Interaction between the critical aromatic amino acid residues Tyr^{352} and Phe^{504} in the yeast Gal2 transporter

Toshiko Kasahara, Michihiro Kasahara*

Laboratory of Biophysics, School of Medicine, Teikyo University, Hachioji, Tokyo 192-0395, Japan

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Abstract Three critical aromatic sites have been identified in the yeast galactose transporter Gal2: Tyr^{352} at the extracellular boundary of putative transmembrane segment (TM) 7, Tyr^{446} in the middle of TM10 and Phe^{504} in the middle of TM12. The relationship between these sites was investigated by random mutagenesis of each combination of two of the three residues. Galactose transport-positive clones selected by plate assays encoded Tyr^{446} and specific combinations of aromatic residues at sites 352 and 504. Double-site mutants containing aromatic residues at these latter two positions showed either essentially full galactose transport activity ($Phe^{352}Trp^{504}$ and $Trp^{352}Trp^{504}$) or no significant activity ($Phe^{352}Tyr^{504}$ and $Trp^{352}Tyr^{504}$), whereas single-site mutants showed markedly reduced activity. These results are indicative of a specific interaction between sites 352 and 504 of Gal2.

Key words: Galactose transport; Gal2; Hxt2; Substrate recognition; Glucose transport; Aromatic amino acid

1. Introduction

Hexose transport across the plasma membrane is an essential step in the utilization of monosaccharides by *Saccharomyces cerevisiae* [1,2]. Analysis of the *S. cerevisiae* genome has implicated more than 20 paralogous genes encoding hexose transporters and related proteins that constitute the Glut transporter family of this species and include Gal2 and Hxt2 [3–5].

Comprehensive studies of chimeras of Gal2, a major high-affinity galactose transporter that also transports glucose with a similar affinity, and Hxt2, a major high-affinity glucose transporter, revealed that two aromatic amino acid residues in transmembrane segment (TM) 10 are essential (Tyr^{446}) or important (Trp^{455}) for galactose recognition by Gal2 [5–7] (Fig. 1). In Hxt2, Phe^{431}, which corresponds to Tyr^{446} of Gal2, is critical for glucose transport [8]. The importance of the aromatic amino acids in these positions was also confirmed with the rat Glut1 glucose transporter: Trp^{388}, which corresponds to Trp^{455} of Gal2, is essential and Phe^{379}, which corresponds to Tyr^{446} of Gal2, is important for glucose transport [9]. Moreover, aromatic amino acids are highly conserved at these positions in the many homologs of these transporters that have been identified in eubacteria, archaea and eukaryotes [10], further emphasizing their importance. Determination of the three-dimensional structures of maltoporins [11] and the sucrose-specific porin ScrY [12] revealed that several aromatic amino acids are located on the same side of an aqueous pore, suggesting that they may perform a substrate relay function.

To examine further the role of aromatic amino acid residues in substrate recognition by Gal2, we previously investigated whether other aromatic amino acids in this transporter are essential. We subjected 14 sites of this protein at which aromatic amino acids are conserved in $>75\%$ of sugar transporters and related proteins to site-directed random mutagenesis. We identified two additional critical aromatic sites: one at the extracellular side of TM7 (Tyr^{352}) and the other in the middle of TM12 (Phe^{504}) [13].

In this respect, to assess the functional roles of Tyr^{352}, Tyr^{446} and Phe^{504}, we randomly replaced pairs of these residues with each of 20 amino acids and examined the effects of such mutations on galactose transport by selecting transport-positive clones. Our results confirm that aromatic amino acids are essential at these three sites and they reveal that specific combinations of residues at positions 352 and 504 are required for galactose transport activity.

2. Materials and methods

2.1. Production of cassette vectors

Construction of the plasmid GAL2-pTV3e, for expression of *GAL2* with a *GAL* expression system in the multicopy plasmid pTV3 (YEp *TRP1 bla*), was previously described [5,13].

2.2. Mutagenesis

Site-directed mutants were prepared with a polymerase chain reaction (PCR)-based approach [7,14]. PCR was performed with native *Pfu* polymerase (Stratagene) and a GeneAmp PCR system 2400 (Perkin-Elmer). After amplification in *Escherichia coli*, *GAL2* plasmids were introduced into *S. cerevisiae* strain LBY416 (*MATα hxt2::LEU2 snf3::HIS3 gal2 lys2 ade2 trp1 his3 leu2 ura3*). Galactose transport-positive clones were selected after the incubation of yeast cells for 4–5 days at 30°C on galactose-limited agar plates (200–400 μg/ml galactose) containing a synthetic medium supplemented with uracil, adenine and amino acids except tryptophan (S(trp) medium) [15]. In parallel, the number of transformants was counted by plating a portion of the LBY416 cell suspension on agar plates containing S(trp) medium supplemented with 2% glucose.

We replaced Tyr^{352}, Tyr^{446} or Phe^{504} with each of the other 19 amino acids [13]. An equal mixture of 20 plasmids corresponding to the wild-type and mutant residues for each site was used for random mutagenesis and the obtained mutants were designated X^{352}, X^{446} or X^{504}. The DNA concentration of each plasmid preparation was determined with a photometer (Gene Spec I, Naka Instruments) and showed little variation: 4.3 ± 0.2, 5.2 ± 0.2 and 5.5 ± 0.2 mg/ml for the X^{352}, X^{446} and X^{504} series, respectively (means ± S.E.M., $n = 20$). Double mutants at X^{352} and X^{504}, designated $X^{352}X^{504}$, were constructed by digesting the mixture of plasmids containing X^{352} with

*Corresponding author. Fax: (81)-426-78 3262.
E-mail: kasahara@main.teikyo-u.ac.jp

Abbreviations: TM, transmembrane segment; PCR, polymerase chain reaction; GFP, green fluorescent protein

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*Eco*RI and *Mlu*I and inserting the released fragments into the corresponding region of an *Eco*RI- and *Mlu*I-digested mixture of plasmids coding for X^{504}. The other double mutants and triple mutants ($X^{352}X^{446}X^{504}$) were similarly constructed. Modified portions of all clones selected in this study were verified by DNA sequencing with an automated sequencer (model 373A, Perkin-Elmer). We checked for any bias in the production of mutants containing specific amino acids by DNA sequencing of 24 unselected clones. Nine of the 72 amino acids at the targeted sites were aromatic residues, indicating no substantial bias toward such amino acids. A plasmid containing the gene for a green fluorescent protein (GFP) variant (F64L, S65T variant), pEGFP (Clontech, CA, USA), was used for the production of Gal2-GFP fusion proteins [13].

2.3. Transport assay

Yeast cells were grown to log phase (OD_{650}, 0.3–0.4) at 30°C in S(trp) medium supplemented with 2% galactose. The transport of galactose or glucose in LBY416 harboring each of the various *GAL2* plasmids was measured at 30°C for 5 s, as described [5,8]. Transport activities in the presence of 0.1 mM D-[^{14}C]galactose or 0.1 mM D-[^{14}C]glucose were expressed as pmol/10^7 cells/5 s. For comparison, the background obtained with control cells harboring the empty vector was subtracted from the transport activity, which was then normalized by expression as a percentage of the corrected activity obtained with cells expressing wild-type Gal2. We considered values $>10\%$ as significant [8].

2.4. Other assays

Immunoblot analysis of yeast homogenates was performed as described [5]. Cell number was determined with a particle counter (Z2, Coulter). Protein concentration was assayed with bicinchoninic acid (Pierce).

3. Results and discussion

Alignment of the amino acid sequences of 63 sugar transporters and related proteins (DM00135) in the DOMO database [16] revealed that 14 sites contain aromatic residues in $>75\%$ of the proteins. Our previous studies have shown that three such sites in Gal2, Tyr^{352}, Tyr^{446} and Phe^{504}, are critical for galactose transport [5–8,13]. To investigate the functional relations between these three positions, we mutated pairs of the sites simultaneously by transformation of yeast cells with a mixture of plasmids coding for each of the 20 amino acids at each position. Galactose transport-positive clones were selected by the growth of cells on galactose-limited agar plates. All of the 120 galactose transport-positive clones obtained encoded aromatic amino acids at these three sites (Table 1), confirming our previous observation that aromatic residues at these positions of Gal2 are critical for galactose transport activity [7,13]. All transport-positive clones encoded Tyr at position 446 with the exception of two clones, both of which encoded $Tyr^{352}Phe^{446}Phe^{504}$; however, because the galactose transport activity of the Phe^{446} mutant ($10 \pm 1\%$ ($n=3$)) was not $>10\%$ of that of wild-type Gal2, we did not consider it a true positive. At site 352, in addition to the wild-type residue (Tyr), Phe and Trp supported galactose transport activity, confirming our previous observations [13]. Similarly, at site 504, in addition to the wild-type residue (Phe), Trp and Tyr supported galactose transport activity, again consistent with our previous data [13].

Since the $X^{352}X^{446}$ and $X^{446}X^{504}$ mutants that showed significant transport activity possessed the wild-type residue (Tyr) at position 446, all of these mutants reverted to single-site mutants. However, in the case of $X^{352}X^{504}$ mutants, in addition to the single-site mutants, two double-site mutants ($Phe^{352}Trp^{504}$ and $Trp^{352}Trp^{504}$) were also positive for galactose transport activity. To elucidate further the role of aromatic amino acids at these two positions, we prepared plasmids encoding $X^{352}X^{504}$ transporters with all nine possible combinations of aromatic residues at these two sites. An assay of galactose transport by the encoded proteins revealed that the five types of mutants identified by plate selection showed significant galactose transport activity ($>15\%$ of that of the wild-type), whereas two mutants not detected by plate selection, $Phe^{352}Tyr^{504}$ and $Trp^{352}Tyr^{504}$, did not possess significant transport activity ($\leq 5\%$ of that of the wild-type) (Fig. 2). Trp^{352}, the other type of mutant not detected by plate selection, showed a galactose transport activity (11%) that

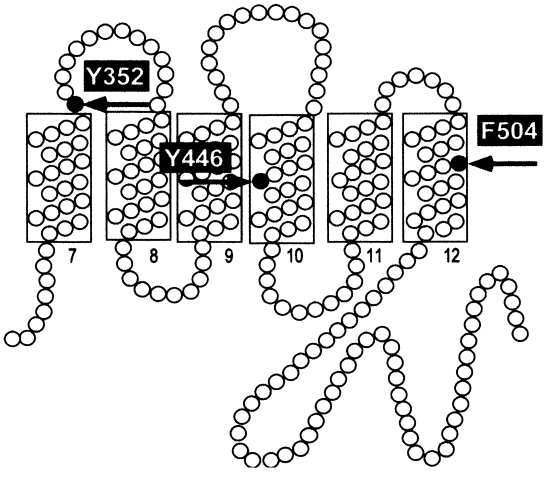

Fig. 1. Three aromatic amino acids in Gal2 critical for galactose transport activity. The structure of Gal2 is based on a model of 12 transmembrane segments [24], with only the COOH-terminal half of the protein being shown.

Table 1
Numbers of galactose transport-positive clones selected from randomly generated double mutants of Gal2 at sites 352, 446 and 504

$X^{352}X^{446}$ mutants			
Site 352	Site 446		
	Tyr	Phe	Trp
Tyr	**25**	1	0
Phe	5	0	0
Trp	5	0	0
$X^{446}X^{504}$ mutants			
Site 446	Site 504		
	Phe	Trp	Tyr
Tyr	**14**	7	9
Phe	1	0	0
Trp	0	0	0
$X^{352}X^{504}$ mutants			
Site 352	Site 504		
	Phe	Trp	Tyr
Tyr	**18**	9	5
Phe	2	10	0
Trp	0	9	0

Mixtures of plasmids encoding Gal2 mutated at sites 352 and 446, sites 446 and 504, or sites 352 and 504 were introduced into LBY416 cells and galactose transport-positive clones were selected. All 120 clones obtained encoded aromatic amino acids at these three sites. Wild-type residues and clones are indicated in bold.

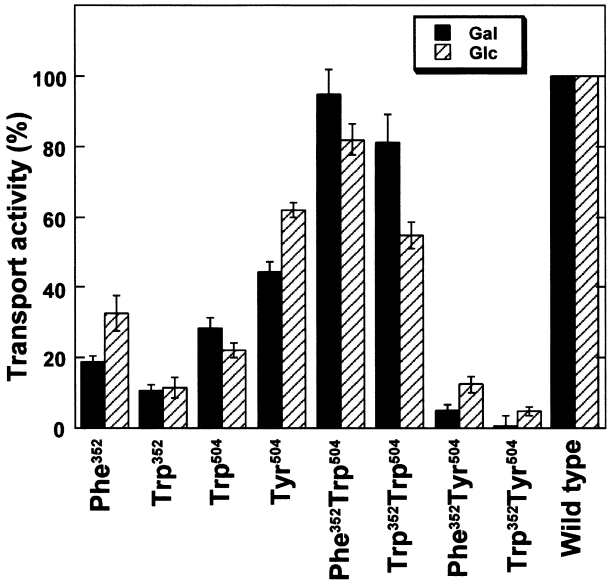

Fig. 2. Galactose and glucose transport activities of yeast cells expressing $X^{352}X^{504}$ mutants of Gal2. LBY416 cells harboring plasmids encoding wild-type Gal2 or $X^{352}X^{504}$ mutants, in which Tyr^{352} and/or Phe^{504} was replaced by each of the other two aromatic amino acids, were cultured to log phase. Transport of 0.1 mM galactose (solid bars) or 0.1 mM glucose (hatched bars) was then measured. After subtraction of the background activity of control cells harboring the empty vector pTV3e (2.8 ± 0.3 and 5.0 ± 0.4 pmol/10^7 cells/5 s (means ± S.E.M., $n = 5$) for galactose and glucose, respectively), the transport activity of experimental cells was expressed as a percentage of the corrected activity of cells expressing wild-type Gal2 (16.5 ± 0.4 and 29.1 ± 0.8 pmol/10^7 cells/5 s (means ± S.E.M., $n = 4$) for galactose and glucose, respectively). Data are means ± S.E.M. ($n = 4$).

just qualified as significant. Whereas all of the single-site mutants (Phe^{352}, Trp^{352}, Trp^{504} and Tyr^{504}) showed reduced but significant galactose transport activities (< 45% of that of the wild-type), the two-site mutants showed either > 80% of the wild-type activity ($Phe^{352}Trp^{504}$ and $Trp^{352}Trp^{504}$) or no significant activity ($Phe^{352}Tyr^{504}$ and $Trp^{352}Tyr^{504}$). Thus, specific combinations of aromatic amino acids are required for galactose transport activity, with the rank order of effectiveness being wild-type, $Phe^{352}Trp^{504}$, $Trp^{352}Trp^{504} \gg Tyr^{504}$, Trp^{504}, Phe^{352}, $Trp^{352} > Phe^{352}Tyr^{504}$, $Trp^{352}Tyr^{504}$ (Fig. 3). A similar tendency was observed with glucose transport activities of these $X^{352}X^{504}$ mutants.

We also attempted to generate three-site mutants by random mutagenesis. However, we detected only seven positive clones out of a total of ~18 000 transformants by plate selection. All seven mutant clones encoded Tyr^{352} and Tyr^{446}, with four encoding Phe (wild-type), two encoding Tyr and one encoding Trp at position 504. Further analysis was hindered by the scarcity of the mutant clones.

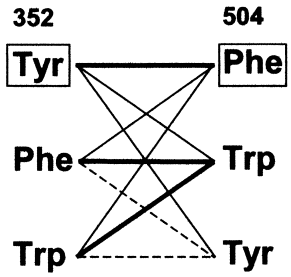

Fig. 3. Relation between galactose transport activity and aromatic amino acids of $X^{352}X^{504}$ mutants of Gal2. The thickness of the line connecting two aromatic amino acids indicates that the mutants containing those residues exhibit high (> 80% of that of wild-type Gal2, thick line), low (> 10%, thin line) or no significant (< 10%, dotted line) galactose transport activity.

The effective combinations of aromatic amino acids in $X^{352}X^{504}$ mutants were site specific; thus, the activity of the wild-type ($Tyr^{352}Phe^{504}$) was different from that of $Phe^{352}Tyr^{504}$, as was the activity of $Phe^{352}Trp^{504}$ from that of Trp^{352} or the activity of Trp^{504} from that of $Trp^{352}Tyr^{504}$. This observation suggested that interaction of the aromatic amino acids with surrounding residues may also play a role in galactose transport. At position 352, situated at the external boundary of TM7, all three aromatic residues supported full galactose transport activity, provided appropriate residues were present at site 504. In this respect, the role of these aromatic amino acids at position 352 may differ from those of the Trp and Tyr residues that are often present near the ends of transmembrane helices in other membrane proteins [17,18].

The kinetic parameters for galactose and glucose transport were measured for six mutant proteins that showed > 10% of the galactose transport activity of wild-type Gal2 (Table 2). The K_m and V_{max} values of $Phe^{352}Trp^{504}$ and $Trp^{352}Trp^{504}$ were similar to those of the wild-type protein, as expected from the results shown in Fig. 2. The single-site mutants examined generally showed increased K_m values. The Tyr^{504} mutant showed a moderate increase in K_m values for D-galactose and D-glucose. The Trp^{504} mutant showed an increased K_m for D-galactose and a decreased V_{max} for D-glucose, whereas Phe^{352} and Trp^{352} showed increased K_m and reduced V_{max} values for both sugars. The substrate specificity of these mu-

Table 2
Kinetic parameters of $X^{352}X^{504}$ mutants

Protein	Galactose		Glucose	
	K_m (mM)	V_{max} (pmol/10^7 cells/5 s)	K_m (mM)	V_{max} (pmol/10^7 cells/5 s)
Wild-type	3.8 ± 0.3	443 ± 12	2.0 ± 0.1	440 ± 7
$Phe^{352}Trp^{504}$	4.9 ± 0.3	408 ± 8	2.0 ± 0.2	481 ± 58
$Trp^{352}Trp^{504}$	6.0 ± 1.0	440 ± 43	3.2 ± 0.1	468 ± 43
Tyr^{504}	6.7 ± 0.9	380 ± 45	4.9 ± 0.8	545 ± 45
Trp^{504}	14.4 ± 2.4	565 ± 80	2.9 ± 0.5	235 ± 35
Phe^{352}	10.0 ± 0.8	370 ± 45	4.6 ± 0.2	300 ± 15
Trp^{352}	11.6 ± 0.8	235 ± 15	6.5 ± 0.8	289 ± 32

LBY416 cells harboring plasmids encoding each of the indicated Gal2 mutants were cultured to log phase, after which glucose and galactose transport activities were measured at substrate concentrations of 1 to 20 mM. The K_m and V_{max} values were determined by the non-linear least-squares method (KaleidaGraph, Synergy Software). Data are means ± S.E.M. ($n = 3$).

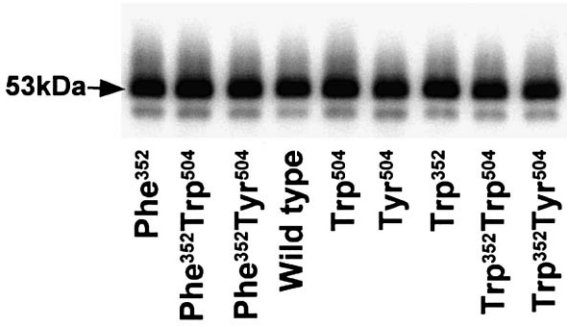

Fig. 4. Immunoblot analysis of the abundance of $X^{352}X^{504}$ mutants in yeast cells. LBY416 cells harboring plasmids encoding Gal2 mutants or wild-type Gal2 were cultured to log phase and then homogenized. A portion of each homogenate (10 μg of protein) was subjected to immunoblot analysis with antibodies to the COOH-terminus of Gal2 and with ^{125}I-labeled protein A. The bands immediately below the 53 kDa bands appear to be degradation products [8].

tants was examined by measuring the transport of 0.1 mM D-[^{14}C]galactose in the presence of a 200-fold molar excess of various sugar analogs: D-glucose, D-galactose, 2-deoxy-D-glucose, D-fructose, D-mannose, 3-*O*-methyl-D-glucose, 6-deoxy-D-glucose, D-allose, D-fucose, D-xylose, L-arabinose, D-arabinose, L-glucose and L-galactose. No marked differences in substrate specificity were detected among the six mutants and wild-type Gal2 (data not shown). Thus, the aromatic amino acids at positions 352 and 504 appear to cooperate in determining the affinity for galactose, but do not appear to be as critical as Tyr^{446} [7].

The extent of expression of the various $X^{352}X^{504}$ mutant proteins was examined by immunoblot analysis of cell homogenates with antibodies to Gal2 (Fig. 4). All of the mutants yielded a predominant immunoreactive band at 53 kDa, corresponding to the position of wild-type Gal2. The wild-type

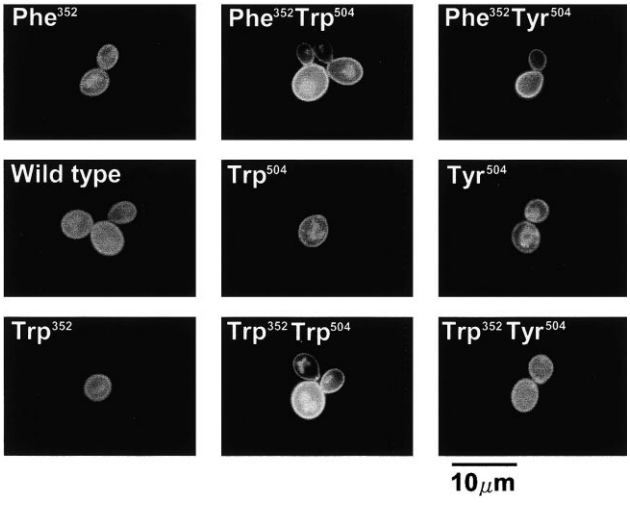

Fig. 5. Localization of $X^{352}X^{504}$ mutants tagged with GFP. LBY416 cells harboring plasmids encoding GFP-tagged $X^{352}X^{504}$ mutants were cultured to log phase and observed with a fluorescence microscope. As noted in the previous study [13], the fluorescence intensity of an individual cell was heterogeneous and intracellular structures probably corresponding to central vacuole or small vesicles emerged when fluorescent intensity was high. Representative cells of each mutant and wild-type are shown.

and mutant proteins were expressed at similar levels. With the use of GFP as a fluorescent tag, we also examined the possibility that the mutant proteins might be mistargeted within the cell. However, all cells expressing the mutant proteins showed a pattern of surface fluorescence similar to that of cells expressing wild-type Gal2 (Fig. 5), indicating that intracellular trafficking of the mutants was normal.

As high-resolution structural information for the Glut transporters is not available, the structure–function relations for such transporters have been investigated by site-directed mutagenesis. Many studies have identified critical amino acids on the basis that their replacement with various other residues results in a loss of activity. However, with such an approach, the possibility cannot be excluded that the loss of activity results indirectly from a change in protein conformation. To exclude this possibility, we adopted a comprehensive 'gain of function' approach to identify critical residues of Gal2 [5–7,13]. Position 352 of Gal2 was already implicated as a critical site from the results of site-directed mutagenesis studies with mammalian Glut1 and Glut4 and yeast Hxt3 [19–22], whereas our studies revealed the importance of positions 446 and 504. Since sites 352 and 504 are the most highly conserved aromatic positions among 63 sugar transporters and related proteins, the importance of these sites and of the interactions between them may also be common to other sugar transporters. The topological location and importance of sites 352, 446 and 504 in Gal2 may be relevant to the model of Widdas [23] in which TM7, TM10 and TM12 form a portion of an opening cleft of Glut1, to a C-terminal channel model of Baldwin [24] where TM7 and TM10 form a part of the glucose permeation pathway and TM7 is adjacent to TM12, or to a model of a lactose permease of *E. coli* (also composed of 12 transmembrane segments) in which TM7 and TM10 are adjacent, with TM12 not far from them [25].

Our present data thus confirm that at least three aromatic amino acids are essential for galactose transport activity of Gal2. Whereas Tyr^{446} is indispensable for such activity, two aromatic amino acids at sites 352 and 504 are also important. In addition, specific combinations of aromatic amino acids at positions 352 and 504 are necessary for transport activity, suggesting that these two sites might be in close proximity and contribute to the galactose permeation pathway.

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