Comprehensive Chimeric Analysis of Amino Acid Residues Critical for High Affinity Glucose Transport by Hxt2 of Saccharomyces cerevisiae

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Hexose transport across the plasma membrane is a necessary step in the utilization of monosaccharides by living cells. The yeast Saccharomyces cerevisiae is able to take up glucose over a wide range of extracellular concentrations with the use of abundant hexose transporters (Hxt1 to -17, Gal2) (1, 2). These transporters belong to the major facilitator superfamily (MFS) and contain 12 transmembrane segments (TMs) (3). Hxt2 is a major high affinity facilitative glucose transporter, whereas Hxt1 is a low affinity facilitative glucose transporter (2, 4). The numbers of amino acids in each putative TM and inter-TM loop of Hxt2 are identical to those in the corresponding regions of Hxt1, and the two proteins share >70% amino acid identity in these regions.

We previously investigated which TMs of Hxt2 are important for high affinity glucose transport by the new approach of TM shuffling (4). We randomly replaced, at the DNA level, each of the 12 TMs of Hxt2 with the corresponding segments of Hxt1. Clones encoding transporters with high affinity for glucose were selected by plating transformants on carbon source-limited agar plates. Our results indicate that Leu-201 in TM5 of Hxt2 is most critical for such activity, with either Cys-195 or Phe-198 also being necessary to support maximal activity.

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We have now investigated which of the amino acid residues in TMs 1, 5, 7, and 8 of Hxt2 are important for high affinity, high capacity glucose transport. Our results indicate that Leu-201 in TM5 of Hxt2 is most critical for such activity, with either Cys-195 or Phe-198 also being necessary to support maximal activity.

EXPERIMENTAL PROCEDURES

Construction of Vectors—Construction of the plasmid Hxt22mnx-pVT, which comprises HXT2 under the control of the ADH1 promoter in the multicopy vector pVT102-U (YEp URA3 bia), was previously described (4). In brief, HXT2 was modified by creating MroI, NheI, XhoI, and ClaI sites in the nucleotide sequences corresponding to the NH2-terminal end of TM4, the loop between TM6 and TM7, the loop between TM9 and TM10, as well as immediately downstream of the termination codon, respectively. The expression vector C1578-pVT, which encodes the chimeric transporter C1578 (in which all of the TMs of Hxt2, with the exception of TMs 1, 5, 7, and 8, have been replaced with those of Hxt1), was also described previously (4).

Mutagenesis—Site-directed mutants were prepared with a PCR-based approach. Because a single mutagenic primer was not sufficiently long to cover all the mutagenic sites in each TM, we performed PCR in two steps with a GeneAmp PCR system 2400 (Applied Biosystems). In the first step, performed with Ex Taq polymerase (Takara, Otsu, Japan), two DNA fragments that were designed to possess 7–10 overlapping nucleotides were prepared for mutants of each TM. These fragments encoded all the possible combinations of amino acid residues at the sites that differ between Hxt1 and Hxt2. Mutagenic primers were constructed by replacing each target codon with degenerate sequences. For example, for TM5 mutants, we used T(K)C for site 198, KTA for site 201, and TWT for site 215 (see Fig. 1); for site 195, we used two primers, one for expressing the Hxt2-derived amino acid, Cys (TGT), and the other for expressing the Hxt1-derived amino acid, Leu (TTG), because degenerate sequences at this site would result in the incorporation of unnatural amino acids. In the second PCR step, performed with native Pfu polymerase (Stratagene), the two fragments produced by the first PCR were joined together without a template, as described previously (4). Each final product was used to replace the corresponding region of Hxt2 in C1578-pVT with the use of two restriction enzymes: EcoRI and MroI for TM5 mutants, MroI and NheI for TM4 mutants, and NheI and XhoI for TM7 or TM8 mutants. After amplification in Escherichia coli, plasmids were introduced into S. cerevisiae strain KY73 (MATa hxt1::HIS3::Δhxt4 hxt5::LEU2 hxt2::ΔHIS3 hxt3::ΔLEU2::Δhxt6 hxt7::ΔHIS3 gln22::Δ ura3-52 MAL2 SUC2 MEL) (5).

Plate Selection—Transformants that possess high affinity, high capacity glucose transport activity were selected after incubation of yeast cells for 3 or 4 days at 30 °C on glucose-limited (glucose, 1 mg/ml) agar plates containing a synthetic medium supplemented with adenine.
and amino acids but not with uracil (S0.1D plates) (4). KY73 cells are not able to grow on S2D plates (glucose, 20 mg/ml), in which glucose is the only carbon source. In parallel, the number of transformants was counted on S2Mal plates (maltose, 20 mg/ml). Modified portions of all clones selected in this study were verified by DNA sequencing with an automated sequencer (model 310, Applied Biosystems).

**Transport Assay**—Cells harboring plasmids were grown to log phase (optical density at 650 nm, 0.3–0.4) at 30 °C in S2Mal synthetic liquid medium. Glucose transport by the cells was measured at 30 °C for 5 s as described (6, 7). Transport activities measured at a n-[14C]glucose concentration of 0.1 mM were expressed as picomoles of glucose per 1 × 10^7 cells per 5 s and were corrected for the background activity determined either in the presence of 0.5 mM HgCl₂ or with 0.1 mM L-[1-14C]glucose. In some experiments, transport activity was calculated as a percentage of that obtained with cells expressing C1578.

**Construction of a Three-dimensional Model of Hxt2**—The crystal structure of LacY (pdb code, 1PV7) was used as the basis for construction of a structural model of Hxt2. Hydrophilic residues in the putative transmembrane helices of Hxt2 were aligned with those of LacY, and the structural model of Hxt2 was generated with the Biopolymer module of Insight II (version 2000; Accelrys, San Diego, CA). The initial model structure was energy-minimized with Discover 3 (version 2000; Accelrys) by fixing the Cα atoms of the model until the final root mean square deviation became <0.1 kcal mol⁻¹ Å⁻¹. The optimized complex structure was selected from 100 energy-minimized structures sampled by the molecular dynamics calculations, which were performed with a temperature of 300 K, a cutoff distance of 10 Å, a distance-dependent dielectric constant, and a time step of 1 fs for 100 ps by sampling the conformation every 1 ps with CVFF (Consistent Valence Forcefield) parameters in Discover 3.

**Other Assays**—A crude membrane fraction was prepared from cells as described (8), and immunoblot analysis of this fraction was performed with rabbit polyclonal antibodies specific for the COOH-terminal region of Hxt2 (6) and with [35S]labeled protein A (IM144, Amersham Biosciences). The intensity of bands corresponding to immune complexes was measured with imaging plates (BAS1800II, Fuji Film) (8), within the range proportional to the amount of protein. Cell number was determined with a particle counter (Z2, Coulter). Protein concentration was measured with bicinchoninic acid (Pierce).

**RESULTS**

**Amino Acid Residues in TMs 1, 5, 7, and 8 of Hxt2 Necessary for High Affinity, High Capacity Glucose Transport**—In a previous comprehensive study, we showed that a minimal combination of TMs 1, 5, 7, and 8 of Hxt2 is necessary for high affinity, high capacity glucose transport (4). To determine which amino acid residues in these TMs are responsible for such activity, we shuffled all of the 20 residues in these regions that differ between Hxt2 and Hxt1 (Fig. 1). TM1 contains six such residues, and we generated 5,700 transformants expressing C1578-based proteins corresponding to all possible combinations of residues that differ between Hxt2 and Hxt1 (Fig. 1). Random saturation mutagenesis of these residues yielded 22,000 transformants and 111 colonies were picked up on S0.1D plates. Subsequent DNA sequencing of 54 clones identified 24 independent mutants that exhibited high affinity, high capacity glucose transport activity. Four residues of Hxt2, Leu-61, Leu-201, Leu-357, and Phe-366, were present in >80% of the transporters selected by plate assay (Table 1), with all high affinity, high capacity transporters possessing Leu-201 of TM5. As a first step in the characterization of amino acid residues responsible for high affinity, high capacity glucose transport, we focused on residues in TM5.

**Characterization of All 16 Shuffled Mutants of TM5**—The amino acid sequence of TM5 of Hxt2 differs at only four positions (sites 195, 198, 201, and 215) from that of the corresponding domain of Hxt1 (Fig. 1). We therefore constructed C1578-based proteins corresponding to all 16 possible combinations of the Hxt1 and Hxt2 residues at these four sites. CFLY and cvf denote the chimeric transporters that contain the Hxt2-derived or Hxt1-derived amino acids at all four of these sites, respectively. The positive clones selected by growth of transformants on carbon source-limited (S0.1D) plates encoded the chimeric transporters CFLY, CFLf, CFLY, IFLf, CeLY, and CeLf (Table II), all of which contain Leu-201. In addition to Leu-201, either Cys-195 or Phe-198 was required for growth on S0.1D plates.

We prepared a crude membrane fraction from cells expressing each of these 16 chimeric transporters and examined the extent of transporter expression by immunoblot analysis with antibodies to the COOH-terminal region of Hxt2 (Fig. 2). All 16 chimeras yielded a predominant immunoreactive band at a position (47 kDa) corresponding to that of wild-type Hxt2. Quantitative analysis of the bands corresponding to the 47-kDa proteins revealed an expression range of 77–148% (n = 3) relative to the intensity of the band yielded by C1578.

We measured the glucose transport activities of all 16 chimeras with 0.1 mM D-glucose as substrate (Fig. 3). All eight chimeric transporters containing Leu-201 possessed substantial glucose transport activity (>50% of that of C1578), whereas the activities of the other eight transporters containing Val-201 did not exceed 30% of that of C1578, when transport activities were normalized on the basis of transporter expression as determined by quantitative immunoblot analysis. Glucose transport activities normalized by cell number did not differ significantly from those normalized by the amount of immunoreactive transporter protein.

Kinetic parameters were determined for all 16 chimeras of TM5 of C1578 (Table II). Whereas all the chimeras possessed Kₘ values in the range of high affinity transport, the Vₘₕ values varied substantially. The values for Vₘₕ/Kₘ ventilated well with growth on agar plates. The 16 chimeras could be divided into three groups characterized by Vₘₕ/Kₘ values of >250, 150–250, or <150. In the first group, all chimeras contained Leu-201 and either Cys-195 or Phe-198 and grew on both S2D and S0.1D plates. In the third group, all chimeras contained Val-201 and grew on neither S2D nor S0.1D plates. The two chimeras in the second group, which contained Leu-201 and Hxt1-derived amino acids at sites 195 and 198, grew only on S2D.

To examine the role of Leu-201 in Hxt2, we constructed Hxt2(CFvY), in which Leu-201 of Hxt2 was replaced with Val. No significant difference between CFvY and Hxt2(CFvY) was
found in plate assays and kinetic parameters of glucose trans-
port (Table II).

**DISCUSSION**

With the use of newly developed TM shuffling, we previously
found that at least four TMs of Hxt2 (TM1, TM2, TM7, and 8) are
required for its high affinity glucose transport activity (4). In
the present study, we also adopted a comprehensive approach
(systematic residue shuffling) to evaluate the contributions of
each of the 20 amino acid residues in TMs 1, TM3, TM7, and 8 of the
high affinity glucose transporter Hxt2 that differ from those of
the low affinity glucose transporter Hxt1. All possible combi-
nations of residues in each TM (2 for TM1, 2 for TM3, 5 for TM5, 2 for TM7, and 2 for TM8) were generated by saturation mutagen-
esis (TMs 1 and 8) or specific construction (TMs 3, 5, and 7). Of
these 20 residues, Leu-201 of TM5 appeared to be the most
important for high affinity, high capacity glucose transport
activity, because all such transporters generated in the present
study possessed this residue. The remaining 19 Hxt2 residues
in TMs 1, TM3, TM5, and 8 were not absolutely required for high

| Clone | TM1 | TM2 | TM3 | TM4 | TM5 | TM6 | TM7 | TM8 |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
| C1879 | I   | C   | L   | L   | I   | V   | C   | F   | L   | Y   | V | I | N |
KY73 cells expressing the 16 chimeras of TM5 of C1578, Hxt2, Hxt2(CFvY), or Hxt1 were subjected to plate assays with S2D and S0.1D medium. Cell growth or no growth after incubation for 3–4 days at 30 °C is indicated by (+) or (−), respectively. The cells were grown to log phase at 30 °C in S2Mal synthetic liquid medium, after which glucose transport activity was measured for 5 s at 30 °C. The $K_m$ and $V_{max}$ values (means ± S.E., n = 3) were determined with 1 to 100 mM d-glucose as substrate.

| Transporter | Agar plate | $K_m$ (mm) | $V_{max}$ (pmol/10^7 cells per 5 s) | $V_{max}/K_m$ |
|-------------|------------|------------|-----------------------------------|---------------|
| S2D         | S0.1D      |            |                                   |               |
| CFLf(C1578) | +          | 5.4 ± 0.6  | 1500 ± 180                       | 277           |
| CFLf        | +          | 3.9 ± 0.5  | 1080 ± 140                       | 277           |
| IFLf        | +          | 4.5 ± 0.7  | 1220 ± 130                       | 271           |
| IFLf(C1578) | +          | 4.0 ± 0.3  | 1030 ± 50                        | 258           |
| CcLf        | +          | 4.3 ± 1.0  | 1110 ± 180                       | 250           |
| IcLY        | +          | 5.8 ± 0.5  | 1410 ± 150                       | 252           |
| IcLf        | +          | 5.4 ± 0.7  | 810 ± 60                         | 150           |
| CcFvY       | +          | 4.2 ± 0.3  | 740 ± 20                         | 176           |
| CFvF        |            | 5.6 ± 0.7  | 650 ± 70                         | 116           |
| IFLfV       |            | 5.0 ± 0.3  | 570 ± 50                         | 114           |
| IFLfV(C1578)| +          | 4.7 ± 0.6  | 500 ± 50                         | 106           |
| CcFvF       | +          | 4.6 ± 0.3  | 440 ± 50                         | 96            |
| CcFvF(C1578)| +          | 3.4 ± 0.5  | 390 ± 40                         | 115           |
| IvCFv       | +          | 4.0 ± 0.6  | 420 ± 50                         | 105           |
| IvCFv(C1578)| +          | 5.0 ± 0.7  | 260 ± 40                         | 52            |
| IvCFv(C1578)| +          | 3.3 ± 0.2  | 170 ± 10                         | 52            |
| Hxt2(CFLf)  | +          | 3.6 ± 0.3  | 1280 ± 50                        | 356           |
| Hxt2(CFvF)  | +          | 3.6 ± 0.3  | 440 ± 40                         | 122           |
| Hxt1(lcFv)  | +          | 44 ± 3     | 2800 ± 270                       | 64            |

* If we assume Michaelis–Menten transport kinetics, $v = \frac{V_{max} \cdot S}{K_m + S}$, $V_{max}$ and $K_m$ represent the maximal velocity and the Michaelis constant, respectively. $V_{max}/K_m$ is a good measure of transport efficiency at low substrate concentrations. A similar term, $k_{cat}/K_m$, has been used as a measure of catalytic efficiency. For the selection condition of growth on S0.1D plates, which contain a glucose concentration of 0.1% (5.5 mM), the glucose concentration surrounding the colonies on the plates would be expected to be substantially lower than the $K_m$.

![Fig. 2](image1.png)

**Fig. 2.** Expression of the 16 chimeras corresponding to all possible combinations of Hxt1- or Hxt2-derived amino acids in TM5 of C1578. KY73 cells harboring plasmids encoding each chimera described in Fig. 3 were cultured to log phase at 30 °C in S2Mal synthetic medium, after which a crude membrane fraction was prepared. A portion of each fraction (10 μg of protein) was subjected to immunoblot analysis with antibodies to Hxt2.

![Fig. 3](image2.png)

**Fig. 3.** Glucose transport activities of the 16 chimeras corresponding to all possible combinations of Hxt1- or Hxt2-derived amino acid residues in TM5 of C1578. Each residue at sites 195, 198, 201, and 215 of C1578 was replaced with the corresponding amino acid of Hxt1, yielding 16 chimeras (left panel); Hxt2- or Hxt1-derived amino acids are indicated in uppercase and lowercase, respectively. KY73 cells expressing these various proteins were grown to log phase at 30 °C in S2Mal synthetic medium, after which glucose transport activity was measured for 5 s at 30 °C with 0.1 mM d-glucose as substrate (right panel); transport activities were normalized by cell number (solid bars) or by the mean level of protein expression as determined by quantitative immunoblot analysis (open bars) and are means ± S.E. of values from three or more experiments.
indicating that Leu at this position is essential and irreplaceable for high affinity glucose transport with a capacity to sustain cell growth under glucose-limited conditions. The reason for this requirement for Leu at position 201 is not immediately clear, but the size of its aliphatic side chain may be important to maintain the protein conformation necessary for high affinity, high capacity transport activity. We previously showed that one specific amino acid residue of Hxt2, Phe-431 in TM10, is absolutely required for recognition of the difference between glucose and galactose, with no other residue being able to perform this task (9). In addition, Tyr-440 in TM10 is necessary for high capacity glucose transport (9). On the other hand, the recognition and transport of glucose with high affinity appear to require a precise coordination of several amino acid residues in the various TMs that form the permeation pathway, because four TMs of Hxt2 are required for high affinity transport.

The critical role of Leu-201 in C1578 was also observed in Hxt2, because Hxt2(CFvY) showed almost the same characteristics as CFvY of C1578. In this respect, it is of considerable interest that Leu in this position is conserved in all the yeast MFS sugar transporters except for Hxt1 (1).

The crystal structures of the bacterial MFS transporters LacY (10) and GlpT (11), both recently determined with a resolution of <4 Å, reveal that the configurations of the TMs in the two proteins are highly similar. TMs 1, 5, 7, and 8 form the central permeation pathway. The aromatic ring of Trp-151 in TM5 of LacY is implicated in interaction with a substrate analog, galactopyranosylthiogalactopyranoside. The importance of TM5 was also demonstrated in the Tet(C/B) tetracycline transporter; mutation of Gly-452 in this segment was found important for tetracycline resistance (12). A structural model for OxlT, an MFS oxalate transporter of Oxalobacter formigenes, based on electron crystallographic analysis placed TM5 along the substrate pathway (13). TM5 of the mammalian glucose transporter GLUT1 is an amphipathic helix that is thought to form part of the sugar permeation pathway (14).

Although the alignment of bacterial and eukaryotic MFS transporters is not without ambiguity (15), we constructed a working structural model of Hxt2 based on the backbone structure of LacY revealed by crystallography. The positions of Leu-201, Phe-198, and Cys-195 of TM5 as well as of Phe-431 and Tyr-440 of TM10 in this model are shown in Fig. 4. All five residues indicated are situated in the cytoplasmic half of the inner core of the protein, and their arrangement suggests that they all likely interact directly with the substrate. Together with the results of previous studies, our present observations indicate that TM5 of yeast MFS transporters is important for the recognition and passage of substrate. Our present results demonstrate that Leu-201 in TM5 plays a central role in the high affinity, high capacity glucose transport activity of Hxt2.

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