Three Aromatic Amino Acid Residues Critical for Galactose Transport in Yeast Gal2 Transporter*

Toshiko Kasahara and Michihiro Kasahara‡

From the Laborary of Biophysics, School of Medicine, Teikyo University, Hachioji, Tokyo 192-0395, Japan

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Tyr^446 in putative transmembrane segment 10 (TM10) of the yeast galactose transporter Gal2 has previously been identified as essential for galactose recognition. In the present study, alignment of the amino acid sequences of 63 sugar transporters or related proteins revealed 14 aromatic sites, including Tyr^446 of Gal2, that are conserved in >75% of these proteins. The importance of the remaining 13 conserved aromatic amino acids was examined individually by random mutagenesis using degenerate primers. Galactose transport-positive clones were identified by plate selection and subjected to DNA sequencing. For those transport-positive clones corresponding to Tyr^352, and Phe^504 mutants, all the amino acid substitutions comprised aromatic residues. The importance of the aromatic residues at these sites was further investigated by replacing them individually with each of the other 19 amino acids and measuring the galactose transport activity of the resulting mutants. Among both Tyr^352 and Phe^504 mutants, the other aromatic amino acids supported galactose transport; no other amino acids conferred high affinity transport activity. Thus, at least three aromatic sites are critical for galactose transport: one at the extracellular boundary of putative TM7 (Tyr^352), one in the middle of putative TM10 (Tyr^446), and one in the middle of putative TM12 (Phe^504).

The yeast Saccharomyces cerevisiae exhibits substantial hexose transport activity (1, 2) and possesses a large number of sugar transporter and sugar transporter-related genes (3–5). The sugar transporter family in S. cerevisiae is not well delineated, and, in the present study, for clarity, the cluster I of 18 sugar permease homologs is referred to as the Glut family of S. cerevisiae (4). This family includes Hxt2, a major high affinity glucose transporter, and Gal2, a major high affinity galactose transporter that also transports glucose with a similar affinity (6). Through comprehensive studies of chimeras of Gal2 and Hxt2, we have previously shown that Tyr^446 is essential and Trp^455 is important for galactose recognition by Gal2 (6–8). Both of these residues are located in putative transmembrane segment 10 (TM10) of Gal2. We also showed that Phe^431 of Hxt2, which corresponds to Tyr^446 of Gal2, is critical for glucose transport; among the other amino acids, only Tyr was able to support transport activity (9). The importance of the aromatic amino acids in this position was confirmed with a mammalian sugar transporter belonging to the Glut family. Thus, in the rat Glu1 glucose transporter, Trp^368, which corresponds to Trp^455 of Gal2, is essential and Phe^509, which corresponds to Tyr^446 of Gal2, is important for glucose transport (10).

Recent crystallographic studies of binding proteins in the periplasm of bacteria (11), of lectins (12), and of sugar-specific porins (13, 14) have shown that the binding of sugars to these proteins is mediated by hydrogen bonding to various amino acids and to H2O as well as by stacking of the sugars with aromatic amino acids. The three-dimensional structures of maltoporins (13) and the sucrose-specific porin ScrY (14) 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. Since we have shown that two aromatic amino acids are important for sugar recognition in Gal2, it is of interest to determine whether other aromatic residues in this transporter, especially those present in transmembrane segments, are also required for activity.

Gal2 possesses 77 aromatic amino acid residues, so it was not feasible to study all of them. Instead, we surveyed conserved aromatic sites with the use of the DOMO data base (15). We identified 14 sites as conserved in >75% of 63 sugar transporter-related proteins. Since one of these 14 conserved aromatic sites in Gal2 was Tyr^446, which has previously been shown to be essential for galactose recognition, we investigated the remaining 13 sites in this study. Mutation and expression analysis revealed that, in addition to Tyr^446, the aromatic sites Tyr^352 and Phe^504 are critical for galactose transport.

Experimental Procedures

Construction of Cassette Vectors—Construction of the plasmid GAL2-pTV3e, for expression of GAL2 by a GAL expression system in the multicopy plasmid pTV3 (YEp TRP1 bha), was described previously (6). Briefly, GAL2 was modified (i) to disrupt an EcoRI site situated at the boundary of Gal2 and the vector, (ii) to create a CloI site immediately downstream of the termination codon, and (iii) to generate a new EcoRI site downstream of the initiation codon, thereby yielding the cassette vector GAL2-pTV3e. This vector was further modified to create or disrupt restriction enzyme sites, without changing the encoded amino acid sequence, as follows. (i) To create a SalI site between the sequences encoding TM8 and TM9 at nucleotides (nt) 1172–1177 (numbering relative to the first nucleotide of the open reading frame of GAL2 as position 1), the nucleotide sequence was changed from GCCGTC to GTGCAG, yielding GAL2A-pTV3e. (ii) To create a SalI site between TM9 and TM10 at nt 1278–1283, the sequence was changed from ATCTTC to GAGCTC, yielding GAL2B-pTV3e. (iii) To disrupt a BamHI site between TM6 and TM7 at nt 873–878, the sequence was changed from GGATCC to GAGCTC, yielding GAL2C-pTV3e. (iv) To create four restriction enzyme sites (SnuI between TM9 and TM10, MfI1 between TM10 and TM11, SpeI between TM11 and TM12, and NcoI in the middle of the COOH-terminal tail), four silent mutations were introduced as described previously (7), yielding Gal2D-pTV3e.

Mutagenesis—We used a polymerase chain reaction (PCR)-based method to prepare site-directed mutants (8, 16). Briefly, three primers

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‡ To whom correspondence should be addressed: Laboratory of Biophysics, School of Medicine, Teikyo University, Hachioji, Tokyo 192-0395, Japan. Tel.: 81-426-78-3261; Fax: 81-426-78-3262; E-mail: kasahara@main.teikyo-u.ac.jp.

The abbreviations used are: TM, transmembrane segment; nt, nucleotide(s); PCR, polymerase chain reaction; GFP, green fluorescent protein.
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were used: one mutagenic primer and two nonmutagenic primers that lie outside both the region to be mutated and the two restriction enzyme sites that sandwich this region. In a first round PCR, the mutagenic primer and one nonmutagenic primer were used to amplify a partial fragment of the target region. In a second round PCR, the product of the first PCR and the second nonmutagenic primer were used to amplify the entire region. PCR was performed with native Pfu polymerase (Stratagene) and a GeneAmp PCR system 2400 (Perkin-Elmer). Two restriction enzyme sites were used to replace wild-type GAL2 with PCR fragments.

Mutagenic primers were constructed by replacing each aromatic amino acid codon with three consecutive equal mixtures of the four nucleotides and are as follows: 5'-GGAACTTNTNNTGGAAGHTTCGTACCAAT (Trp352), 5'-CTCGAATCCCCGTTNTTGGAGTTCGTGTG (Trp352), 5'-ACTGACNNNNAAAAAAAAATNTGTGAC (Tyr314), 5'-AAGGCTACNNGTGGAGAATAG (Arg241), and 5'-CACTAGCCATTNTNNGTGAGTTTCGTACCAAT (Trp352). These primers were shown to produce the correct replacement of the PCR template and the two nonmutagenic primers 5'-AACGGTACCNNNGTAGAAAAAATAATTG (Tyr352), 5'-TNNNGTCTTTTTCTTTG (Tyr514), and 5'-NNNATTTTCTGTTATG (Tyr442), 5'-GCCATGTTTTTTTATGTCN (Trp455), 5'-CCATGCTTTTTTCTGTTATG (Trp455), 5'-AGGGCCGCGTTCGGCGG (Ser242). The PCR products were digested with BglII and SalI (between TM6 and TM7) and ligated to the COOH terminus of the initiation codon of EGFP in order to shift the NcoI site to the outside of the open reading frame. A CiaI site was created immediately downstream of the termination codon of EGFP by changing TAAAGCGG to TATTCGAT. The sequence of a NcoI site of GAL2D-pTV3es was changed to CTCTTG, and a new NcoI site was created immediately upstream of the termination codon of GAL2 by adding CCATGG. EGFP was cut out with NcoI and CiaI and ligated to the COOH terminus of GAL2 in GAL2D-pTV3es to obtain pGAL2-EGFP. As a result, pGAL2-EGFP has a NcoI site at the end of GAL2, and the sequence coding for two amino acids, Pro-Trp, was inserted at the boundary. Galactose transport activity of GFP-tagged wild-type Gal2 was not significantly different from untagged Gal2 (93 ± 5.8%, mean ± S.E., n = 5). To make GFP-tagged Gal2 mutants, mutated GAL2D-pTV3es was cut out with EcoRI and MutI for Tyr352 mutants or with EcoRI and SmaI for Phe357 mutants, and those replaced the corresponding part of pGAL2-EGFP.

Modified portions of all clones selected in this study were verified by sequencing with an automated DNA sequencer (model 373A; Perkin-Elmer).

Transport Assay—All cells were grown to log phase (optical density at 650 nm, 0.3–0.4) at 30 °C in S(trp) medium supplemented with 2% galactose. Transport of galactose or glucose in LBY416 harboring the various GAL2 plasmids was measured at 30 °C for 5 s as described (6, 9). Transport activities with 0.1 mM D-[14C]glucose were expressed as pmol/107 cells/5 s. For comparison, the background obtained with control cells harboring the empty vector was subtracted from the transport activity obtained with each clone; the activity was then normalized by expression as a percentage of that obtained with cells expressing wild-type Gal2. We considered values of >10% as significant (9).

Fluorescence Microscopy—Living cells in early exponential to midexponential phase were examined with an epifluorescence microscope (DMR, Leica) equipped with a filter cube L5. Micrographs were recorded on film (T-MAX p3200, Kodak) and processed for display with Adobe Photoshop and Canvass (Deneva Software). It was observed that the fluorescence intensity of an individual cell was heterogeneous as noted previously (18, 19), and representative cells are shown here in the figures. As a control, ANS6-2D (MATa sec6-4 ura3-52 leu2-3, 112 trp1-289 his3/4) was used to confirm that Gal2-GFP is localized at the plasma membrane. At the restrictive temperature, the secretary vesicle.
RESULTS

Alignment of the amino acid sequences of 63 sugar transporters and sugar transporter-related proteins (DM00135) in the DOMO database (15) revealed that 23 sites contained aromatic residues in >50% of the proteins (Fig. 1). Fourteen of these sites contained aromatic amino acids in >75% of the proteins (Fig. 2). Since one of these 14 sites, Tyr446, has already been identified as essential for galactose transport by Gal2 (8), we investigated the remaining 13 sites in the present study.

Each codon corresponding to these 13 sites was replaced individually with a mixture of the four nucleotides, and each of the resulting 13 series of mutants was expressed in LBY416 cells, in which Gal2 is defective and the two glucose transporter-related genes HXT2 and SNF3 are disrupted. With the use of plate selection, galactose transport-positive clones were collected and subjected to DNA sequencing. To ensure a sufficient number of clones, each series was expressed in at least four independent experiments. The numbers of amino acid residues present at these 13 conserved aromatic sites of Gal2 in galactose transport-positive clones are shown in Table I.

The numbers of amino acid residues present at 13 highly conserved aromatic sites of Gal2 in galactose transport-positive clones

Table I

| Replaced residue | Mutated residue |
|------------------|----------------|
| Gly              | Trp241 | Tyr268 | Tyr351 | Tyr352 | Phe357 | Trp442 | Phe462 | Trp479 | Phe483 | Phe504 | Tyr514 | Phe516 |
| Asl              | 1      | 2      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      |
| Leu              | 1      | 2      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      |
| Tyr              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Trp              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Cys              | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      | 1      |
| Pro              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Ser              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Thr              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Asn              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Gln              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Asp              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Gld              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| His              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Lys              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Arg              | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     |
| Total            | 29     | 21     | 23     | 19     | 19     | 19     | 19     | 19     | 19     | 19     | 19     | 19     |
| Aromatic (%)     | 38     | 52     | 13     | 100    | 5      | 63     | 33     | 94     | 14     | 44     | 100    | 41     | 38     |

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Table I
number of mutants, we used >700 (most often, 1000–2000) of the transformants for selection. The numbers of galactose transport-positive clones possessing various amino acids in the targeted positions of Gal2 are shown in Table I. For Tyr^{352} and Phe^{504} mutants, only aromatic amino acids were detected among the galactose transport-positive clones. For Phe^{462} mutants, all but one (94%) of the replacement residues were aromatic. We therefore selected these three sites as candidates for critical aromatic residues.

Each of these three amino acids was individually replaced with each of the other 19 amino acids, and the resulting mutants were expressed in LBY416. Only 2 of the 19 Tyr^{352} mutants, those containing Phe or Trp, exhibited significant (>10%) transport activity (Fig. 3). The galactose and glucose transport activities of the Tyr^{352} → Phe mutant were 34 and 42%, respectively, relative to the values for wild-type Gal2, whereas the Tyr^{352} → Trp mutant mediated galactose transport at a level of 13% of the wild-type activity.

Among the Phe^{504} mutants, only those containing aromatic amino acids exhibited galactose transport activity; the activities of the Phe^{504} → Tyr and Phe^{504} → Trp mutants were 60 and 42%, respectively, of the value for wild-type Gal2 (Fig. 4). With regard to glucose transport, in addition to Tyr (59%) and Trp (27%), several other amino acids (Leu, Ile, Met, Gln, and Glu) at position 504 conferred low, but significant, activity (<20%). Thus, an aromatic amino acid at position 462 is not essential for galactose transport activity.

The differences in transport activities of the various Gal2 mutants were not due to differences in the extent of protein expression (Fig. 6), as revealed by immunoblot analysis of cell homogenates with antibodies to the COOH terminus of Gal2. All of the mutants were detected as a predominant immunoreactive band of 53 kDa, corresponding to the position of wild-type Gal2. Although the levels of expression were not identical, they were similar for most mutants. The extents of expression of the Asp mutant of Tyr^{352} and Phe^{504}, as well as of the Asp, Lys, and Arg mutants of Phe^{462} were substantially lower than those of the other mutant proteins.

Another possibility that mutated Gal2s were subjected to mistrafficking was checked by GFP-tagged Gal2. Fig. 7 shows that the cell contour of all of the GFP-tagged Tyr^{352} mutants was fluorescent. In addition, when fluorescence intensity was high, intracellular structures probably corresponding to central vacuole or small vesicles also emerged. As a control, expression of GFP-tagged wild-type Gal2 in sec6 cells was observed. At the restrictive temperature, fluorescence was accumulated in intracellular vesicles (sec6, 37 °C) and at the permissible temperature, it shifted to cell contour (sec6, 25 °C), indicating localization of Gal2 in plasma membrane. Fig. 8 shows that, similar to GFP-tagged Tyr^{352} mutants, the cell contour of all of the GFP-tagged Phe^{504} mutants was fluorescent, indicating also that trafficking of Phe^{504} mutants was normal.

The kinetic parameters of some of the Gal2 mutants that showed significant transport activity were measured (Table II). Compared with wild-type Gal2, all of the mutants examined, with the exception of the Phe^{462} → Tyr mutant, showed either increased $K_m$ values or decreased $V_{max}$ values or both; the kinetic parameters of the Phe^{462} → Tyr mutant were similar to those of the wild-type protein, as was expected from the data shown in Fig. 5. Galactose transport in Tyr^{352} mutants was measured with a high substrate concentration of 10 mM. Only Tyr, Phe, and Trp supported galactose transport even with 10 mM. On the other hand, most of the Phe^{504} mutants (except for Gly, Ala, Asn, Glu, His, and Lys) exhibited galactose transport activity with 10 mM galactose and consisted of three groups:

![Graph showing transport activity of different mutants](image_url)
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FIG. 7. Localization of Tyr<sup>352</sup> mutants of Gal2 tagged with GFP. LBY416 cells harboring plasmids encoding each of GFP-tagged Tyr<sup>352</sup> mutants (shown by single-letter code) were cultured to log phase and observed with a fluorescence microscope. Representative cells are shown. As a control, sec6 cells expressing GFP-tagged wild-type Gal2 were cultured overnight at 25 °C, shifted from S(trp) medium supplemented with 2% glucose to S(trp) medium supplemented with 2% galactose, and cultured at the permissive temperature (25 °C) or at the restrictive temperature (37 °C) for 12 h, and fluorescent images and Nomarski image were photographed.

We have previously shown that two aromatic amino acids in putative TM10 are important for sugar transport not only in the yeast transporters Gal2 and Hxt2 (8, 9) but also in the mammalian transporter Glut1 (10). These three proteins belong to the Glut family, which includes passive sugar transporters of mammalian tissues and passive or active sugar/H<sup>+</sup> transporters from higher plants, green algae, protozoa, yeast, cyanobacteria, and eubacteria (21–24). To investigate further the role of aromatic amino acids in sugar transport by members of the Glut family, we searched for aromatic sites that are conserved among sugar transporters and related proteins in the DOMO data base. Fourteen such sites were conserved in >75% of the 63 proteins listed. One of these 14 sites corresponded to Tyr<sup>446</sup> in Gal2, which we have previously shown to be essential for galactose transport (8). We therefore investigated the remaining 13 sites in Gal2, including Trp<sup>455</sup>, a position at which aromatic amino acids as well as several others are tolerated (8).

Random site-directed mutagenesis at each of the 13 conserved sites and selection of galactose transport-positive clones identified three residues, Tyr<sup>352</sup>, Phe<sup>504</sup>, and Phe<sup>462</sup>, as candidates for essential aromatic sites. We then prepared three series of mutants by replacing each of these three residues individually with each of the other 19 amino acids and measured the transport activities of these mutants. At position 352, located at the extracellular boundary of putative TM7, aromatic amino acids were essential not only for galactose transport but also for glucose transport. All 63 proteins whose sequences were aligned contain an aromatic amino acid at this site (Fig. 1). Replacement of Tyr at this site in the mammalian glucose transporter Glut1 with Ile rendered the protein inactive, whereas substitution with Phe had no effect on transport activity (25). Olsowski et al. also showed that Tyr at this site was irreplaceable using Cys-less Glut1 (26). In Glut4, replacement of Tyr at this site with Phe abolished transport activity but did not affect iodo-4-azidophenetyl-amido-7-O-succinyl-deacetyl-forskolin binding (27). Deletion of Tyr abolished glucose transport activity of yeast glucose transporter Hxt3 (28). These observations are consistent with our results showing that an aromatic amino acid at this site is essential for transport activity. In addition, the region between TM7 and TM8 has been proposed as a substrate recognition site in mammalian glucose transporters. Doege et al. (29) suggested that Ser<sup>294</sup> and Thr<sup>295</sup> in the exofacial loop between TM7 and TM8 of Glut4 are important in the conformational changes that occur during transport. Seatter et al. (30) have proposed that a conserved motif, QLS, in TM7 contributes to substrate recognition on the basis of results obtained by exchanging this motif between Glut2 and Glut3. Thus, the TM7–TM8 region may be important for sugar transport, particularly for substrate recognition.

Position 504 (Phe) in Gal2 was also shown to be a critical aromatic site for galactose transport. Consistent with the results obtained by plate selection, with a low substrate concentration, only aromatic amino acids at this site were able to support galactose transport, although some nonaromatic residues (Leu, Ile, Met, Gln, and Glu) conferred reduced, but significant, glucose transport activity. With the high galactose concentration of 10 mM, some of the Phe<sup>504</sup> mutants showed significant transport activities. In accord with these observations, in yeast Hxt3 (28), replacement of Phe<sup>490</sup>, which corresponds to this site, with Ile conferred significantly weaker glucose uptake as observed by cell growth on glucose plate. These results suggest that aromatic amino acids at this site contribute to discrimination between galactose and glucose, although this site does not appear as critical as is Tyr<sup>446</sup> in this regard (8).

Despite the fact that Phe<sup>462</sup> appeared to be a candidate for an essential aromatic site on the basis of characterization of the galactose transport-positive clones, several nonaromatic amino acids exhibiting more than 75% of the activity (Trp and Tyr), those of 30–45% (Leu, Val, and Cys), and those of 10–20% (Ile, Met, Pro, Ser, Thr, Gln, Asp, and Arg). These results suggest that a major role of Phe<sup>504</sup> in galactose transport is on K<sub>m</sub>, but kinetic parameters consistent with the above observations were not obtained, due to low activities of most mutants.

DISCUSSION

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DISCUSSION

We have previously shown that two aromatic amino acids in putative TM10 are important for sugar transport not only in the yeast transporters Gal2 and Hxt2 (8, 9) but also in the mammalian transporter Glut1 (10). These three proteins belong to the Glut family, which includes passive sugar transporters of mammalian tissues and passive or active sugar/H<sup>+</sup> transporters from higher plants, green algae, protozoa, yeast, cyanobacteria, and eubacteria (21–24). To investigate further the role of aromatic amino acids in sugar transport by members of the Glut family, we searched for aromatic sites that are conserved among sugar transporters and related proteins in the DOMO data base. Fourteen such sites were conserved in >75% of the 63 proteins listed. One of these 14 sites corresponded to Tyr<sup>446</sup> in Gal2, which we have previously shown to be essential for galactose transport (8). We therefore investigated the remaining 13 sites in Gal2, including Trp<sup>455</sup>, a position at which aromatic amino acids as well as several others are tolerated (8).

Random site-directed mutagenesis at each of the 13 conserved sites and selection of galactose transport-positive clones identified three residues, Tyr<sup>352</sup>, Phe<sup>504</sup>, and Phe<sup>462</sup>, as candidates for essential aromatic sites. We then prepared three series of mutants by replacing each of these three residues individually with each of the other 19 amino acids and measured the transport activities of these mutants. At position 352, located at the extracellular boundary of putative TM7, aromatic amino acids were essential not only for galactose transport but also for glucose transport. All 63 proteins whose sequences were aligned contain an aromatic amino acid at this site (Fig. 1). Replacement of Tyr at this site in the mammalian glucose transporter Glut1 with Ile rendered the protein inactive, whereas substitution with Phe had no effect on transport activity (25). Olsowski et al. also showed that Tyr at this site was irreplaceable using Cys-less Glut1 (26). In Glut4, replacement of Tyr at this site with Phe abolished transport activity but did not affect iodo-4-azidophenetyl-amido-7-O-succinyl-deacetyl-forskolin binding (27). Deletion of Tyr abolished glucose transport activity of yeast glucose transporter Hxt3 (28). These observations are consistent with our results showing that an aromatic amino acid at this site is essential for transport activity. In addition, the region between TM7 and TM8 has been proposed as a substrate recognition site in mammalian glucose transporters. Doege et al. (29) suggested that Ser<sup>294</sup> and Thr<sup>295</sup> in the exofacial loop between TM7 and TM8 of Glut4 are important in the conformational changes that occur during transport. Seatter et al. (30) have proposed that a conserved motif, QLS, in TM7 contributes to substrate recognition on the basis of results obtained by exchanging this motif between Glut2 and Glut3. Thus, the TM7–TM8 region may be important for sugar transport, particularly for substrate recognition.

Position 504 (Phe) in Gal2 was also shown to be a critical aromatic site for galactose transport. Consistent with the results obtained by plate selection, with a low substrate concentration, only aromatic amino acids at this site were able to support galactose transport, although some nonaromatic residues (Leu, Ile, Met, Gln, and Glu) conferred reduced, but significant, glucose transport activity. With the high galactose concentration of 10 mM, some of the Phe<sup>504</sup> mutants showed significant transport activities. In accord with these observations, in yeast Hxt3 (28), replacement of Phe<sup>490</sup>, which corresponds to this site, with Ile conferred significantly weaker glucose uptake as observed by cell growth on glucose plate. These results suggest that aromatic amino acids at this site contribute to discrimination between galactose and glucose, although this site does not appear as critical as is Tyr<sup>446</sup> in this regard (8).

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acids were subsequently shown to support transport activity. It is not clear why mutants with Met, Pro, or Ser at this position, which showed much higher transport activity than did the mutant containing Ala, were not detected in the original screening. It may be possible that the four nucleotides used for preparing random primers for the Phe462 mutants were not equally mixed. We found that the first nucleotide of the codon corresponding to Phe462 was biased toward T (2 A, 12 T, 8 G, and 4 C) when 26 clones from E. coli transformants were sequenced, which might explain the low frequencies of Met and Pro but not that of Ser.

The role of Trp in mammalian Glut1 has been investigated with the use of cytochalasin B, a potent inhibitor of this transporter that covalently binds to one or more Trp residues of the protein after photolysis. Two of the six Trp residues of Glut1 were shown to be important for transport activity (31–33). Substitution of Trp412 in putative TM11 markedly reduced glucose transport activity, whereas substitution of Trp388 in putative TM12 resulted in a less pronounced decrease in activity. Substitution of both Trp residues resulted in a complete loss of covalent labeling by cytochalasin B but did not result in complete inhibition of cytochalasin B binding or of glucose transport activity; the precise roles of these residues thus remained unclear (34). In Gal2, Trp455 and Trp479, the residues corresponding to Trp388 and Trp412 of Glut1, were not identified as essential in the present study. The reason for this apparent difference between Glut1 and Gal2 is not clear, but it may be attributable to the difference in fine structure between the two proteins. In this regard, whereas cytochalasin B inhibits its glucose transport by Glut1, it does not inhibit that mediated by Hxt2 (35). Moreover, unlike the corresponding residues in Gal2 or Hxt2, we have previously shown that Trp388 of Glut1 is essential for transport activity (10).

In conclusion, our present and previous data indicate that at least three aromatic sites in Gal2 are critical for galactose transport activity: Tyr446 (at the extracellular boundary of TM7), Tyr446 (in the middle of TM10), and Phe504 (in the middle of TM12). The positions corresponding to Tyr382 and Phe462 in Gal2 represent the two most highly conserved aromatic sites in the 63 sugar transporters and sugar transporter-related proteins examined (Fig. 1), which suggests that they also may be critical in these other proteins. At the site corresponding to Tyr446 of Gal2, only 4 of the 63 proteins contain a nonaromatic residue: His in human and rabbit Glut5, Glu in YFIG_BACSU, and Leu in SGTP2. Histidine is occasionally present in sugar binding sites (12), and the functions of YFIG_BACSU and SGTP2 are not known. The conservation of these three aromatic sites among sugar transporters of this family may indicate that they coordinate the transmembrane passage of sugars.

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FIG. 8. Localization of Phe504 mutants of Gal2 tagged with GFP. LBY416 cells harboring plasmids encoding each of GFP-tagged Phe504 mutants were cultured to log phase and fluorescent images were observed as described in Fig. 7 and under “Experimental Procedures.”

| Protein         | Galactose | Glucose |
|-----------------|-----------|---------|
|                 | $K_m$     | $V_{max}$ | $K_m$     | $V_{max}$ |
| Wild type       | 4.8 ± 0.4 | 450 ± 10 | 2.4 ± 0.3 | 385 ± 45 |
| Tyr352 → Phe    | 10.0 ± 0.8| 370 ± 45 | 4.6 ± 2.2| 300 ± 15 |
| Trp382 → Trp    | 11.6 ± 0.8| 235 ± 15 | ND        | ND       |
| Phe462 → Met    | 5.4 ± 0.9 | 110 ± 15 | 1.2 ± 0.1 | 90 ± 5   |
| Phe462 → Tyr    | 5.7 ± 0.3 | 445 ± 35 | 2.6 ± 0.3 | 400 ± 35 |
| Phe504 → Trp    | 14.4 ± 2.4| 565 ± 80 | 2.9 ± 0.5 | 235 ± 35 |
| Phe504 → Tyr    | 6.7 ± 0.9 | 380 ± 45 | 4.9 ± 0.8 | 545 ± 45 |

*ND, not determined due to low activity.*
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