Importance of the first external loop for substrate recognition as revealed by chimeric *Chlorella* monosaccharide/H^+ symporters**

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Abstract It had been shown previously by heterologous expression in *Schizosaccharomyces pombe*, that the two monosaccharide/H^+ symporters HUP1 and HUP2 of *Chlorella kessleri* differ significantly concerning their substrate specificity: HUP1 transports predominantly D-glucose while HUP2 prefers D-galactose. Several chimeric transporters were constructed and their substrate specificities determined. Surprisingly, it is sufficient to replace the first part of the external loop 1 of the HUP1 symporter by the corresponding portion of HUP2 to improve transport and also to decrease the K_m value for D-galactose. Additional data indicating the importance of the first loop for substrate recognition and binding are discussed.

Key words: Sugar transport; HUP1; Chimeric transporter; *Chlorella kessleri*; D-galactose; *Schizosaccharomyces pombe*

1. Introduction

The unicellular green alga *Chlorella kessleri* possesses an inducible hexose transport system [1], capable of accumulative uptake of a variety of monosaccharides and their analogues using a proton gradient for electrogenic secondary active transport [2–4]. The cDNA coding for a *Chlorella* monosaccharide/H^+ co-transporter was cloned by differential screening [5] and named HUP1 (hexose uptake protein 1). Its identity has been confirmed by heterologous expression in *Schizosaccharomyces pombe* [6] and in *Xenopus* oocytes [7]. Furthermore, sugar uptake was detectable in an in vitro vesicle system consisting of plasma membranes of transgenic yeast fused with cytochrome-*c* oxidase containing proteoliposomes [8]. Immunochemical studies on cross-sections of *Chlorella* cells localized the majority of the HUP1 protein in the plasma membrane as well [9].

The HUP1 symporter belongs to a large family of substrate transporters, called major facilitator superfamily (MFS) [10]. The members of this family are thought to consist of 12 putative α-helical transmembrane segments connected by internal and external loops. This topological model, originally developed solely from hydropathy plots, is in good agreement with data derived from alkaline phosphatase fusion protein analysis of the *Escherichia coli* lactose permease lacY [11] and N-glycosylation scanning mutagenesis studies on the human glucose facilitator GLUT1 [12]. However, hard structural data of transport proteins are still missing, therefore, information on the binding sites and translocation pathways of substrates and co-substrates can only be obtained indirectly, for example by mutagenesis.

Structure–function analysis of the HUP1 transporter was carried out in *S. pombe* YGS-B25, a sugar uptake-deficient mutant [13]. Several mutants with increased K_m value for glucose were found by site-directed mutagenesis [14] and/or PCR random mutagenesis with subsequent selection for decreased sensitivity towards the toxic sugar 2-deoxyglucose [15]. The amino acids affected clustered in the middle of the transmembrane helices V (Q179), VII (Q298 and Q299) and XI (V433 and N436), with the exception of D44, which is located at the beginning of the first external loop (Fig. 1A).

The presence of the HUP1 protein alone does not cover the broad specificity of monosaccharide transport in *Chlorella*. Recently, it has been demonstrated that indeed two other monosaccharide/H^+ symporters are co-induced by glucose [9]. They were designated HUP2 and HUP3 due to their high homologies to the HUP1 transporter (74 and 92%, respectively). Comparison of HUP1 and HUP2, both functionally expressed in *S. pombe* YGS-B25, showed that the transporters differ significantly concerning their substrate specificity [9]. All the previously identified residues of HUP1 probably involved in the glucose recognition/transport (see above) are also present in HUP2. This raises the question how the different substrate specificities are determined in the two transporters. To answer this, a set of chimeric proteins was constructed and their substrate specificities were characterized. The results clearly point to a participation of the first extracellular loop of HUP2 in specific galactose recognition.

2. Materials and methods

2.1. Strains and growth conditions

The strain *E. coli* TG1 was used as host for the phagemid pUC118 and the helper virus M13KO7 in site directed mutagenesis. The sugar transport-deficient mutant *S. pombe* YGS-B25 (leu$^-$) [13] was grown in 2% gluconate/2% yeast extract and used for heterologous expression of the HUP1, HUP2 and chimeric cDNAs. Transformed *S. pombe* cells were cultivated in minimal medium containing 2% gluconate and 0.67% yeast nitrogen base without amino acids.

2.2. Construction of the different chimeric cDNAs

The 1770-bp *Sac*I/*Bam*HI fragment, containing the full-length cDNA of *HUP1* [6], and the 1642-bp *Hin*dIII fragment, containing the full-length cDNA of *HUP2* [9], were ligated into the vector pUC18. Generation of a cDNA encoding a chimeric HUP2/HUP1 transporter with the fusion point at the end of helix VII (C1) was possible, since a unique *Bsa*AI restriction site appears at homologous positions in the wild-type transporter genes. The fragment coding for the N-terminal part of HUP1 was eliminated by digestion with *Sac*I/*Bsa*AI and replaced by the appropriate *HUP2* fragment. The chimeric cDNAs fused at the beginning of helix IV (C3) and the middle of the

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Table 1
Comparison of the initial uptake rates given as sugar/glucose ratios of wild-type and chimeric transporters

	HUP2	C1	C2	C4	C5	HUP1	C6
Mannose	0.04	0.01	0.17	0.26	0.12	0.67	0.33
Fructose	0	0	0.07	0.35	0.17	0.41	0.66
Xylose	0.09	0.05	0.05	0.03	0.01	0.05	0.02
Galactose	1.31	0.29	0.97	0.48	0.01	0.02	0.50

first external loop (C4) were created in the same way, using unique *Eag*I and *Bsg*I restriction sites, respectively. We previously reported [14] that an *Eco*RV site could be introduced in the big central loop by site directed mutagenesis without changing the amino acid sequence. The same was now done at the homologous position in *HUP2*. Preparation of single-stranded *HUP2* template DNA and site-specific mutagenesis with the Sculptor Kit were performed as described in the Amersham manual and in [14]. Again, the chimeric cDNA (C2) was constructed by exchanging the N-terminal *Sac*I/*Eco*RV fragment of *HUP1* for that of *HUP2*. The chimeric cDNA with the fusion site at the end of helix I (C5) was generated in a two-step PCR [16] and sequenced afterwards to exclude the possibility of undesired mutations. The PCR conditions were the same as indicated in the standard protocol of the Perkin-Elmer/Cetus Gene Amp DNA amplification kit. Finally, a chimeric *HUP1/2/1* cDNA was constructed in which only the sequence coding for the front part of the first external loop of HUP1 was exchanged for that of HUP2 (C6). This was achieved by introducing an *Eco*RV site at the beginning of loop 1 in HUP1 and HUP2, digestion with *Eco*RV/*Bsg*I and ligation of the appropriate fragments.

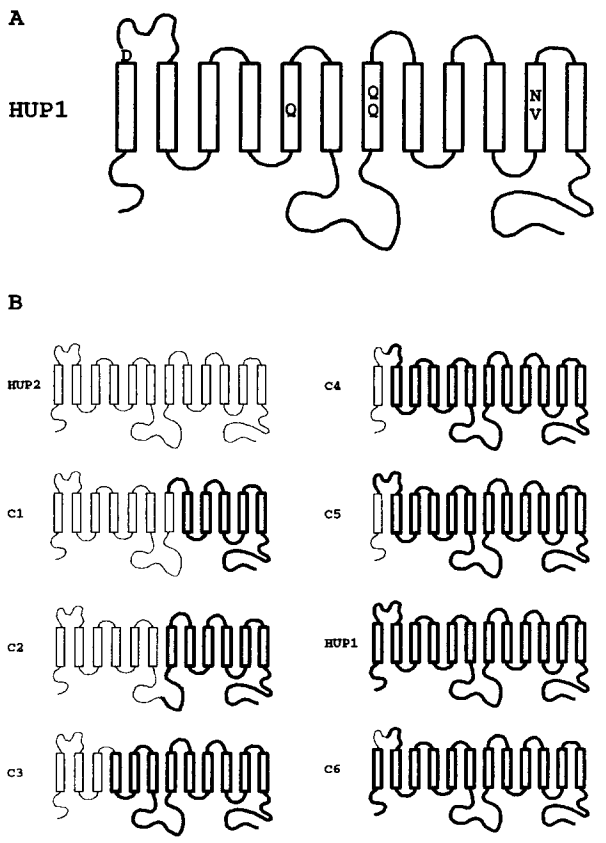

Fig. 1. (A) Topology of the *Chlorella* HUP1 monosaccharide/H^+ symport protein. Rectangles represent the 12 putative transmembrane α-helices. Exchange of the indicated amino acids increases the K_m value for D-glucose [14,15]. (B) Schematic drawing of the wild-type and chimeric transporters. The HUP1 portion (thick line) of the HUP2/1 transporters increases from C1 to C5. The chimeric HUP1/2/1 symporter C6 will be discussed in section 3.4.

2.3. Transformation of S. pombe mutant

All chimeric *HUP2/1* cDNAs (C1–5) were cloned via *Hin*dIII into the expression vector pEVP11 [17], whereas the *HUP1/2/1* cDNA (C6) was cloned via *Sac*I/*Bam*HI into the same vector. *S. pombe* YGS-B25 was transformed as described [6].

2.4. Affinity purification of anti-HUP1-A antibodies

Small pieces of nitrocellulose filters were incubated in the following way: 1 h at 4°C with 20 μg of purified HUP1-Bio-His-6 protein (Caspari et al., in prep.), 1 h at room temperature with blocking buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% skim milk powder, 0.1% Triton X-100) and 2 h at 4°C with 1 ml of 1:10 diluted anti-HUP1-A antiserum. Bound antibodies were removed from the filters as described in [9]. The procedure was repeated 2× and the eluates were pooled. Although anti-HUP1-A antiserum was raised against a fusion protein of ß-galactosidase and the 186 C-terminal amino acids of the HUP1 protein, mainly the last 27 residues are immunogenic [14].

2.5. Isolation of total membranes, SDS–PAGE and immunoblotting

S. pombe cells of a 30-ml culture (OD_{578} ~1) were pelleted by centrifugation. Their membranes were isolated as described [14]. The protein content was assayed by the method of Bradford [18]. SDS–PAGE was carried out according to Laemmli [19]; proteins were transferred electrophoretically to nitrocellulose and incubated overnight with affinity purified anti-HUP1-A antibody. The blot was immunodetected with the ECL kit of Amersham.

2.6. Transport tests

Five to twenty OD_{578} units of *S. pombe* cells were harvested, washed once in 5 ml of 100 mM potassium phosphate buffer, pH 6.0, and resuspended in the same buffer to a final volume of 1 ml. Cells were optimally energized by adding ethanol to a final concentration of 120 mM. After 2 min of shaking at 30°C the test was started by adding radioactive sugar (and competing sugar when indicated). Samples were withdrawn at given intervals, filtered through nitrocellulose filters (0.8 μm pore size) and washed once with destilled water. Incorporation of radioactivity was determined by scintillation counting. All radioactive compounds were purchased from Amersham.

3. Results

3.1. Construction of chimeric HUP2/1 transporters

Five different chimeric cDNAs with the N-terminal part of HUP2 and the C-terminal part of *HUP1* were generated (C1–C5 in Fig. 1B) and cloned into the expression vector pEVP11. *S. pombe* YGS-B25 (leu^-) was transformed with the constructs and transformants were selected for growth on minimal medium.

3.2. Analysis of the substrate specificities

The initial uptake velocities for various monosaccharides at external concentrations of 10 μM have been determined in the yeasts transformed with the wild-type or chimeric *HUP2/1* cDNAs. Chimera C3 showed no measurable uptake activity (as the untransformed control), whereas all the other transformants were active and exhibited their own characteristic substrate specificities (given as sugar/glucose ratios in Table 1). In addition, the order of sugar preference for each carrier has been confirmed by uptake competition experiments using

Table 2
Comparison of the K_m and V_{max} values for D-glucose and D-galactose of wild-type and chimeric transporters as well as the K_i values for D-glucose uptake inhibition by D-galactose

	HUP2	C1	C2	C4	C5	HUP1	C6
Glucose							
K_m [M]	4.5×10^{-6}	2.5×10^{-5}	3×10^{-5}	2.5×10^{-5}	1.5×10^{-5}	1.5×10^{-5}	2.5×10^{-5}
V_{max}[a]	13	0.25	3	3	2.5	150	80
Galactose							
K_m [M]	2.5×10^{-5}		2.5×10^{-5}	2×10^{-4}		3×10^{-3}	2×10^{-4}
V_{max}[a]	20		2	8		240	125
K_i [M][b]	4×10^{-5}	1×10^{-4}			9×10^{-3}	$3\times10-3$	

[a] V_{max} is given as μmol of sugar taken up/g fresh weight/h.
[b] K_m values of C1 and C5 can not be measured directly, since galactose uptake is too low. Therefore, the uptake of glucose given at a concentration which corresponds to the appropriate K_m value is inhibited with different galactose concentrations. The K_i value is that concentration which inhibits glucose uptake by two-thirds.

5 μM radioactive glucose and a 10,000-fold excess of non-labeled sugars (data not shown). The different substrate specificities of the wild-type transporters are obvious: HUP1 is predominantly a glucose transporter with fairly good affinities for fructose and mannose but not galactose, whereas HUP2 seems to be rather a galactose carrier, which transports glucose well, mannose poorly and no fructose at all.

When comparing the relative uptake rates for each sugar (Table 1), it becomes evident that with increasing HUP1 portion (from the left to the right) the mannose/glucose and the fructose/glucose ratios rise more or less continuously, while the xylose/glucose ratio is nearly unaffected. Surprisingly, the galactose/glucose ratio stays relatively high upto C4, but diminishes abruptly to the level of HUP1 in C5. This difference in galactose recognition between C4 and C5 is the more amazing, if one considers that these chimeras differ only in the front part of the first external loop (Fig. 1B).

3.3. K_m measurements

The K_m values for glucose and galactose uptake of all symporters have been determined (Table 2). HUP1, HUP2 and the HUP2/1 chimeras have nearly the same affinity for glucose (K_m 5–30 μM), indicating that homologous regions of HUP1 and HUP2 can functionally replace each other without changing the glucose-binding. On the other hand, concerning galactose there are remarkable differences between the wild-type transporters from the outset: HUP2 has a low K_m (25 μM), HUP1 a high one (3 mM). For some of the chimeras (C1 and C5), the K_m could not be measured directly since galactose transport was too low. However, measuring the K_i values for inhibition of glucose uptake in comparison with the wild-type transporters gives an indirect clue to their affinities towards galactose. Regarding this C1 strongly resembles HUP2, while C5 is very much like HUP1 (Table 2). C2 and most probably also C1 possess the same K_m value for galactose as HUP2, hence the N-terminal half of HUP2 is sufficient for good galactose recognition. C4, which encompasses nothing but the first 75 amino acids of HUP2, still has a more than 10-fold lower K_m than HUP1. In contrast, the affinity for galactose seems to be totally reduced to the level of HUP1 in C5, which still contains the first 46 amino acids of HUP2.

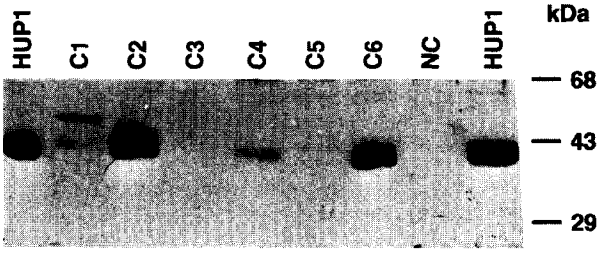

Fig. 2. Western blot of crude total membranes (30 μg/lane) containing wild-type HUP1 or the various chimeric transporters (C1–C6). NC stands for the negative control transformed with the vector pEVP11 only. The blot was incubated with purified anti-HUP1-A antibody, which is directed against the C-terminal 186 amino acids of HUP1 [14] and does not cross-react with the HUP2 protein (R. Stadler, pers. commun.).

3.4. Construction of a chimeric HUP1/2/1 transporter

The results presented above could be interpreted in two ways: either co-operation of helix I and loop 1 of HUP2 enhances galactose affinity or the residues of the first loop are alone responsible for that. To distinguish between these possibilities, another chimeric symporter named C6 was constructed, where solely the front loop part of HUP1 was exchanged for that of HUP2 (Fig. 1B), and introduced into the *S. pombe* mutant. The galactose/glucose ratio (Table 1) as well as the K_m values for glucose and galactose (Table 2) of C6 are close to those found for C4, therefore, the first part of loop 1 is sufficient for the improved galactose binding.

3.5. Expression of the chimeric transporters

The relative concentration of each chimeric transport protein in total membranes of *S. pombe* YGS-B25 was estimated by Western blot analysis with purified anti-HUP1-A antibodies (Fig. 2). For all symporters except C2, the signal intensity on the blot correlates well with the glucose uptake activity (Table 2). In the case of C5, the amount of transporter is even too low to be detected. The same could hold for C3, which shows no measurable uptake activity. The additional signal in the membrane extract of C1 can not be explained at the moment. Although carrying a wild-type level of transport

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          *                *
HUP1  DNGVTGGVVSLEAFEKKFFPDVWAKKQEVH
HUP2     I        T MPE LQ     SIYDRT QPS
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Fig. 3. Sequence alignment of the segments of HUP1 and HUP2 that were interchanged in order to create the chimeric HUP1/2/1 transporter C6. Only those amino acids of HUP2 are listed that differ from the HUP1 sequence. Asterisks mark the residues D44 and K60 of HUP1 (see section 4).

protein, C2 has dramatically decreased V_{max} values. This could be due either to reduced catalytic activity or to partial mistargeting.

4. Discussion

The studies of chimeric HUP transporters presented above demonstrate that the front part of the first extracellular loop is critical for determining substrate specificity. In this region, 16 out of 30 residues are different between the sequences of HUP1 and HUP2 (Fig. 3). If the concept were right that substrate-binding and transport is mediated by a concerted action of transmembrane helices and loops, it should be possible in the future to identify the crucial residues in the loop 1 by separate site directed exchanges.

Additional findings support the unique importance of loop 1: Insertion of four amino acids (GIPE) after K60, which was moreover changed to T, resulted in a HUP1 mutant with an expression comparable to wild type but exhibiting only 2% of its activity. The K_m for glucose, however, was not affected [14]. Substitution of D44 (Fig. 1) for E decreased not only the rate of uptake to about 10% but also increased the K_m for glucose by a factor of 15 as compared with HUP1 [14]. Moreover, this amino acid exchange specifically alters the K_m for glucose but not for mannose, fructose, xylose or galactose (T. Caspari and A. Will, unpubl. data). Aspartate 44 is also present in HUP2 (Fig. 3). The HUP1/2/1 symporter C6 is very well expressed. On the other hand, expression of all chimeric HUP2/1 transporters (except C2) is reduced as compared with the wild-type HUP1 (Fig. 2). Of course, the overall structure in these chimeras could be perturbed so that normal integration into the membrane or correct intracellular targeting is affected. However, one could also imagine that the HUP2 moiety may in part determine the amount of chimeric transport protein. Due to the lack of a HUP2-specific antibody, it can not be tested, whether the wild-type HUP2 carrier is less abundant in the plasma membrane, too, although this is suggested from a comparison of the V_{max} values with those of HUP1 (Table 2).

Nishizawa et al. [20] have constructed various chimeras between two facilitators of *Saccharomyces cerevisiae*, namely Gal2, which transports galactose and glucose, and Hxt2, which is specific for glucose. The region from putative helix X up to the 18th residue of the C-terminal tail was identified as the galactose recognition domain of Gal2. This could mean that the mechanism of galactose recognition may differ among different members of the MFS family. Alternatively, the first extracellular loop (this paper) and the last extracellular loop ([20]) may well be located close to each other and cooperate in galactose-binding.

Based on the results of various chimeric and point mutation studies performed on opioid receptors, Metzger and Ferguson [21] recently proposed an alternative model for the determination of selectivity. They suggest a high-affinity ligand-binding region common in all opioid receptor subtypes, which resides in the transmembrane domain. The extracellular loops are thought to act as a gate which allows passage of certain ligands while excluding others. In analogy, this would mean for the HUP proteins that galactose selectivity is conferred by unfavourable interactions of galactose with loop 1 of HUP1, rather than by specific contacts with loop 1 of HUP2. Similarly, a part of the C-terminal region is thought to function as a barrier to the homologous sugar-binding site in the transmembrane domain of Hxt2. The concept of a rather unspecific binding site is supported by the finding that all transmembrane amino acids of HUP1 shown in Fig. 1A are also present in HUP2. Moreover, most of these HUP1 mutants possess increased K_m values for all other sugars tested (A. Will, unpubl. data).

The construction of chimeric monosaccharide transporters presented here can serve as a valuable tool to investigate how the substrate specificity is determined. Future work will probably help to discriminate between the different hypotheses of selectivity.

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