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 transport 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).1 TM11, and TM12 segments and the proximal half of the C-terminal hydrophilic 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 SmalI and EcoRI sites in a multicloning site of pTV3, a YEp vector (3). The nucleotide sequence immediately following the initiation codon was modified from ATGGCAGTTGAG to ATGGGAAATCC to create an EcoRI site, which changed the amino acid sequence from Met-Ala-Val-Glu to Met-Ala-Val-Glu-Phe. The nucleotide sequence immediately following the termination codon, TAATGCCATT, was modified to TAATCGATT to create a ClaI 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 TAATCGATT to create a ClaI 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 ClaI site were used to replace GAL2 with HXT2. Plasmids were introduced into LBY416 (MATa hxt2::LEU2 snf3::His5 gal2 lys2 ade2 trp1 his3 leu2 ura3) (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 (2^84·3^56) 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 codons 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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1 The abbreviations used are: TM, transmembrane; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid.
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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 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 red, those in native Hxt2 are shown in blue, and amino acids that are not found in Gal2 or Hxt2 are shown in green.

degenerate PCR primers possessed a 10-nucleotide-long complementary overlap. The reactions were started with a preincubation for 4 min at 94 °C, and this was followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 5 min, and elongation at 72 °C for 2 min with and without incubation at 72 °C for 4 min. After cutting both ends with SacI and MluI, the resulting PCR products were used to replace the corresponding TM10 region in HXT2 in the plasmid Hxt2-0 (4). After amplification in E. coli, the plasmid mixture was introduced into LBY416 and the galactose transport-positive clones were selected after incubation for 4–5 days at 30 °C on galactose limited agar plates containing a synthetic medium (15) supplemented with 200 μg/ml galactose. When random mutagenesis of 1 or 2 amino acids in Gal2 or Hxt2 was necessary, a degenerate PCR primer was prepared in which nucleotides were mixed to produce codons for 20 amino acids at the appropriate position(s) in the sequence.

Other Assays—The nucleotide sequence of the replaced fragment and the surrounding regions in each of the clones used in this study was verified by sequencing both strands using a DNA sequencer (373A, Applied Biosystems). Immunoblotting of yeast homogenates (4) and the surrounding regions in each of the clones used in this study was verified by sequencing both strands using a DNA sequencer (373A, Applied Biosystems). Immunoblotting of yeast homogenates (4) and the surrounding regions in each of the clones used in this study was verified by sequencing both strands using a DNA sequencer (373A, Applied Biosystems). Immunoblotting of yeast homogenates (4) and the surrounding regions in each of the clones used in this study was verified by sequencing both strands using a DNA sequencer (373A, Applied Biosystems). Immunoblotting of yeast homogenates (4) and the surrounding regions in each of the clones used in this study was verified by sequencing both strands using a DNA sequencer (373A, Applied Biosystems). Immunoblotting of yeast homogenates (4) and the surrounding regions in each of the clones used in this study was verified by sequencing both strands using a DNA sequencer (373A, Applied Biosystems).

RESULTS AND DISCUSSION

We have devised a novel method to systematically identify amino acid residue(s) in TM10 that are critical for the substrate 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 encode all the possible combinations of different amino acid residues between Gal2 and Hxt2. The PCR products were substituted for the corresponding TM10 region of HXT2, and these constructs were introduced into the galactose transport-negative strain LBY416. Comparison of the deduced amino acid sequences of 19 clones selected as galactose transport-positive clones revealed that Tyr446 was the only location where an amino acid residue from Gal2 was always found (Fig. 1). In addition, the Trp455 of Gal2 was present in 14 of 19 clones, with Cys present at this location in the remaining 5 clones. LBY416 cells harboring each of the 19 clones showed galactose transport (Fig. 1) at levels 8–36% of the original Gal2 transporter, a level of transport comparable with the 17% observed for the chimeric transporter Hxt2-2 (4) containing TM10 derived from Gal2 and the remaining parts from Hxt2.

To confirm that Tyr446 and Trp455 are important for galactose recognition, several experiments were performed. 1) A random mixture of Gal2 clones encoding 20 different amino acid substitutions at Tyr446 and Trp455 was subjected to galactose transport-positive selection. Each of the 15 clones picked up possessed Tyr446 and Trp455. 2) Similarly, when Phe431 and Tyr440 of Hxt2 (corresponding to Tyr446 and Trp455 of Gal2, respectively) were randomly substituted with 20 amino acids, 13 galactose transport-positive clones were found to contain Tyr431. By contrast, 5 Trp440, 3 Cys440, 4 Thr449, and 1 Leu449 clones were observed among the galactose transport-positive clones. 3) In addition, random mutagenesis of Tyr440 in a modified Hxt2 containing Tyr343 yielded 13 Trp440, 3 His440, and 1 Met440 galactose transport-positive clones. 4) Tyr446 of Gal2 was replaced with the other 19 amino acids, and galactose and glucose transport activities were measured (Fig. 2). Only the Tyr446 clone was active in galactose transport, whereas several
Thus, changing two critical amino acids (Phe431 to Tyr and "idences in substrate specificity were found in these cells (Fig. 4). critical amino acid residues within TM10. No significant differ-
ing to galactose recognition in Gal2 but that there are no other
of Gal2, suggests that there is some other region(s) contribut-
to transport galactose in other organisms including GalP in
(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 of the mammalian Glut1 transporter (corresponding to
Trp55 of Gal2) has been pointed out previously (20–23). Re-
placement of Trp with other amino acids changed several as-
pects 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 particu-
lar 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 Tyr446 and Trp455 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 interpreta-
tion, 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 resi-
dues play an indirect but crucial role in determining the con-
formation of amino acid residue(s) directly interacting with
galactose is not excluded by this study. It is also possible that

![Fig. 2. Galactose and glucose transport in Gal2 containing 20 different amino acid residues at the position of 446. Site-directed
mutagenesis of Gal2 at residue 446 was performed using PCR. Galactose (Gal) and glucose (Glc) transport in LBY416 cells harboring one
of these clones was measured for 5 s. After subtracting the value obtained with
the vector only (pTV3e), the values relative to Gal2 (in the case of
galactose transport) or Hxt2 (in the case of glucose transport) were
calculated, and the average and standard error are indicated (n ≥ 3).](image)

![Fig. 3. Expression of Gal2 (X-Trp) series transporters. Gal2 was modified to create 19 clones coding for different amino acids at
the position 446. Cell harboring 20 different clones were cultured in the
same conditions as the transport assays. Cell homogenates (10 μg each)
were immunoblotted with antibody to the Hxt2 C terminus. Autoradiography
of [125I]protein A (IM144, Amersham Corp.) was performed using imaging plates (BAS2000, Fuji Film). The 53-kDa band (53K)
corresponds to Gal2, and the bands immediately below the 53-KDa
band seem to be degradation products, because they increased when
freeze-thawing was repeated.](image)

![Fig. 4. Substrate specificity of Hxt2 (Y-Trp), Hxt2-8, and
Gal2. PCR reaction was used to replace Phe431 and Tyr440 of Hxt2 with
Tyr and Trp, respectively (corresponding to Tyr446 and Trp555 in Gal2).
Galactose transport at 0.1 mM in the presence of a 200-fold excess of the
indicated nonradioactive monosaccharide was performed for 5 s. The
average and S.E. from more than three assays are expressed relative to
the value in the presence of sorbitol, which was used to adjust osmola-
arity. Hxt2-8 indicates plasmid containing HXT2–0, TM10 of which was
replaced with Gal2 (4). For many sugars Gal2 showed stronger inhibi-
tion than Hxt2-8 or Hxt2 (Y-Trp), which may be due to higher
affinity for galactose in Gal2 (4).](image)
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 and yeast or differences in the transport substrates used; Arbuckle et al. used fructose as a discriminating sugar, whereas we used galactose. 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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