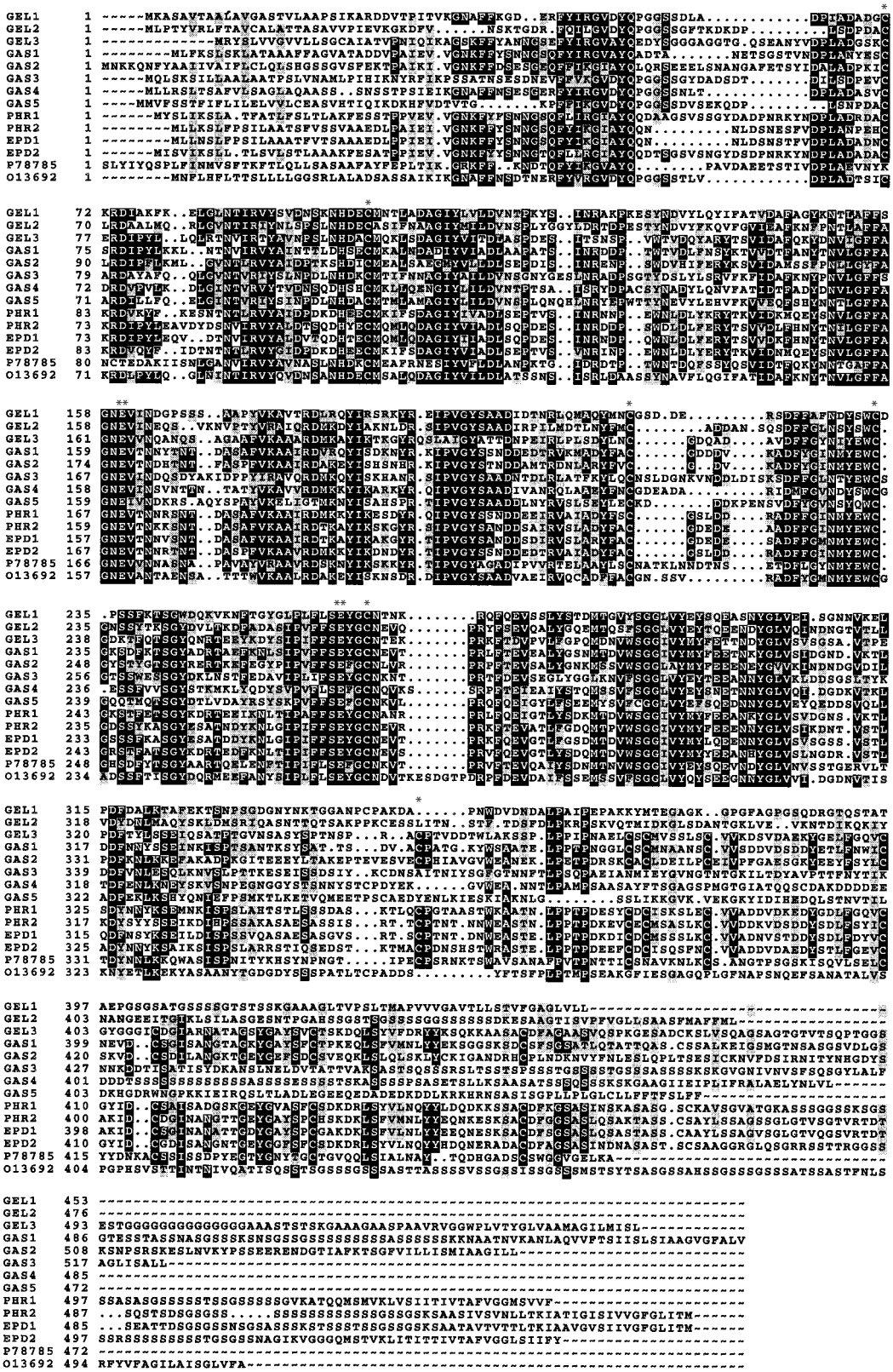

Figure 2 Comparison of the predicted amino acid sequences of Gel1p, Gel2p and Gel3p of *A. fumigatus* with Gas1p and its homologues in *S. cerevisiae*, Phr1p and Phr2p of *C. albicans*, Epd1p and Epd2p in *C. maltosa*, and P78785 and O13692 in *S. pombe*

Identical residues are enclosed in black boxes; similar residues are shown by grey-boxed shading. The two catalytic residues are indicated by double asterisks; the conserved cysteine residues are indicated by an asterisk.

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it is not based solely on the maximization of alignment scores, as in most classical methods. The analysis involves a comparison of the size, distribution and shape of the clusters of hydrophobic residues on the helical representation (for details, see [16,17]. HCA has indeed proved to allow the detection of similarities between proteins sharing low amino-acid identity (typically 10–20 %) [16,17].

Site-directed mutagenesis of Gel1p and construction of the mutant strain

Plasmid pISAB2 was used for the production of a recombinant, truncated $Gel1p_{419}$ lacking the hydrophobic C-terminus [6]. Previous studies have shown that the absence of the C-terminus partly permits the secretion of the protein in the culture medium, without altering its enzymic activity [6].

Mutagenesis of amino acid Glu-160

To construct Glu-160-mutated Gel1p, two fragments of *GEL1* were amplified with two sets of primers: a pairing of the forward primer ISAB1 (5′-TATCTCGAGCCCCCTCCATCAAGGCT-CGTGACGACGTTACTCCCATCACT-3′) and reverse primer Mut1A (5′-GTTTCCGGAGAAGAAAGCGAGGGT-3′, and the forward primer Mut1B (5′-TTCTCCGGAAAC**CTC**GTTA-TCAACGATGGCCCTTCC-3′) and reverse primer ISAB2 (5′-GTAGGATCCCTAAGCGCCCTTGGAAGAGGTGGA-3′). ISAB1 is complementary to nts +58 to +99, with an *Xho*I (underlined) site incorporated at the 5′ end. The primers Mut1A and Mut1B were complementary to nts +454 to +473 and +469 to +501 respectively of the coding region of *GEL1*; for these primers, the restriction site *Bse*AI (underlined) and a Leu codon (shown in bold in above sequence), instead of a Glu codon, were incorporated (Figure 1). The reverse primer ISAB2 is complementary to nts +1237 to +1257 of the coding region, incorporating an in-frame TAG stop codon and a *Bam*HI site (underlined in the above sequence) at the 3′-end. Total DNA, obtained as described previously [19] from strain CBS 144.89 of *A. fumigatus*, was used as the template for PCR amplification. Thirty cycles, consisting of a 1-min melting step at 95 °C, a 1-min annealing step at 60 °C and 1 min extension at 70 °C, were performed. The PCR-generated ISAB1–Mut1A product was digested with *Xho*I and *Bse*AI, whereas the PCR product Mut1B–ISAB2 was digested with *Bse*AI and *Bam*HI. These two digested fragments were then cloned into the expression vector pKJ113 [20], and digested with *Xho*I and *Bam*HI, generating the plasmid $pMut_{Glu\text{-}160}$.

Mutagenesis of amino acid Glu-261

To construct the Glu-261-mutated Gel1p protein, two fragments of *GEL1* were amplified with two set of primers: the forward primer ISAB1 with the reverse primer Mut2A (5′-TAGAAGCTTAGGAAGAGAGGAAGACCGTAGCC-3′), and the forward primer Mut2B (5′-CCTAAGC**TT**CTACGG-CTGCAACACCAACAAG-3′) with the reverse primer ISAB2. The primers Mut2A and Mut2B were, respectively, complementary to nts 754–776 and 784–804 of the coding region, and led to the incorporation of the *Hin*dIII restriction site (underlined above) and a Phe codon instead of a Glu codon (shown in bold in above sequence) (Figure 1). Amplification was performed as described above. The PCR-generated ISAB1–Mut2A product was digested with *Xho*I and *Hin*dIII, and the PCR product of Mut2B–ISAB2 was digested with *Hin*dIII and *Bam*HI. These two digested fragments were cloned into the expression vector pKJ113 [20] digested with *Xho*I and *Bam*HI, generating the plasmid $pMut_{Glu\text{-}261}$. DNA sequencing confirmed the specific mutagenesis of Glu-160 and Glu-261.

Production of recombinant mutated proteins

Pichia pastoris GS115 (Invitrogen, Leek, The Netherlands) spheroplasts were transformed with $pMut_{Glu\text{-}160}$ or $pMut_{Glu\text{-}261}$ linearized by *Eco*RI. Transformants were selected on a histidine-deficient medium, and screened on minimal-methanol plates for insertion of the construct in the *P. pastoris* GS115 genome, as described previously [20]. Production of $Gel1Mut_{Glu\text{-}160}$ and $Gel1Mut_{Glu\text{-}261}$, placed under the control of the alcohol oxidase promoter in *P. pastoris*, were secreted into the culture medium in the presence of 0.7 % (v/v) methanol [following the manufacturer's instructions (Invitrogen)].

Enzymic analysis of Gel1p and mutated Gel1p

The recombinant Gel1p (lacking the attachment of GPI) was purified as described by Mouyna et al. [6]. Culture filtrates containing the recombinant $Gel1Mut_{Glu\text{-}160}$ and $Gel1Mut_{Glu\text{-}261}$ proteins were frozen and kept at −20 °C prior to purification. For the assay of the enzymic activity, purification of the recombinant proteins was necessary, since endogeneous β(1–3)glucanase and β(1–3/1–6)glucanosyltransferase activities [19,21] were always secreted into the culture medium by the yeast heterologous host. Although released directly in only low amounts in comparison with the recombinant protein of interest, their presence would interfere with the determination of the activity, since these contaminating enzymes acted on the laminarioligosaccharide substrate and/ or reaction products (results not shown). Recombinant Gel1p was purified as follows: after dialysis against a 10 mM Tris/HCl buffer (pH 7), the culture filtrates were applied to an 8 mm × 75 mm DEAE-5PW anion-exchange chromatography column (TosoHaas, Zettachring, Germany) equilibrated with 20 mM Tris/HCl, pH 8, at a flow rate of 0.7 ml/min. The recombinant proteins were eluted with a NaCl gradient (0–375 mM in 50 min, followed by 375–500 mM in 10 min). β(1–3)Glucanosyltransferase activity was analysed as previously described [5]. Briefly, the purified proteins were incubated at concentrations of 0.05–0.16 mg/ml with 3 mM reduced laminarioligosaccharide G_{13} in a 50 mM sodium acetate buffer, pH 5.5, at 37 °C. Aliquots of 2.5 μl, supplemented with 40 μl of 50 mM NaOH, were analysed sequentially by high-performance anion-exchange chromatography (HPAEC) with a 4.6 mm × 250 mm CarboPAC-PA1 column (Dionex, Idstein, Germany), as described previously [5].

SDS/PAGE of Gel1p, $Gel1Mut_{Glu\text{-}160}$p and $Gel1Mut_{Glu\text{-}261}$p proteins was performed on a 10 % (w/v) separating gel with a 4 % stacking gel. Electrotransfer of proteins to a nitrocellulose membrane (0.2-μm pore size, cellulose nitrate; from Schleicher and Schuell through Ceralabo, Paris, France) was accomplished at 30 V in a 50 mM Tris/200 mM glycine/20 % (v/v) methanol buffer [22]. The antiserum used for Western blotting analysis was directed against Gel1p. To obtain anti-Gel1p, rabbits were immunized against a peptide (INRAKPESYNDVYC), designed on the basis of the sequence data [6]. Coupling of the peptide via cysteine with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, immunization of the animal and titre determination of the antiserum were performed by Eurogentec (Seraing, Belgium). Immunopurification of the specific anti-peptide antibodies was accomplished after coupling the peptide with epoxy-activated Sepharose (Pharmacia, Orsay, France), following the manufacturer's instructions. Immunolabelling of blots was performed

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using the enhanced chemiluminescence (ECL®) Western blotting detection procedure of Amersham (Les Ulis, France).

RESULTS AND DISCUSSION

The families of Gas/Phr/Epd/Gel proteins share common characteristics

GEL1 has been characterized recently [6], and, in addition, two new members of the *GEL* families (*GEL2* and *GEL3*) have also been isolated recently (I. Mouyna, unpublished work). Figure 2 shows the alignment of the new member of Gelp (Gel2p and Gel3p) with sequences which present significant similarity found in *S. cerevisiae* (Gas1p–Gas5p), *C. albicans* (Phr1p, Phr2p), *C. maltosa* (Epd1p, Epd2) and *S. pombe* (O13692 and P78785). Gelp family proteins are organized into the three domains, as described by Popolo and Vai [23], i.e. (i) an N-terminal catalytic domain that was further divided into six conserved blocks; (ii) a cysteine-rich region containing six highly conserved cysteine residues; and (iii) a serine-rich C-terminal segment and a hydrophobic C-terminus, characteristic of GPI-anchored proteins [6,23]. The size of all the proteins of this family was variable (ranging from 452 to 559 amino acids). The levels of identity observed among all these proteins varied from 28–81 % (Table 1). The 'best' identities were not associated with gene families inside a fungal species. For example, the highest percentage of identity was found between Epd1p and Phr2p from *C. maltosa* and *C. albicans* respectively, whereas only 30 % identity was observed between Gel3p and both Gel1p and Gel2p from *A. fumigatus*.

Table 1 Percentages of identity among all the GAS family proteins of *S. cerevisiae*, the PHR family proteins of *C. albicans*, the EPD family proteins of *C. maltosa* and the GEL family proteins of *A. fumigatus*

Bold values indicate the highest percentage of homology found for each protein listed in the left column.

	GEL1	GEL2	GEL3	GAS1	GAS2	GAS3	GAS4	GAS5	PHR1	PHR2	EPD1	EPD2	P78785	O13692
GEL1		38	30	37	33	36	**49**	36	36	33	35	35	35	45
GEL2			30	37	32	**46**	40	37	35	35	35	36	34	39
GEL3				43	32	28	33	28	41	41	**46**	39	41	37
GAS1					44	33	37	32	55	**59**	58	52	44	39
GAS2						29	36	31	41	**43**	42	40	37	34
GAS3							**37**	37	33	32	32	32	31	37
GAS4								35	35	36	37	36	37	**49**
GAS5									33	32	**34**	32	33	34
PHR1										55	56	**74**	40	37
PHR2											**81**	54	39	38
EPD1												**57**	42	38
P78785														37

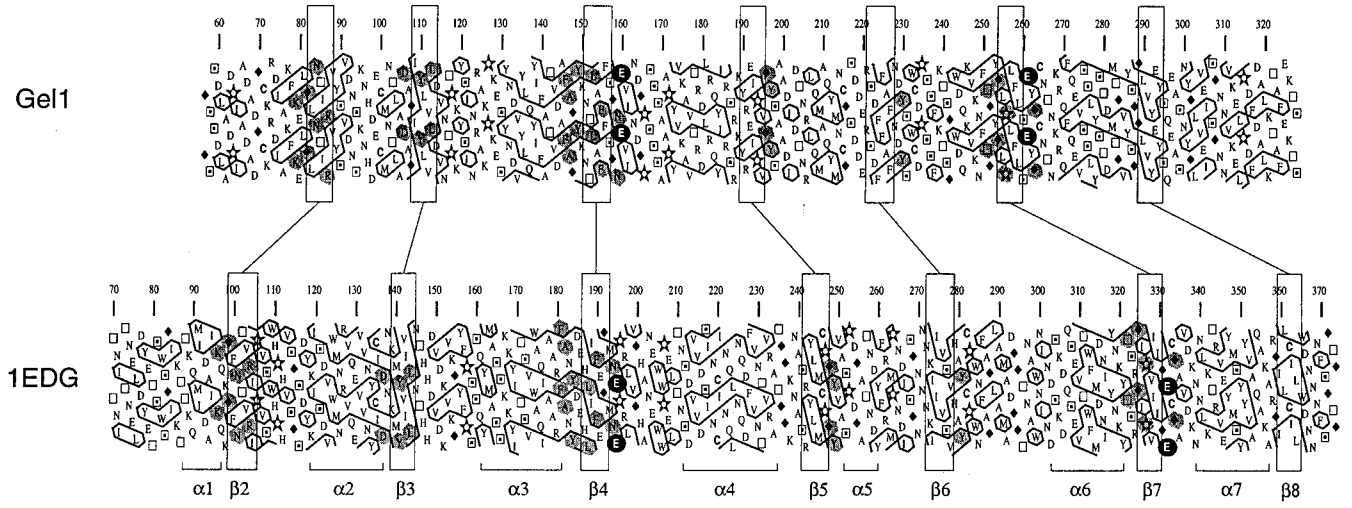

Figure 3 HCA comparison of Gel1 (upper panel) and endoglucanase A of *Cl. cellulolyticum* (1EDG; bottom panel)

The plots were prepared, edited and analysed as described previously [23]. The symbols ☆, ◆, ⊡, □, coding for proline, glycine, serine and threonine respectively, are used to aid visual inspection of the plots. The location of the various secondary-structure elements of endoglucanase A was inferred from the three-dimensional structure [27], and is noted at the bottom. The correlation between the β-strands involved in the $(\beta/\alpha)_8$ barrel is shown. The two catalytic residues located at the C-terminal ends of strands $\beta 4$ and $\beta 7$ are shown in white lettering on black circles. The other conserved residues are printed on a grey background.

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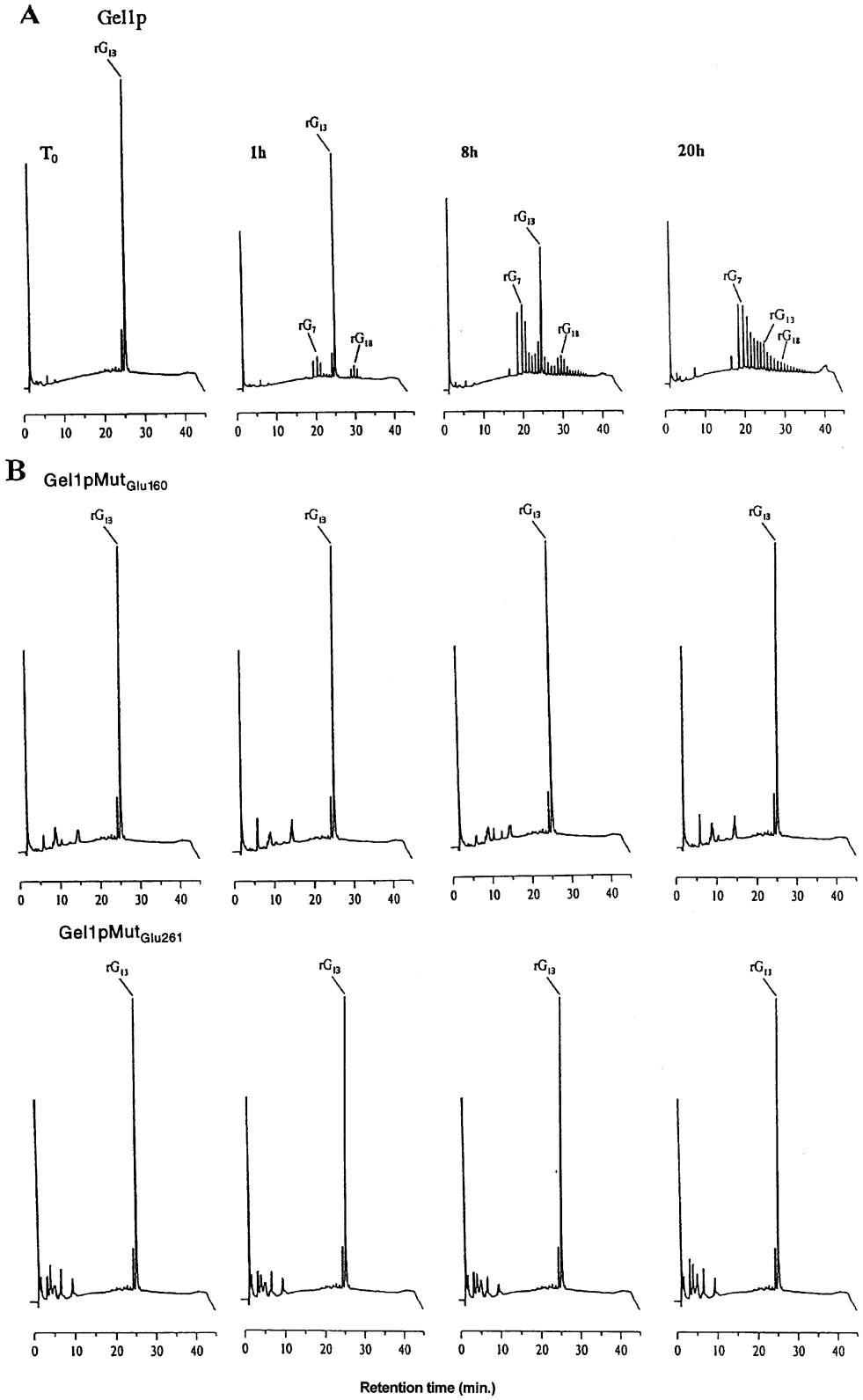

Figure 4 HPAEC analysis of products from the incubation of the recombinant Gel1p, and mutated Gel1p proteins Gel1pMut$_{Glu-160}$ and Gel1pMut$_{Glu-261}$, with reduced laminarioligosaccharides

Samples (3 μg) of respective purified recombinant proteins were incubated with 3 mM reduced laminarioligosaccharide containing 13 glucose units (+rG13) in 20 μl of 50 mM sodium acetate, pH 5.5, at 37 °C. Aliquots (2.5 μl) supplemented with 40 μl of 50 mM NaOH were analysed by HPLC with a CarboPAC PA-1 column, and a pulsed electrochemical detector. (**A**) Analysis of product after 0, 1, 8 and 20 h of incubation with Gel1p. (**B**) Analysis of products obtained with mutated Gel1 proteins.

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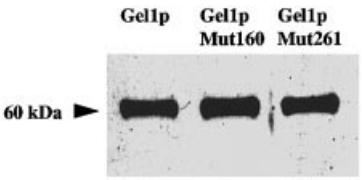

Figure 5 Western blot of Gel1p and mutated proteins Gel1pMut$_{Glu-160}$p and Gel1pMut$_{Glu-261}$p immunolabelled with anti-Gel1p antibody

The same amount of protein (1 μg) was loaded in each lane.

Identification of the catalytic sites of these multigenic family

HCA allows the classification of glycoside hydrolases and transglycosidases to be made [13,14]. Three-dimensional structural analysis, as well as HCA of the regions around the catalytic residues of glycosidases, have shown that several of the sequence-based families can be grouped in superfamilies or 'clans' [14,24,25] sharing not only the same global fold, but also the same molecular mechanism and the same catalytic machinery. The largest of these clans, clan GH-A, grouped together families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 51 and 53 [14], where the catalytic acid–base and nucleophilic residues were located respectively at the C-termini of strands β4 and β7 of a common $(\beta/\alpha)_8$ barrel structure [14,24,25]. Since the Gas/Gel/Phr/Epd proteins did not display obvious overall similarities to any of the previously defined families, they were assigned to a new family, family 72. However, HCA showed that this family also belongs to clan GH-A. In clan GH-A, hydrolysis occurs with an overall retention of the anomeric configuration. The β(1–3)-glucanosyltransferase activities of all members of family 72 are in perfect agreement with this retaining mechanism. Enzymic hydrolysis of the glycosidic bound by retaining glycoside hydrolases takes place via general acid catalysis that requires two critical residues, functioning as an acid–base and a nucleophile. For several representatives of clan GH-A, the catalytic acid–base and nucleophilic residues have been identified unambigously by site-directed mutagenesis, mechanism-based inhibitor studies and by examination of the three-dimensional structure of the protein in complex with oligosaccharides [25–27].

The HCA plot of a member of family 72, Gel1p, was compared with HCA plots of the endoglucanase A of *Clostridium cellulolyticum*, a member of family 5 of the GH-A clan with a known three-dimensional structure (Brookhaven Protein Databank; endoglucanase A of *Cl. cellulolyticum* [28]) (Figure 3). Gel1p, like all the members of the clan GH-A, is described by an HCA plot typical of proteins with alternating β-strands (short vertical clusters) and α-helices (longer, more horizontal clusters; Figure 3). With the exact position of the β-strands of the $(\beta/\alpha)_8$ barrel of the endoglucanase A of *C. cellulolyticum* being known from the three-dimensional structure [28], these have been readily traced into Gel1p. Although sequence identity between the two proteins was only approximately 8.5 % (21 residues conserved in approx. 250 residues), the correspondence was unambigous, and its significance was substantiated by the fact that the conserved residues were all localized around the β-strands of the endoglucanase A of *C. cellulolyticum*, i.e. the regions forming the core of the endoglucanase A structure (Figure 3). Two glutamate residues of Gel1p were found in the regions corresponding to the C-terminal ends of strands β-4 and β-7 of endoglucanase A. These glutamate residues, which form the catalytic machinery (acid–base and nucleophile respectively) of GH-A clan members [14,24,25], were also invariant among all members of family 72. This suggested that residues Glu-160 and Glu-261 of Gel1p were the acid–base and nucleophilic residues responsible for the transglycosylation mechanism.

In order to confirm biochemically the involvement of Glu-160 and Glu-261 in the active site of Gel1p, we constructed mutated Gel1p, where Glu-160 and Glu-261 were replaced with Leu-160 and Phe-261 respectively. Analysis of the enzymic activity of Gel1p and the recombinant Gel1p proteins produced in *P. pastoris*, Gel1pMut$_{Glu-160}$ and Gel1pMut$_{Glu-261}$, is shown in Figure 4. All these recombinant proteins were secreted in the culture medium in the same amounts, indicating that they are correctly folded, but no β(1–3)glucanosyltransferase activity was observed for the mutated proteins Gel1pMut$_{Glu-160}$ and Gel1pMut$_{Glu-261}$ (Figure 5). These results confirmed that Glu-160 and Glu-261 were involved in the catalytic site of this β(1–3)glucanosyltransferase.

In conclusion, our results demonstrate that Glu-160 and Glu-261 are located in the catalytic site giving rise to the β(1–3)glucanosyltransferase activity of family 72 of the glycosylhydrolases, which play a role in fungal morphogenesis. This work may help in the design of specific inhibitors for this β(1–3)glucanosyltransferase activity, which could serve as putative new anti-fungal therapeutic agents.

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