Amino Acid Residues Responsible for Galactose Recognition in Yeast Gal2 Transporter*

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A novel, systematic approach was used to identify amino acid residues responsible for substrate recognition in the transmembrane 10 region of the Gal2 galactose transporter of Saccharomyces cerevisiae. A mixture of approximately 25,000 distinct plasmids that encode all the combinations of 12 amino acids in transmembrane 10 that are different in Gal2 and the homologous glucose transporter Hxt2 was synthesized. Selection of galactose transport-positive clones on galactose limited agar plates yielded 19 clones, all of which contained the Tyr446 residue found in Gal2. 14 of the 19 clones contained Trp455 found in Gal2, whereas the other 5 contained Cys455, a residue not found in either Gal2 or Hxt2. When Tyr446 of Gal2 was replaced with any of the other 19 amino acids, no galactose transport activity was observed in the resulting transporters, indicating that Tyr446 plays an essential role in the transport of this sugar. Replacement of 2 amino acids of Hxt2 with the corresponding Tyr446 and Trp455 of Gal2 allowed the modified Hxt2 to transport galactose. The Km of galactose for the modified transporter was 8-fold higher than that of Gal2. These results and other evidence unequivocally show that Tyr446 is essential and Trp455 is important for the discrimination of galactose versus glucose.

Site-directed mutagenesis has been extensively used in attempts to determine functional sites in transporters (1, 2). This approach is limited, however, by the fact that it is usually not possible to mutate every amino acid and replacements that are made often yield results that are negative in nature (3, 4). As an alternative method, the use of chimeras to identify functional domains of transporters has proved highly fruitful (5–9). We have used chimeras to analyze two homologous sugar transporters in the yeast Saccharomyces cerevisiae (3, 4): Gal2, a high affinity galactose transporter (10) that was unexpectedly found to transport glucose with nearly the same affinity (3), and Hxt2, a major glucose transporter that does not transport galactose (3, 10). These two transporters belong to the Glut transporter family, the largest known organic solute transporter family comprising more than 80 transporters found in prokaryotes through mammals (11, 12). Creating chimeras between the Gal2 and Hxt2 transporters gave us an opportunity to study the galactose recognition site in Gal2 and, to gain insights into the substrate recognition sites in Glut family transporters in general. To unequivocally determine the substrate recognition site, we have taken two steps. In the first step (3), three types of systematic chimeras were made using the Escherichia coli homologous recombination system. The site responsible for differentially recognizing galactose and glucose was localized to a 101-amino acid region that includes the transmembrane 10 (TM10).† TM11, and TM12 segments and the proximal half of the C-terminal hydrophobic tail. In the second step (4), the 101-amino acid region was subdivided into the above four regions by introducing five restriction enzyme sites into the corresponding segments of each gene without changing the amino acids encoded. By analyzing plasmids containing all the possible combinations of these segments inserted into the corresponding parts of Hxt2, we identified TM10 as the domain where galactose and glucose are differentially recognized. TM10 contains 35 amino acid residues, of which only 12 are different between Gal2 and Hxt2. Thus, it is reasonable to assume that the amino acid residue(s) essential for the substrate recognition can be found among these 12 residues. We employed a new comprehensive approach and found that 2 amino acid residues in TM10 are important for substrate recognition.

EXPERIMENTAL PROCEDURES

Production of GAL2 and HXT2 Cassette Vectors—A DNA fragment containing GAL2 was cut out by PmaI and EcoRI and ligated to SmaI and EcoRI sites in a multicloning site of pTV3, a Yep vector (3). The nucleotide sequence immediately following the initiation codon was modified from ATGGCCAGTTGAG to ATGGCCAGAATTC to create an EcoRI site, which changes the amino acid sequence from Met-Ala-Val-Glu to Met-Ala-Glu-Phe. The nucleotide sequence immediately following the termination codon, TAATGCCGTT, was modified to TAATCGGATT to create a CiaI site. These two restriction enzyme sites were used to replace the open reading frame of GAL2 with HXT2. To do this, the nucleotide sequence immediately following the termination codon of HXT2 was modified from TAAGAGATT to TAATCGGATT to create a CiaI site. Because HXT2 has an EcoRI site extending from the 7th to the 12th nucleotides of the coding sequence, the EcoRI site and the aforementioned CiaI site were used to replace GAL2 with HXT2. Plasmids were introduced into LBY416 (MATa hxt2::LEU2 snf3::HIS3 gal2 lys2 ade2 trp1 his3 leu2 wu3) (3).

Multiple Mixed Mutagenesis—Two set of degenerate PCR primers (see Fig. 1), a forward primer containing 84 nucleotides, and a reverse primer containing 56 nucleotides were synthesized to create a mixture of 24,576 (243 × 241) clones that encode all the possible combinations of amino acid residues in the TM10 region that are different in Gal2 and Hxt2. When designing PCR primers, the codons were changed to reduce the number of distinct primers: 1) at locations where the codons for the same amino acid in Gal2 and Hxt2 were different, only one of the codons was adopted, and 2) at locations where corresponding amino acids were different, the codon that required the synthesis of the minimal number of degenerate primers were employed. This is a modification of the method of random mutagenesis using degenerate oligonucleotides (13, 14). PCR reactions using Taq polymerase were performed (2400, Applied Biosystems) without adding a DNA template because the two

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incubation for 4–5 days at 30 °C on galactose limited agar plates con-
LBY416 and the galactose transport-positive clones were selected after
idues between Gal2 and Hxt2. The PCR products were substi-
ted for the corresponding TM10 region of HXT2, and these
clones were observed among the galactose transport-positive
clones. 3) In addition, random mutagenesis of Tyr440 in a mod-
ified Hxt2 containing Tyr431 yielded 13 Trp440, 3 His440, and 1
Leu440. No appreciable differences in transport characteristics were
measured for the galactose transport-positive clones. 4) Tyr 446 of Gal2
was necessary, a degenerate PCR primer was prepared in which nucle-
able to both genes are omitted. Galactose transport activity was measured at 30 °C
for s with 0.1 mM [14C]galactose as a substrate (3, 4), and after subtracting the values obtained with cells harboring the vector only (pTV3e),
values relative to the original Gal2 transporter were calculated, and the averages of more than three assays are shown at the right. Also shown are galactose transport activities of Hxt2-15 (Hxt2 having Gal2-derived TM10, TM11, and TM12 and the beginning half of the C-terminal region)
(4), Hxt2-8 (Hxt2 having Gal2-derived TM10) (4), and the original Hxt2. Amino acids found in native Gal2 are shown in blue, and amino acids that are not found in Gal2 or Hxt2 are shown in green.

FIG. 1. Galactose transport-positive clones obtained by multiple mixed mutagenesis. The nucleotide and deduced amino acid sequences of TM10 in GAL2 are shown, and when these sequences are different from those of HXT2, the differences are also indicated. Selection of galactose transport-positive clones on galactose limited agar plates yielded 19 clones. Deduced amino acid sequences obtained by nucleotide sequencing of the TM10 region in these 19 clones are shown. Amino acids common to both genes are omitted. Galactose transport activity was measured at 30 °C (5) for 5 s with 0.1 mM [14C]galactose as a substrate (3, 4), and after subtracting the values obtained with cells harboring the vector only (pTV3e),
values relative to the original Gal2 transporter were calculated, and the averages of more than three assays are shown at the right. Also shown are galactose transport activities of Hxt2-15 (Hxt2 having Gal2-derived TM10, TM11, and TM12 and the beginning half of the C-terminal region)
(4), Hxt2-8 (Hxt2 having Gal2-derived TM10) (4), and the original Hxt2. Amino acids found in native Gal2 are shown in blue, and amino acids that are not found in Gal2 or Hxt2 are shown in green.

RESULTS AND DISCUSSION
We have devised a novel method to systematically identify amino acid residue(s) in TM10 that are critical for the sub-
strate recognition. We have tentatively named this method “multiple mixed mutagenesis.” We first used PCR to prepare a mixture of approximately 25,000 distinct sequences that en-
code all the possible combinations of different amino acid resi-
dues between Gal2 and Hxt2. The PCR products were substi-
clones including Gal2 containing Phe^{446}, Trp^{446}, and Tyr^{446} were active in glucose transport. Expression of all the 20 clones in this series was confirmed by immunoblotting of cell homogenates (Fig. 3). Thus, with the change of Tyr^{446} to Phe, the Gal2 transporter is changed to a glucose-specific transporter, the activity of which in the presence of 0.1 mM glucose is about half that of the Hxt2 transporter. 5) Hxt2 containing Tyr^{431} and Trp^{455}, designated Hxt2 (Tyr-Trp), showed almost the same characteristics of galactose transport as Hxt2-8: the $K_m$ and $V_{max}$ of galactose transport in Hxt2 (Tyr-Trp) cells were $39 \pm 6 \text{ mM}$ (mean $\pm$ S.E., $n = 3$) and $1.0 \pm 0.7 \text{ nmol/10}^7 \text{ cells/5 s}$, and those of Hxt2-8 cells were $41 \pm 2 \text{ mM}$ and $0.69 \pm 0.03 \text{ nmol/10}^7 \text{ cells/5 s}$. By contrast, galactose transport in Gal2 cells has a $K_m$ of $5.3 \pm 0.3 \text{ mM}$ and a $V_{max}$ of $0.97 \pm 0.1 \text{ nmol/10}^7 \text{ cells/5 s}$ (4). The fact that Hxt2 (Tyr-Trp) showed almost the same $K_m$ as that of Hxt2-8, which was 8-fold higher than that of Gal2, suggests that there is some other region(s) contributing to galactose recognition in Gal2 but that there are no other critical amino acid residues within TM10. No significant differences in substrate specificity were found in these cells (Fig. 4).

Thus, changing two critical amino acids (Phe^{431} to Tyr and Tyr^{446} to Trp) causes the Hxt2 transporter to recognize galactose in addition to glucose.

Previous studies on the Glut family transporters have overlooked the importance of the amino acid situated at Tyr^{446} in Gal2 and the equivalent residues in other transporters. When TM10 is depicted as forming an a-helix (Fig. 5), Tyr^{446} is situated in the middle of the amino acids that are different in Gal2 and Hxt2, whereas the other side of the a-helix consists of amino acid residues that are common to both Gal2 and Hxt2. Tyr^{446} is not conserved in Glut family transporters that also transport galactose in other organisms including GalP in E. coli (16), STP1 in Arabidopsis thaliana (17), HUP2 in Chlorella kessleri (18), and SGTP1 in Schistosoma mansoni (19). This suggests that there are subtle changes in substrate recognition sites among these homologous transporters. The importance of Trp^{365} of the mammalian Glut1 transporter (corresponding to Trp^{455} of Gal2) has been pointed out previously (20–23). Replacement of Trp with other amino acids changed several aspects of transporter physiology including expression levels (20), targeting (20), reduction in intrinsic activity under certain conditions (21), and forskolin binding (22, 23). It is of particular interest to determine whether the replacement of Trp with other amino acids brings any change in various functional aspects of Gal2 in addition to the substrate recognition. The role of Tyr^{446} and Trp^{455} of Gal2 in substrate recognition may be interpreted in many ways. These amino acid residues may relieve steric hindrance, so that galactose that is excluded from Hxt2 may be accepted by Gal2. However, the fact that Gal2 transporters possessing different amino acids at residue 446 show more strict selectivity for galactose transport compared with glucose transport (Fig. 2) may not be consistent with this idea. It is possible that the two aromatic side chains of Tyr and Trp form hydrogen bonds with galactose. With this interpretation, the structure around C4 of galactose may form a bond primarily with Tyr. Another possibility is that these aromatic residues lie close to galactose (“stacking effect”) as is the case in galactose binding lectins (24). The possibility that these residues play an indirect but crucial role in determining the conformation of amino acid residue(s) directly interacting with galactose is not excluded by this study. It is also possible that
two amino acids may not form a single recognition site but instead function independently. Considerable evidence supports the notion of two substrate binding sites in Glut family transporters (2).

Recently Arbuckle et al. (9) constructed eight chimeras of human Glut2 and Glut3 and used them to show that TM7 is important for substrate recognition. The reason why the region identified for substrate recognition is different from ours is not known. The difference in the results of their study and ours may reflect difference in homologous transporters in human Glut2 and Glut3 as well as other functionally important sites. If the latter possibility proved to be the case, it would suggest that both regions are necessary for the recognition of sugar molecules. It is also possible that TM7 and TM10 contribute to two different binding sites that have different substrate specificities, TM7 for exofacial binding site and TM10 for endofacial binding site (2).

Using a three-step chimera approach, we were able to identify the amino acid residues responsible for the differential recognition of galactose and glucose. At each step we have tried to avoid making assumptions about particular locations. It seems reasonable to expect that this multiple mixed mutagenesis method will be generally applicable to other classes of proteins that have homologous counterparts. This method is an alternative to site-directed mutagenesis and is an appropriate approach for determining not only the substrate recognition site but also other functionally important sites.

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