Purification by Ni²⁺ Affinity Chromatography, and Functional Reconstitution of the Transporter for N-Acetylglucosamine of Escherichia coli*

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The N-acetyl-o-glucosamine transporter (IIIGlcNAc) of the bacterial phosphotransferase system couples vectorial translocation to phosphorylation of the transported GlcNAc. IIIGlcNAc of Escherichia coli containing a carboxyl-terminal affinity tag of six histidines was purified by Ni²⁺ chelate affinity chromatography. 4 mg of purified protein was obtained from 10 g (wet weight) of cells. Purified IIIGlcNAc was reconstituted into phospholipid vesicles by detergent dialysis and freeze/thaw sonication. IIIGlcNAc was oriented randomly in the vesicles as inferred from protein phosphorylation studies. Import and subsequent phosphorylation of GlcNAc were measured with proteoliposomes preloaded with enzyme I, histidine-containing phosphocarrier protein, and phosphoenolpyruvate. Uptake and phosphorylation occurred in a 1:1 ratio. Active extrusion of GlcNAc entrapped in vesicles was also measured by the addition of enzyme I, histidine-containing phosphocarrier protein, and phosphoenolpyruvate. Uptake and phosphorylation occurred in a 1:1 ratio. Active extrusion of GlcNAc entrapped in vesicles was also measured by the addition of enzyme I, histidine-containing phosphocarrier protein, and phosphoenolpyruvate to the outside of the vesicles. The K₅₀ for vectorial phosphorylation and non-vectorial phosphorylation were 66.6 ± 8.2 µM and 750 ± 19.6 µM, respectively. Non-vectorial phosphorylation was faster than vectorial phosphorylation with K₅₀ at 15.8 ± 0.9 s⁻¹ and 6.2 ± 0.7 s⁻¹, respectively. Using exactly the same conditions, the purified transporters for mannose (IIABMan, IICMan, IIDMan) and glucose (IICBGlc, IIAGlc) were also reconstituted for comparison. Although the vectorial transport activities of IICBGlcNAc and IIICBGlc are inhibited by non-vectorial phosphorylation, no such effect was observed with the IIABMan, IICMan, IIDMan complex. This suggests that the molecular mechanisms underlying solute transport and phosphorylation are different for different transporters of the phosphotransferase system.

N-Acetylglucosamine (GlcNAc) is the monomer building block of chitin, which forms the organic matrix of the exoskeletons of arthropods (insects, spiders, crabs) and the cell walls of fungi and of zooplankton. Chitin is the second most abundant biopolymer of N-acetylglucosamine (GlcNAc) transporter. Based on this strong structural similarity, IICBA(GlcNAc) can be characterized as follows: (Weigel et al., 1982a, 1982b; Dörsgang et al., 1984; Peri and Waygood, 1988; Plumbidge, 1990; Plumbidge and Kolb, 1991, 1993). IICBA(GlcNAc) has 40% sequence identity and is colinear with the IICBGlc and IIA(Glc) subunits of the glucosetransporter. Based on this strong structural similarity, IICBA(GlcNAc) can be characterized as follows: (Weigel et al., 1982a, 1982b; Dörsgang et al., 1984; Peri and Waygood, 1988; Plumbidge, 1990; Plumbidge and Kolb, 1991, 1993). The amino-terminal IIC domain of 370 residues spans the membrane eight times, contains the substrate specificity determinants, and provides the interface for dimerization. The IIB and the IIA domains are globular and

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1The abbreviations used are: PTS, phosphoenolpyruvate-sugar phosphotransferase system; IICBA(GlcNAc), N-acetylglucosamine transporter; IICBGlcNAc, N-acetylglucosamine transporter; IICBGlc, transmembrane subunit of the glucosetransporter; IIA(Glc), cytoplasmic subunit of the glucosetransporter; IIABMan, hydrophilic subunit of the mannose transporter; HPr, histidine-containing phosphocarrier protein of the PTS; PEPl, phosphoenolpyruvate; DM, N-decyl-β-D-maltopyranoside; MOPS, 3-(N-morpholino)propanesulfonic acid.

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exposed on the cytosolic face of the membrane. IIB (residues 370–480) mediates phosphoryltransfer between IIA and O-6' of GlcNAc. In this process IIB becomes transiently phosphorylated on Cysɛ12. The carboxyl-terminal IIA domain (residues 480–648) mediates phosphoryltransfer between HPr and IIB through a phospho-Hisɛ69 intermediate. The IIA and IIB domains of IICBAGlc are linked through an Ala-Pro-rich peptide segment, which is characteristic for structurally independent domains (Erni, 1989; Perham, 1991). The IIB and IIC domains are linked by the invariant sequence LKTPGRED.

IICBGlcIICGlcNAc and IICBGlcIICGlc can functionally complement each other. IIA IICGlc can complement a truncated GlcNAc transporter (IICBGlc, Vogler and Lengeler, 1988), and IICBGlcGlcNAc can suppress IIA Glc defects (Vogler et al., 1988). A chimeric protein between the IIC domain of IICBGlc and the IIA and IIB domains of IICBGlcGlcNAc was active and glucose-specific (Hummel et al., 1992). Expression from the IIC domain of IICBGlcGlcNAc inhibits glyceral kinase and maltose uptake, but in contrast to IIA Glc it does not inhibit adenylcyclase (catabolite repression; van der Vlag and Postma, 1995).

This functional interaction between two homologous but not identical membrane transporters poses questions with respect to the mechanism of the underlying protein-protein interactions. Does complementation occur between different domains on two homodimeric transporters (e.g., between IIB IICGlc and IICGlc in a transient tetramer intermediate), or is there subunit exchange with concomitant formation of IICBGlcIICBGlcNAc heterodimers? The complexity of native membranes and interactions with other membrane constituents severely limit the elucidation of these aspects. Thus it is vital to reconstitute the purified membrane proteins in artificial phospholipid vesicles to study these functions. The transporters for mannitol and mannose have already been reconstituted into phospholipid vesicles (Efferink et al., 1990; Mao et al., 1995). However, they belong to structurally unrelated families of PTS transporters. In this paper, we describe the purification of IICBGlcGlcNAc by Ni2⁺ chelate affinity chromatography and its functional reconstitution in phosphatidylethanolamine vesicles. As a prerequisite for further work and for comparison, IICBGlc and the structurally unrelated mannose transporter were also reconstituted using the same procedure.

**MATERIALS AND METHODS**

Bacterial Strains and Growth Media—In E. coli K12 LR2-16BΔGl the unstable ptsG allele was deleted from strain LR2-168 manI-4 ptsG lacY galT6 xyl-7 (Lengerl et al., 1981) by plasmid deletion to cat as described (Buhr et al., 1994).

Plasmid Construction—Plasmid pFEH6 (see Fig. 1A) for the controlled expression and purification by Ni2⁺ chelate affinity chromatography of IICBGlcGlcNAc plasmid was constructed as follows. The 5′ upstream region of nagE in plasmid pT5SE2 (Hummel et al., 1992) was trimmed with Bal31, the truncated nagE cloned into the Smal site of pFEH119, and one plasmid (pF NagE) containing only a 20-nucleotide upstream noncoding sequence was selected as described (Buhr et al., 1994). To append six histidines to the carboxyl terminus of IICBGlcGlcNAc the polymerase chain reaction was used. A primerase chain reaction fragment was amplified with primers GGTGCCAGCGCAAGAGC (pICCCGCAGATTTGGTGA-1) and GGTGATCTTACCTTATTGGTCAGGGTGGATGATCTTACCTTATTGGTCAGGCG (pICCCGCAGATTTGGTGA-2). The polymerase chain reaction product was digested with NdeI and HindIII and the 270-base pair fragment was used for plasmid purification, restriction analysis, ligation, and transformation (Sambrook et al., 1989).

Expression and Purification of IICBGlcGlcNAc—E. coli LR2-168GΔ (pFEH6) was grown in LB broth. When the cells had reached A600 = 1.5, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and incubation continued for 3 h. Cells were harvested by centrifugation (16,000 × g; 4°C; 15 min), and the cell pellet was resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 2 ml/g wet weight, of cells). Cells were broken by two passages through a French pressure cell, cell debris was removed by low speed centrifugation (12,000 × g; 4°C; 10 min), and large size beads collected. High speed centrifuged cells were collected at 4°C; 4 h; 1 h), resuspended in buffer B (10 mM Tris glycine, pH 9.3, 10 mM β-mercaptoethanol), shock frozen in liquid N2, and stored at −80°C. Membrane proteins were solubilized with 2% n-dodecyl-β-D-maltoside (DM, Sigma). The mixture was sonicated in a bath-type sonicator (Tec 40, Tecsonic, Switzerland) for 1 min, stirred for 15 min at 4°C, and cells were broken by low speed centrifugation (by centrifugation at 16,000 g; 1 h). Without delay, the pH of the extract was adjusted to 8.3 with 1 M acetic acid, mixed with Ni2⁺-nitrilotriacetic acid-agarose (Qiagen, GmbH, Germany; 3 ml of resin for the membrane extract from 1 g wet weight, of cells; equilibrated with buffer C: 50 mM MOPS, pH 7.5, 300 mM NaCl, 10 mM β-mercaptoethanol, 0.5% DM) and incubated for 1 h at 4°C with gentle shaking. The slurry was transferred to a chromatography column, washed with 5 bed volumes of buffer C, and eluted stepwise with 10, 25, and 100 mM imidazole in buffer C. IICBGlcGlcNAc eluted in the 100 mM imidazole step. The active fractions were pooled, supplemented with 10% glycerol (final concentration), shock frozen in liquid N2, and stored at −80°C. Protein concentrations were determined by a modified Lowry assay (Markwell et al., 1978) using bovine serum albumin as standard.

Assay for PEP-Sugar Phosphotransferase Activity—Phosphorylation of GlcNAc was assayed by the ion exchange method of Kundig and Rosenman (1971) modified as described (Erni et al., 1982). The reaction mixture contained per 100 μl: 50 mM KPi, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM NaF, 5 mM MgCl2, 1 mM PEP (Sigma), 0.5 mM [14C]GlcNAc (New England Nuclear, 56.3 mCi/mmol, diluted to 1,000 dpm/nmol), 2 μl (20 μg) of a cytoplasmic extract as a source of soluble phospho carrier proteins (enzyme I, HPr), and either the indicated amount of IICBGlcGlcNAc plus 1 μg of phosphatidylglycerol (Sigma), or IICBGlcGlcNAc in proteoliposomes prepared as described below. Incubation was for 30 min at 37°C.

Reconstitution of IICBGlcGlcNAc—Purified IICBGlcGlcNAc (75 μl in 0.5 M) was dialyzed for 6 h against 2 × 500 ml of buffer D (50 mM KPi, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM NaF) containing 0.75% octyl-β-D-glucopyranoside. 30 mM MgI, E. coli L-arabinofuranoside (type 1X, Sigma) was suspended in buffer D containing 0.75% octyl-β-D-glucopyranoside and briefly sonicated in a bath-type sonicator. 0.5 ml of IICBGlcGlcNAc was mixed with 0.5 ml of phosphatidylinositol membrane detergent solution (lipid/protein molar ratio 37:100), washed 3 times 30 s at room temperature, and briefly sonicated. Octyl glucoside was dialyzed against 500 ml of buffer D without detergent-containing SM-2 Bio-Beads (Bio-Rad) (3 mg/ml). Dialysis was for 24 h with four buffer changes. 0.25 ml aliquots of proteoliposomes were shock frozen in liquid N2 and stored at −80°C.

Assay for Vectorial Import and Phosphorylation of GlcNAc—Proteoliposomes were loaded with PEP, cytosolic PTS proteins, and with 0.5 mM L-3H]Glc (45,000 dpm/nmol) as aqueous phase marker. A 250-μl aliquot of proteoliposomes was thawed at room temperature, and MgCl2, Mg2+·PEP, enzyme I, and HPr were added to a final concentration of 1 mM, 10 mM, 0.1 μM, and 0.1 μM, respectively. The mixture was sonicated for 45 s in a bath-type sonicator. The sonicated proteoliposomes were freeze-thawed six times (liquid N2/room temperature water bath) and sonicated for 20 s in a bath-type sonicator. The proteoliposomes were separated from free components by gel filtration on Sephacryl S-300 (Pharmacia; 12-ml bed volume, buffer D). The peak liposome-containing fractions were pooled. To measure GlcNAc uptake, membrane proteins were diluted 10-fold in buffer D at 37°C, and incubated for 30 min 4°C; 15 min with ADP (2 mM, Serva) and pyruvate kinase (25 μCi, Fluka) to destroy residual external PEP. The import reaction was started by adding [14C]GlcNAc (New England Nuclear, 5.0 mCi/mmol) to the desired concentration. 50-μl aliquots were withdrawn at the indicated time points, diluted into 1 ml of ice-cold buffer D, and immediately sonicated through a 24 g, 35-μm pore size). The filters were washed with 2 × 1 ml of buffer D, and the radioactivity retained on the filters was determined by liquid scintillation counting. To measure the concomitant formation of GlcNAc-6P, 50-μl aliquots were diluted into buffer D containing 0.2% Triton X-100, and GlcNAc-6P was separated from free GlcNAc by anion exchange chromatography (Erni et al., 1982).
ether incompletely solubilized the activity. IICBAGlcNAc could not be solubilized in 2% Triton X-100 and pentaethylene glycol octyl ether, whereas Triton X-100 and dodecyl maltopyranoside were added to final concentrations of 10 mM, 0.1 mM, and 0.1 mM, respectively. The export reaction was started by adding PEP to a final concentration of 5 mM. 50% of the activity was solubilized at different time points, and the radioactivity retained in the vesicles as well as the formation of GlcNAc-6P were measured as described above. To measure competition between vectorial export and non-vectorial phosphorylation, 5 and 10 mM [12C]GlcNAc was added to the external compartment together with enzyme I and HPr. The reaction was started by the addition of PEP (10 mM) at 37 °C, and 50-μl aliquots were withdrawn at different time points, and the radioactivity retained in the vesicles as well as the formation of GlcNAc-6P were measured as described above. To measure competition between vectorial export and non-vectorial phosphorylation, 5 and 10 mM [12C]GlcNAc was added to the external compartment together with enzyme I and HPr. The reaction was started by the addition of PEP (10 mM) at 37 °C, and 50-μl aliquots were withdrawn at different time points, and the radioactivity retained in the vesicles as well as the formation of GlcNAc-6P were measured as described above.

Protein Phosphorylation Assay—20 μl of incubation mixture in buffer E (50 mM NaPi, pH 7.5, 10 mM MgCl2, 2.5 mM dithiothreitol, 2.5 mM NaF) contained 18 pmol of purified IICBAGlcNAc reconstituted in phosphatidylethanolamine vesicles, 10 pmol of purified HPr, and 4 pmol of enzyme I. The reaction was started by adding 400 pmol of [32P]PEP (39 cpn/pmol, 80 pmol/μl) at 37 °C. After a 5-min incubation, the reaction was stopped by adding phenylmethylsulfonyl fluoride to 3 mM final concentration). [32P]PEP was prepared as described by Roosien et al. (1983).

Reconstitution of IICBAGlcNAc and IICBMan—IICMan—The IICBAGlcNAc was solubilized and purified by Ni2+ chelate affinity chromatography in the presence of 0.2% DM and 1% C14100 in the presence of 0.02% dodecyl maltoside (Waeben et al., 1993; Huber, 1996). Reconstitution and all subsequent procedures were done exactly as described above for IICBAGlcNAc.

RESULTS AND DISCUSSION
Purification of IICBAGlcNAc by Ni2+ Chelate Affinity Chromatography—Exploratory experiments indicated that IICBAGlcNAc, like the transporters for mannose and glucose, could be purified by isoelectric focusing. However, the yield and purification were not satisfactory. To facilitate purification nagE was cloned under the control of the inducible Ptac promoter, and the carboxyl terminus of the protein was extended with a hexahistidine tag for purification by metal chelate affinity chromatography (Fig. 1A). 95% of the membrane-bound GlcNAc phosphotransferase activity could be solubilized in 2% DM at pH 9.3. Besides DM, octyl glucoside was also satisfactory, whereas Triton X-100 and pentaethylene glycol octyl ether incompletely solubilized the activity. IICBAGlcNAc could be eluted with 100 mM imidazole in 50 mM MOPS, 300 mM NaCl, pH 7.5. 80% of the phosphotransferase activity present in the membranes was recovered, and the protein was more than 95% pure as judged by polyacrylamide gel electrophoresis (Fig. 1B). Approximately 4 mg of purified IICBAGlcNAc was obtained from 10 g, wet weight, of cells.

Preparation and Characterization of Proteoliposomes—Purified IICBAGlcNAc was reconstituted with E. coli phospholipids by the β-ocyt glucoside detergent dialysis method based on the dilution procedure of Racker et al. (1979). The IICBAGlcNAc subunit of the glucose transporter and the IICMan—IICMan complex of the mannose transporter could be reconstituted by the same method. SM-2 Bio-Beads were added in the dialysis buffer to facilitate the removal of detergent and reduce the dialysis time and number of buffer changes (Phillipott et al., 1988). The preformed, concentrated proteoliposomes were loaded by freeze-thaw sonication with either [14C]GlcNAc or PEP, enzyme I, and HPr. [3H]Glc was added as a marker to measure the included aqueous space and to control the impermeability of the proteoliposomes. The loaded proteoliposomes were separated from nonincluded components by gel filtration chromatography. The uranyl acetate-stained vesicles obtained after gel filtration had diameters between 150 and 450 nm (electron micrographs not shown). The internal volume of the vesicles is approximately 0.8 μl/mg phospholipid as calculated from the amount of L-[3H]Glc coeluting with the proteoliposomes from the gel filtration column.

The orientation of membrane proteins in the bilayer depends on the method by which proteoliposomes are formed (Levy et al., 1990, 1992). The orientation of IICBAGlcNAc was determined by phosphorylation, exploiting the fact that the IIA and IIB domains are surface-exposed but covalently linked to the transmembrane IIC domain and can specifically be phosphorylated by HPr and enzyme I.

IICBAGlcNAc was phosphorylated before and after detergent solubilization. Twice as much IICBAGlcNAc is phosphorylated in the presence of Triton X-100, indicating that only 50% is accessible in intact proteoliposomes (Table I). When the intact proteoliposomes were first treated with trypsin, phosphorylation was strongly reduced. When the trypsin-treated proteoliposomes were solubilized, about 50% of the total protein could again be phosphorylated (Table I). Phosphorylation after pro-
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TABLE I

Random orientation of IICBA\(^{\text{GlcNAc}}\) in proteoliposomes
IICBA\(^{\text{GlcNAc}}\) in proteoliposomes was phosphorylated with \(^{[32P]}\)PEP in the presence of enzyme I and HPr. The reaction was carried out with intact proteoliposomes and after detergent solubilization. Prior to phosphorylation, from each preparation one aliquot was treated with trypsin to destroy accessible IIA and IIB domains. \(^{[32P]}\)IICBA\(^{\text{GlcNAc}}\) was analyzed by quantitative binding to nitrocellulose filters. For details see “Materials and Methods.”

|           | I       | II      | III     | IV      |
|-----------|---------|---------|---------|---------|
| Triton X-100 (first) | –       | +       | –       | +       |
| Trypsin   | –       | –       |       | +       |
| Triton X-100 (second) | –       | –       | +       | +       |
| Phosphorylation (with \(^{[32P]}\)PEP) | +      | +       | +       | +       |
| \(^{[32P]}\)IICBA\(^{\text{GlcNAc}}\) (pmol on filter) | 34.8 ± 1.6 | 60.6 ± 14 | 10.9 ± 0.4 | 36.5 ± 2 |

When GlcNAc and the cytosolic components are both added to the outside, non-vectorial phosphorylation but no transport is observed. Non-vectorial phosphorylation is concentration-dependent with a \(K_m\) of 750 ± 19.6 \(\mu M\) and a \(K_m'\) of 15.8 ± 0.9 \(s^{-1}\) (Fig. 3, A–C). The \(K_m\) for non-vectorial phosphorylation is 10 times higher than for vectorial transport. This result will be further discussed below.

Comparison between Transport and Non-vectorial Phosphorylation—If proteoliposomes are loaded with \(^{[14C]}\)GlcNAc and the cytosolic proteins are added to the outside, inside-out transport can be measured. The imposed orientation allows to manipulate the system from the “cytosolic face.” However, only qualitative changes can be monitored. The system is of limited value for the quantitative determination of kinetic parameters because intravesicular GlcNAc is depleted quickly. The rapid extrusion of GlcNAc is strictly coupled to phosphorylation, and there is no diffusion of GlcNAc through the phospholipid layer and no facilitated diffusion via the unphosphorylated carrier (Fig. 4).

As demonstrated above, IICBA\(^{\text{GlcNAc}}\) catalyzes two reactions: vectorial transport with concomitant phosphorylation, and non-vectorial phosphorylation. The \(K_{cat}/K_m\) values of the two reactions are 0.09 and 0.025 \(s^{-1} \mu M^{-1}\), respectively. This raises the question of whether the two reactions compete. Therefore the export of encapsulated \(^{[14C]}\)GlcNAc was measured in the presence of increasing concentrations of external GlcNAc. GlcNAc inhibits transport of \(^{[14C]}\)GlcNAc in a concentration-dependent manner (Fig. 5A). Concentrations of 5 and 10 \(mM\) inhibit the export by 40 and 55%, respectively. Because no competition was observed in previous experiments with the mannose transporter of the bacterial phosphotransferase system (Mao et al., 1995), these experiments were repeated. For comparison they were also done with the glucose transporter IICB\(^{\text{Glc}}\)-HPr. The two transporters were purified by metal chelate affinity chromatography (Waeger et al., 1993; Huber, 1996), reconstituted by octyl glucoside dialysis, and loaded with \(^{[14C]}\)Glc exactly as IICBA\(^{\text{GlcNAc}}\). Consistent with the observation of Mao et al. (1995) and in striking contrast to IICBA\(^{\text{GlcNAc}}\), the transport activity of IICB\(^{\text{Man}}\)-IICMan is not inhibited by external glucose (Fig. 5B). Glucose transport by the IICB\(^{\text{Glc}}\)-HPr complex, on the other hand, is inhibited exactly as IICBA\(^{\text{GlcNAc}}\) (Fig. 5C).

Conclusions—The transporter for GlcNAc is the fourth membrane transporter of the bacterial phosphotransferase system that has been purified to homogeneity (Jacobson et al., 1979; Erni et al., 1982; Erni and Zanolari, 1985). IICBA\(^{\text{GlcNAc}}\) could be purified in a single step by metal chelate affinity chromatography. This method appears generally suitable for the purification of phosphotransferase transporters (Waeger et al., 1993; Huber, 1996), possibly because these proteins have large hydrophilic domains to attach to the histidine tag. This can be visualized with the x-ray structure of the IIA domain of the Bacillus subtilis glucose transporter (Liao et al., 1991), to which IICBA\(^{\text{GlcNAc}}\) is homologous. The carboxyl terminus is exposed on the protein surface, at 21 \(\AA\) from the amino terminus and 23 \(\AA\) from the active site His\(^{B3}\) (equivalent to His\(^{E69}\)). Over these distances, the His tag is unlikely to interfere with docking between the IIA active site and either HPr or the IIB domain. Indeed, the carboxyl-terminal His tag does not affect the phosphotransferase activity of IICBA\(^{\text{GlcNAc}}\). Phosphorylation of substrates without transport was first observed in vivo (Thompson and Chassy, 1985; Thompson et al., 1985; Nueffer et
and was also found after reconstitution of the transporters for mannitol and mannose (Elferink et al., 1990; Mao et al., 1995). In all cases the Km for transport is lower than for non-vectorial phosphorylation. This difference (66 μM versus 750 μM for IICBAGlcNAc) suggests that phosphotransferase transporters have different affinities depending on what side of the membrane the binding site is oriented to (assuming that the transporter has only one substrate binding site that can isomerize between inward and outward orientations).

Of particular interest is the difference between IICBAGlcNAc and IICBGlc or IICManzIIDMan on the one hand and IIABManzIICManzIIDMan on the other hand with respect to competition between transport and non-vectorial phosphorylation. To our knowledge this
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is the first experiment that indicates that the two families of PTS transporters have not only different structures but that they also function differently. The homologous IICBAGlcNAc and IICBMan1IDMan1 complex has a broad substrate specificity (including Glc, Man, GlcNAc), and transphosphorylation proceeds through two phosphohistidine intermediates. It is a heterooligomeric composed of two membrane-spanning subunits (stoichiometry 1:2) and a hydrophilic complex of two structurally interwined polypeptide chains (Nunn et al., 1996). The mechanistic basis of this differences remains to be discovered.

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